Effect of Mixed and Single Crops on Disease Suppressiveness of Soils

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ABSTRACT

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The effect of mixed cropping on disease suppressiveness of soils was tested for two cropping systems, Brussels sprouts–barley and triticale–white clover. Disease suppressiveness of field soils was evaluated in bioassays for the soilborne pathogens *Rhizoctonia solani*, *Fusarium oxysporum* f. sp. *lini*, and *Gaeumannomyces graminis* var. *tritici*. For both cropping systems, mixed cropping did not enhance disease suppressive-

ness of the soils. In some cases, soil cropped to barley alone was significantly more suppressive to *F. oxysporum* f. sp. *lini* than soils cropped to Brussels sprouts or the mixture of Brussels sprouts and barley. Analyses of the diversity of the indigenous bacterial and fungal microflora by denaturing gradient gel electrophoresis of amplified 16S- and 18S-rDNA fragments, respectively, revealed, in most cases, no significant differences between mixed and mono-cropped soils. In conclusion, in this study, mixed cropping of soils with Brussels sprouts and barley or with triticale and white clover did not enhance microbial diversity or disease suppressiveness of soils to three different soilborne plant pathogens.

Intensification of agriculture has contributed substantially to the increase in world food production over the past 50 years (27). However, the ecological side effects, including soil erosion, water pollution, eutrophication, and reduced soil fertility, may be as profound as the increase in food production. Concerns have developed over the long-term sustainability and environmental consequences of the intensification of the agricultural system (27). Therefore, a change to a more sustainable way of food production has been advocated over the past decade.

One of the constraints to future food production might be the occurrence of soilborne pathogens as serious threats of crops. Most soilborne pathogens are difficult to control and resistance in plants to many soilborne pathogens is lacking or has not been fully explored (48). Crop management strategies, including mixed cropping, might provide a way to reduce deleterious effects from soilborne pathogens.

There are some examples that show that monoculture can lead to specific disease decline in some pathosystems when a susceptible crop is grown continually in the absence of rotation (48). In general, however, continuous cropping of one specific crop leads to an increase in disease incidence caused by soilborne and other plant pathogens. Most natural and agricultural soils exhibit some degree of suppressiveness to soilborne plant pathogens. This phenomenon is referred to as general suppression or general antagonism (14,21,47), which appears to be nonspecific and active against a wide range of soilborne pathogens. Mixed cropping might stimulate these general disease suppressive properties of soils.

Mixed cropping is a way of farming practiced by many traditional farmers (13,43). In contrast to single cropping, two or more crops are grown in the same field at the same time (43). Farmers using mixed crops could be less dependent on the input of pesticides because of lower pest and disease pressure and fertilizer inputs due to higher efficiency of nutrient use (13). Therefore,

mixed cropping has been proposed to lead to a more sustainable farming system (43).

Much effort has been made to investigate mixed cropping systems, especially with respect to the ecological processes involved in crop performance (13). The relation between crop or cultivar mixtures and disease severity has been investigated extensively, but has focused mostly on disease caused by above-ground pathogens like rice blast (52) and foliar pathogens of small grains (29). Effects of mixed cropping on soilborne pathogens have been described less frequently (1,2,8,44). For example, Burdon and Chilvers (8) showed reduced spread of *Pythium* sp. in a garden cress–ryegrass mixture and Vilich (44) reported a reduction in disease severity of soilborne pathogens in barley–wheat mixtures. In these experiments, disease suppressiveness was investigated for pathogens specific to one or both of the crops included in the mixed cropping. It is not known, however, if mixed cropping also stimulates the general (nonspecific) disease suppressiveness of soils to a variety of soilborne pathogens.

Soil biological processes underlying suppressiveness to soilborne pathogens have not been investigated extensively for soils with mixed crops. Suppressiveness to soilborne pathogens is affected by biological, chemical, and physical characteristics of the soil (48). Soil biological characteristics are strongly influenced by plant species composition, diversity, and sequence. Plant species composition and specific plant traits have more important effects on soil ecological processes than species richness of crop mixtures (20,46). Increased plant diversity can stimulate microbial community biomass, respiration, and fungal abundance (51), and removal of particular plant species from mixed plant communities can influence soil microbial community composition and diversity, the associated food webs, and as a result ecosystem properties and processes (46) in bulk soil. Some of these processes, including microbial activity, are known to affect soilborne pathogen behavior (21,47,49). In a limited number of cases, soil microbial diversity has been directly linked to root disease suppression (31,49). For example, corky root of tomato was more suppressed in organic soils containing a more diverse population of actinomycetes than in conventional soils containing a less diverse population (49). More diverse microbial populations have a higher

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chance to harbor particular species that can suppress soilborne plant pathogens.

The objectives of the present study were to determine the effect of mixed cropping on (i) general disease suppressiveness of bulk soils to three different soilborne pathogens, (ii) microbial diversity and activity in bulk soils, and (iii) relation between microbial diversity, activity, and general disease suppressiveness in those soils. Field soils cropped to Brussels sprouts, barley, triticale, white clover, and the mixtures Brussels sprouts–barley and triticale–white clover were evaluated in greenhouse assays for their suppressiveness to *Rhizoctonia solani* Kühn (AG1), *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *lini* (Bolley) W.C. Snyder & H. N. Hans., and *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier [senso lato] var. *tritici* J. Walker. Three different pathosystems were used because different pathogens generally react differently to biotic and abiotic properties of a soil (16). We used two pathosystems with crops (carrot with *R. solani* and flax with *F. oxysporum* f. sp. *lini*) not grown in the field to circumvent the effect of adaptation of the microbial community, including soilborne pathogens, to a certain host. Results of tests for general disease suppressiveness could otherwise be confused with specific disease suppression phenomena. Diversity of the indigenous bacterial and fungal microflora was determined with denaturing gradient gel electrophoresis (DGGE) of amplified 16S- and 18S-rDNA fragments, respectively, and related to disease severity determined in the different bioassays.

MATERIALS AND METHODS

General set-up. At two locations near Wageningen (The Netherlands), Wageningen-Hoog (WH) and Achterberg (AB), barley, Brussels sprouts, and their mix were grown for two consecutive years. At another location in Wageningen, Bornsesteeg (BSW), triticale, white clover, and their mix were grown for three consecutive years. Crops were grown organically (details described below). Soil was sampled from the field at various points in time and bioassays were performed to determine disease suppressiveness of the soils against the plant pathogens *R. solani* (using carrot as test plant), *F. oxysporum* f. sp. *lini* (flax as test plant), and *G. graminis* var. *tritici* (triticale or barley as test plant) (Table 1).

Field experiments. Barley (*Hordeum aestivum* L. cv. Video, Samenwerkende Graankweekbedrijven Wierum-Zelder, The Netherlands), Brussels sprouts (*Brassica oleracea* L. cv. Maximus, Syngenta, Basel, Switzerland), and their mix were grown at WH and AB for two consecutive years (2000 and 2001) in two replicates at each location. Plots measured 25 by 25 m. Fields were fertilized annually in spring with 25 ton ha⁻¹ pig manure (NPK kg ha⁻¹, 180:105:180) and plowed to a depth of 30 cm. Organically grown, 6-week-old Brussels sprouts plants obtained from a plant nursery, ‘West Plant Group’ (Venlo, The Netherlands), were planted in May at a distance of 50 and 75 cm within and between rows, respectively. Barley was sown the same day at an inter-row spacing of 12.5 cm, an intra-row spacing of approximately 2.5 cm, and a quantity of 125 kg of seed ha⁻¹. In the crop

mixture, five rows of Brussels sprouts were alternated with five rows of barley, each at the same inter- and intra-row distances as the respective single crops. Mechanical weed control was performed in the single crops two to three times during the growing season. Mechanical weeding was not possible in the mixed crops but was done manually when necessary. Later in summer both single and mixed crops were hand-weeded.

Triticale (*X Triticosecale* Wittm. cv. Galtjo, Svalöf-Weibull B.V., Emmeloord, The Netherlands), white clover (*Trifolium repens* L. cv. Pertina, Cebeco Zaden, Vlijmen, The Netherlands), and their mix were grown at BSW in three replications during three growing seasons (2000 to 2003). Fields were fertilized with 30 ton of farmyard manure ha⁻¹ (NPK kg ha⁻¹, 207:114:222) incorporated with a rotary spade digger to a depth of 30 cm. Triticale and white clover were sown on the same day between 28 September and 11 October. Triticale and white clover single crops were each sown at an inter-row distance of 25 cm and intra-row distance of 2.5 and 0.5 cm, respectively (125 kg ha⁻¹ for triticale and 15 kg ha⁻¹ for white clover). For the mixed crops, triticale and white clover were sown additively in alternating rows at the same distances and amounts as the single crops. Triticale plots were harrowed several times to remove weeds. White clover and the mixed crop were not harrowed to prevent damage to the white clover. In late spring hand weeding was performed in all plots.

Soil sampling. At various times (Table 1) and from 10 to 15 random sampling points in each plot, soil was sampled from the upper 15 cm and pooled per plot. A small spade was used to sample the soil (200 to 300 g of soil per subsample) and collect it in plastic bags. The spade was cleaned between plots. The same day, the soils were sieved through a 4-mm-mesh sieve, thoroughly mixed, and used for one bioassay. From this batch, subsamples were taken, pooled, and stored at –20°C until DNA extraction. Soil samples for general soil and nutrient analyses (approximately 100 g) were air-dried at room temperature for 7 days and stored in closed containers until further analysis. General soil characteristics (Table 2) were determined by the Department of Soil Quality, Wageningen University, (Wageningen, The Netherlands) and were similar for all three locations.

***R. solani* bioassays (RS1 and RS2).** Pipes (PVC, polyvinyl chloride) were longitudinally cut in half (4.4 × 2.2 × 40 cm) and holes were made at every 10 cm in the bottom to allow water uptake when necessary. The pipes were placed horizontally on a greenhouse bench and filled with field soil. The greenhouse temperature was maintained at ±20°C. Carrot seeds (*Daucus carotas* L. cv. Amsterdamse Bak, Pieterpikzonen, Heerenveen, The Netherlands) were surface-sterilized for 1 min in 1% NaOCl, rinsed with running tap water for 5 min, and sown in a 35-cm-long row with 5 to 10 carrot seeds every 2.5 cm. Pipes were placed on a bench covered with a moist irrigation mat (BWM 300 plus [water holding capacity 300 g m⁻¹]; Henofa bv, The Netherlands) in the greenhouse in a randomized complete block design with four replications and covered with plastic to maintain a relative humidity (RH) of 100%. *R. solani* (AG1, isolate 55, originally isolated from *Cotoneaster* sp.) (40) was grown on malt extract agar for

TABLE 1. Host–pathogen systems used to test disease suppressiveness in soil samples collected from fields with mixed and single crops

Pathogen	Host	Bioassay ^x	Mixed crops	Date ^y	Years of crop growth ^z
<i>Rhizoctonia solani</i> (AG1)	Carrot	RS1	Brussels sprouts–barley	05-11-01	1
		RS2	Triticale–white clover	06-12-01	1
<i>Fusarium oxysporum</i> f. sp. <i>lini</i>	Flax	FOL1	Brussels sprouts–barley	07-16-01	2
		FOL2	Triticale–white clover	06-23-03	3
<i>Gaeumannomyces graminis</i> var. <i>tritici</i>	Barley	GGT1	Brussels sprouts–barley	02-22-02	2
	Triticale	GGT2	Triticale–white clover	06-25-02	2

^x Bioassays with soils from different locations and cropping history were performed with *R. solani* on carrots (RS1 and RS2), *F. oxysporum* f. sp. *lini* on flax (FOL1 and FOL2), and *G. graminis* var. *tritici* on barley or triticale (GGT1 and GGT2).

^y Starting date of the bioassay.

^z Number of years of the cropping history at the start of the bioassay.

2 to 3 days at 25°C in darkness. Immediately after seedling emergence (approximately 5 days), an agar plug (5-mm diameter) with actively growing mycelium of a 2- to 3-day-old colony of *R. solani* was placed, 1 cm below the soil surface, at one end of the row of carrots in each pipe. Pipes that were not infested served as controls. When necessary the growth blanket was wetted with water, no fertilizer was added during the experiment. Progress of the disease was determined by counting the infected seedlings twice per week. The experiment was terminated when the pathogen reached the end of a row of carrots in one of the treatments.

***F. oxysporum* bioassays (FOL1 and FOL2).** *F. oxysporum* f. sp. *lini* (isolate FOLn3, provided by P. Lemanceau, INRA, Dijon, France) inoculum was prepared by growing the fungus in malt extract broth (Oxoid Ltd., Basingstoke, Hampshire, England) for 3 weeks at 25°C on a rotary shaker. The conidial suspensions were centrifuged (4,000 rpm) and washed three times with sterile deionized water. The conidia were dried for 2 weeks in talc (Merck KGaA, Darmstadt, Germany) in a container with a sterile airflow. Conidial density was determined by dilution plating on Komada medium (25) with 0.15 g of oxytetracycline liter⁻¹ instead of streptomycin sulfate. Soils were mixed with talc inoculum to a final density of 2×10^5 conidia g⁻¹ of soil. Control soils were mixed with talc only. Plastic pots (7 by 7 by 8 cm) were filled with 150 g (dry weight) of infested or control soil and placed in the greenhouse (20°C, RH 70%) in a randomized complete block design with six replicates. Flax seeds (*Linum usitatissimum* L. cv. Opaline, provided by P. Lemanceau, INRA, Dijon, France) were surface-sterilized as described previously. Seeds were left overnight at 20°C in a sterile petri dish on moistened sterile filter paper to germinate. The next day, 10 germinated seeds were sown per pot and covered with a layer of 25 g of uninfested soil. Plants were watered on dishes when necessary and 10 ml of full-strength Hoagland solution (0.005 M CaNO₃, 0.005 M KNO₃, 0.001 M KH₂PO₄, 0.002 M MgSO₄ × 7H₂O, 0.5% Fe tartrate, and 1 ml of micronutrient solution [2.86 g liter⁻¹ H₃BO₃, 1.81 g liter⁻¹ MnCl₂ × 4 H₂O, 0.22 g liter⁻¹ ZnSO₄ × 7H₂O, 0.08 g liter⁻¹ CuSO₄ × 5H₂O, and 0.02 g liter⁻¹ H₂MoO₄ × H₂O]) was added once a week. The number of diseased plants per pot, scored once a week, was used to calculate the area under the disease progress curves (AUDPCs) (9). The AUDPCs from the noninfested pots were subtracted from AUDPCs calculated for the infested pots.

***G. graminis* bioassays (GGT1 and GGT2).** *G. graminis* var. *tritici* (isolate R3-111-a-1) (32) inoculum (ground oats, particle size 0.2 to 0.5 mm) was mixed with the soils to a final concentration of 0.5% (wt/wt). Control pots were infested with autoclaved inoculum at the same concentration. Pots were filled with 150 g (dry weight) of infested soil and covered with 25 g of uninfested soil. In every pot, five surface-sterilized (1% NaOCl for 1 min and rinsed 5 min in running tap water), pregerminated seeds of barley or triticale were sown and covered with 25 g of uninfested soil (in total, 50 g of uninfested soil). Pots were placed in the greenhouse in a completely randomized block design with four replicates. Plants were watered when necessary and 10 ml of full-strength Hoagland solution was added once per week. Root disease severity was scored after 4 weeks of plant growth on a scale from 0 to 8 (0 = healthy, 1 = small lesions on one root, 2 =

larger lesions on two or more roots, 3 = one lesion reaching the stem base, 4 = two or more lesions reaching the stem base, 5 = stem base infected, 6 = infection reaching the stem, 7 = plants yellowing and wilting, and 8 = dead plant) (38).

DNA extraction. From the soil samples stored at -20°C, DNA was extracted using the FastDNA SPIN Kit for soils (Bio101 Systems, Carlsbad, CA), according to manufacturer's protocol with some small adaptations. All buffers used, were provided by the manufacturer in the kit. In short, 500 mg of soil was added to 978 µl of phosphate buffer (pH 8.0) and 122 µl of MT buffer; and the mix was subjected to a bead-beater (Braun Celhomogenisator Model MSK, B. Braun Biotech International, Germany) three times for 90 s and centrifuged 5 min at 14,000 × g. The supernatant was added to 250 µl of protein precipitation solution reagent, mixed 10 times, and centrifuged again for 5 min at 14,000 × g. The supernatant was transferred to 1-ml binding matrix, shaken for 2 min on a rotator (Heidolph Unimax 2010, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany, 60 rpm), and centrifuged for 1 min at 14,000 × g. The supernatant was discarded, except for approximately 500 µl that was used to dissolve the matrix again. The solution was transferred to a spin filter and centrifuged for 1 min at 14,000 × g. To the filter containing the matrix, 500 µl of salt ethanol washing solution-M was added and centrifuged for 1 min. After discarding the flow-through, the filter was centrifuged for 2 min, put in a new tube, and left to air dry for 5 min at room temperature. To the dry matrix, 100 µl of Dnase/Pyrogen-free, ultrapure water, 65°C) was added, vortexed, left at 65°C for 20 min in a water bath, and centrifuged for 1 min at 14,000 × g. Quality and concentration of DNA in the flow-through was determined on a 1.2% agarose gel using diluted Lambda-DNA (Roche Diagnostics GmbH, Mannheim, Germany). Samples were diluted to a final concentration of approximately 1 ng µl⁻¹ before polymerase chain reaction (PCR).

PCR and DGGE. The PCR amplifications were performed using the U968 (40-bp GC clamp) and L1401 universal eubacterial primers (18) and primers FR1 (58-bp GC-clamp) and FF390 for amplification of fungal small-subunit (SSU) rDNA (41). For both primer sets, each PCR was performed as described by Rosado et al. (33) with small adjustments. Each reaction contained 31.34 µl of deionized water, 5 µl of 10× Stoffel buffer, 1 µl of 10 µM DNTP mix, 7.5 µl of 25 mM MgCl₂, 1 µl of 10 µM U968 (or FR1), 1 µl of 10 µM L1401 (or FF390), 0.5 µl of formamide 100%, 0.16 µl of T4 gene 32 protein (1.19 µg µl⁻¹, USB Corporation, Cleveland, OH), 0.5 µl of AmpliTaq Stoffel fragment (10 units µl⁻¹) (Applied Biosystems, Foster City, CA), and 2 µl of diluted DNA extract (1 ng µl⁻¹). Reactions were carried out in a PTC-200 (MJ Research, Waltham, MA) with the following program for the eubacterial primers: 94°C (3 min), 94°C (1 min), 60°C (1 min), and 72°C (2 min) (2 cycles). The annealing temperature dropped 1°C per two cycles until 55°C was reached, and then the program was repeated for 29 cycles followed by a final extension step of 72°C for 10 min. The PCR program for the fungal SSU rDNA primers was 94°C (4 min), 92°C (0.5 min), 55°C (1 min), 68°C (2 min) (2 cycles), and then the temperature dropped 2°C every two cycles until 47°C was reached. The program was run for an additional 29 cycles at 92°C for 0.5 min, 47°C for 1 min, 68°C for 45 s + 1 s cycle⁻¹, and a final extension step at 68°C for 10 min. The amount and quality

TABLE 2. Properties of the soil samples used in the bioassays

Location ^x	Soil type	OM% ^y	N%	C%	P%	K%	C/N ^z	Clay%	Silt%	Sand%
Wageningen Hoog (WH)	Sand	3.4	0.11	1.68	0.09	0.12	14.7	1.4	7.5	91.1
Achterberg (AB)	Sand	3.5	0.12	2.01	0.05	0.14	16.6	1.3	6.1	92.6
Bornsesteeg Wageningen (BSW)	Sand	4.9	0.18	2.63	0.07	0.21	14.9	2.1	11.9	86.0

^x Locations are all within 10 km from the city of Wageningen, The Netherlands.

^y Organic matter content, determined by glow loss method (4).

^z C to N ratio.

of the obtained PCR products were checked on 1.2% agarose gel prior to DGGE analysis.

The DGGE analysis was carried out using the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). Vertical denaturing gradients 45 to 60% (16S-rDNA) and 30 to 60% (18S-rDNA) (100% denaturant is defined as 7 M urea plus 40% formamide) were used. Samples (20 µl) were loaded in duplicate gels in a random order. Gels were run at 60°C at 100 V for 16 h and bands were visualized using the Bio-Rad Silver Stain Kit according to manufacturer's protocol. Gels were analyzed with Phoretix 1D Advanced version 4.00 (Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK). Background was subtracted using the rolling disk method (radius 40 pixels). Minimum peak height was set at 2 pixels and width was fixed at 5 pixels. Shannon-Weaver (SW) indices were calculated using the relative band intensity (peak height × fixed width) according to the following equation: $H' = -\sum P_i \log P_i$, where $P_i = n_i/N$ and n_i = peak intensity of the i th band and N is the sum of the intensities of all peaks in one lane profile (11). Similarity indices were calculated as described by McCaig et al. (28).

Nitrogen and pH. A portion of each soil sample (100 g) collected for use in bioassays was air-dried for 7 days, sieved through a 1-mm mesh sieve, and stored in plastic containers until further processing. For nitrogen analysis and pH measurement, 2 g of soil was added to 100 ml of 0.01 M CaCl₂ and shaken for 2 h. The pH was measured in this solution with an Inolab pH Level 1 (WTW GmbH, Weilheim, Germany). Nitrate and ammonium concentrations were determined with an Autoanalyzer II (Technicon Instrument Corp., Tarreytown, NY) according to Houba and Novozamsky (22).

Microbial respiration. Soil samples were stored for 24 h at room temperature before further processing. Microbial respiration was performed according to the protocol described by Heinemeyer et al. (17) for an infrared gas analyzer. Fifty grams of moist soil was placed in closed containers connected to a continuous flow system where moisture saturated air was blown over

the soil. Released CO₂ was measured using an infrared gas analyzer (ADC 7000 Gas Analyzer, Hoddesdon, England). Production of CO₂ was calculated by subtracting CO₂ concentration of an unfilled control tube from the tubes filled with soil. Microbial respiration was only measured in soils used for the FOL2 and GGT2 bioassays from the triticale–white clover system.

Statistical analysis. All data were analyzed using the SAS system for Windows version 8.00 (SAS Institute, Cary, NC). Disease scores from the bioassays (average AUDPCs for FOL1, FOL2, RS1, and RS2 and disease indices for GGT1 and GGT2) were analyzed by split-plot analysis of variance (ANOVA) (PROC-MIXED, Brussels sprouts–barley) (36) for FOL1, GGT1, and RS1 or one-way ANOVA (PROC-GLM, triticale–white clover) for FOL2, GGT2, and RS2 (pooled samples). Crop effects on disease scores were tested by contrast analysis, and crop–location interaction effects were compared by least square means. Residuals were tested for normality. SW indices obtained from DGGE analyses also were analyzed with one-way ANOVA or split-plot analyses. Log-transformed band intensities were subjected to principal component analyses (PCA) (28) to distinguish between soils grown to the different crops. Relative disease scores, diversity indices, and nitrogen concentrations were calculated as a fraction of the highest value to be able to compare crop effects within a cropping system using all bioassays as replicates. Fractions were square root-arcsine transformed to obtain normality for both disease scores and diversity indices. Simple and multiple linear regressions were used to describe the relation between disease scores, nitrogen data, and diversity indices.

RESULTS

Effect of mixed cropping on disease suppressiveness.

R. solani. In containers not infested with *R. solani*, no carrot plants were infected during the course of the bioassay. In infested containers, the rate of disease spread (expressed as AUDPC, Table 3) was not significantly different between the soils with the mono- and mixed crop treatments for both the Brussels sprouts–barley and the triticale–white clover systems. Disease severity was neither significantly different between the locations AB and WH nor for the crop–location interaction effect. The bioassay was terminated after 17 days when carrot plants at the end of the row showed damping-off and the fungus had spread 35 cm from the point of inoculation.

F. oxysporum. The AUDPCs for the noninfested pots were low and flax plants had no wilting. In infested pots, the AUDPCs for *Fusarium wilt* were significantly affected by the cropping history of the soil collected at locations AB and WH (Table 3). Soils cropped to barley showed on average 16% lower AUDPC values than soils cropped to Brussels sprouts alone ($P = 0.01$) or to the mixed crop ($P = 0.01$). Location was not important for the AUDPC in this bioassay and there was no interaction effect of crop–location. For the soils from the BSW location, grown to triticale, clover, or the mix, no significant crop effects were found on disease development caused by *F. oxysporum*.

G. graminis var. tritici. In the noninfested soils, *G. graminis* var. *tritici*-like infections were low. In artificially infested soils, disease scores were lowest in the barley soils for both locations (Table 3) but crop effects were not significant ($P = 0.16$). Location had no significant effect on take-all disease severity in the GGT1 bioassay and there was no crop–location effect. No differences were observed between single crops and the mixed triticale–white clover crop at the BSW location.

When relative disease scores were pooled and analyzed for all three bioassays together at locations AB and WH, a significant crop effect was obtained ($P = 0.04$, data not shown). Contrast analyses indicated that soils grown to barley were more disease suppressive than soils grown to Brussels sprouts ($P = 0.013$). Soils grown to the mixed crop did not differ in disease suppressiveness.

TABLE 3. Disease scores in bioassays used to test disease suppressiveness of soils cropped to Brussels sprouts, barley, and their mix, or triticale, white clover, and their mix

Location ^v	Crop	Bioassay ^w		
		RS	FOL	GGT
AB	Brussels sprouts	75.3 ^x	11.1 ab	4.7
	Barley	57.1	9.5 a	3.9
	Mix	56.4	11.3 b	4.2
WH	Brussels sprouts	104.9	10.6 a	3.7
	Barley	109.0	8.8 b	1.6
	Mix	86.2	10.5 a	3.3
AB and WH ^y	Brussels sprouts	90.1	10.8 a	4.2
	Barley	83.0	9.2 b	2.8
	Mix	71.3	10.9 a	3.7
BSW	Triticale	46.8 ^z	10.1	3.6
	White clover	56.3	9.9	4.2
	Mix	42.7	10.6	3.3

^v Disease suppressiveness bioassays were executed with soils from different locations and crops. AB = Achterberg, WH = Wageningen Hoog, and BSW = Bornsesteeg Wageningen.

^w Disease scores for the bioassays with *Rhizoctonia solani* on carrots (RS) and *Fusarium oxysporum* f. sp. *lini* on flax (FOL) are averaged AUDPCs, and those for the bioassays with *Gaeumannomyces graminis* var. *tritici* on barley or triticale (GGT) are averaged disease indices (0 = healthy, 1 = small lesions on 1 root, 2 = bigger lesions on 2 or more roots, 3 = 1 lesion reaching the stem base, 4 = 2 or more lesions reaching the stem base, 5 = stem base infected, 6 = infection reaching the stem, 7 = plants yellowing and wilting, and 8 = dead plant).

^x Numbers followed by different letters within the same columns per location differ at $P = 0.05$. No letter indicates not significantly different at $P = 0.05$.

^y Data from locations AB and WH were pooled before analysis.

^z Three field plot samples were pooled. The P value was calculated from four blocks in the bioassay.

siveness from those grown to Brussels sprouts or barley. Disease suppressiveness was not different between the two locations; however, a significant bioassay–soil effect was present ($P = 0.04$). No crop–location effect was present when all bioassays were analyzed together. Analyses of the relative disease scores of all bioassays together from the BSW location (triticale–white clover) indicated no differences between the single and mixed cropped soils with respect to disease suppression.

Effect of mixed cropping on microbial diversity. SW indices of 16S- (bacterial) and 18S-rDNA (fungal) DGGE bands were, in general, similar for most bulk soils tested (Table 4). The bacterial SW indices were higher than fungal SW indices in all bioassays. The number of detectable bands was 38 ± 7 bands per lane for the bacterial gels in the Brussels sprouts–barley system and 34 ± 6 in the triticale–white clover system. Brussels sprouts–barley had 21 ± 6 bands on the fungal gels and triticale–white clover had 27 ± 6 bands. There was no indication that the mixed cropped soils had a greater number of bands or higher diversity indices than the mono-cropped soils for both bacterial and fungal communities when analyzed per bioassay. Overall, there was no effect of crop or location based on the bacterial SW indices for soils used in the FOL1 bioassay. Similarly, in the soils used for the GGT1 or RS1 bioassays, crop or location effects were not significant. The SW indices of soils collected from the BSW location and used in the bioassays FOL2, GGT2, and RS2 were not significantly different for both bacterial and fungal community.

Analyses of relative bacterial SW indices for all bioassays per cropping system together (Brussels sprouts–barley or triticale–white clover) indicated a significant crop effect ($P = 0.04$) in the Brussels sprouts–barley system. Mixed cropped soil had a higher relative SW index than soil cropped to Brussels sprouts ($P = 0.02$). Differences between barley and Brussels sprouts and the mixed cropped soil were not significant. Also, the comparison between locations AB and WH showed no differences. Relative

SW indices of fungal communities were not significantly different between crops and between soil types in the Brussels sprouts–barley system. Overall, analyses of relative SW indices showed no crop effect in the triticale–white clover system. A bioassay–crop interaction was near significant ($P = 0.06$), indicating that time of sampling might have an effect on the soil microbial diversity.

In all bioassays, soils from locations AB and WH could be separated by PCA based on band number and intensity of bacterial DGGE profiles (data only shown for the GGT1 bioassay, Fig. 1A). After PCA of the fungal community, locations AB and WH could also be separated, except for those used in the GGT1 bioassay, where the soils grown to Brussels sprouts clustered together more than soil type (Fig. 1B). Barley probably induced the observed effect, because the mixed cropped bulk soil was more similar to the mono-cropped barley soil than to the mono-cropped Brussels sprouts soil (Fig. 1B). This separation only occurred in the GGT1 bioassay and the moment of sampling might be the reason for this. In general, closest neighboring field

TABLE 4. Shannon-Weaver indices of 16S-rDNA denaturing gradient gel electrophoresis profiles of soils used to test disease suppression^u

Location ^v	Crop	Bioassay ^w		
		RS	FOL	GGT
AB	Brussels sprouts	1.48 ^x	1.20 a	1.27
	Barley	1.47	1.33 b	1.28
	Mix	1.55	1.26 ab	1.35
WH	Brussels sprouts	1.48	1.30	1.32
	Barley	1.44	1.34	1.43
	Mix	1.52	1.29	1.43
AB and WH ^y	Brussels sprouts	1.45	1.25	1.29
	Barley	1.48	1.33	1.36
	Mix	1.54	1.28	1.39
BSW	Triticale	1.38 ^z	1.45	1.52
	White clover	1.41	1.47	1.41
	Mix	1.09	1.46	1.45

^u The soils originated from fields grown to Brussels sprouts, barley, and their mix, or triticale, white clover, and their mix.

^v AB = Achterberg, WH = Wageningen Hoog, and BSW = Bornsesteeg Wageningen.

^w Shannon-Weaver indices belonging to the bulk soil samples from disease suppressiveness bioassays with soils from different locations and crops. Bioassays were executed with *Rhizoctonia solani* on carrots (RS), *Fusarium oxysporum* f. sp. *lini* on flax (FOL), and *Gaeumannomyces graminis* var. *tritici* on barley or triticale (GGT). Indices were calculated as $H' = -\sum p_i (\log p_i)$, where $p_i = n_i/N$ and n_i = the area of a peak in intensity and N = sum of all peaks in a lane profile (11).

^x Shannon-Weaver indices with the same letter within columns per soil type are not significantly different at $P = 0.05$. No letter means not significantly different at $P = 0.05$.

^y Data from the locations AB and WH were pooled before analysis.

^z Pooled samples, only one value.

Fig. 1. Principal component analysis on similarity between lanes of A, 16S-rDNA or B, 18S-rDNA denaturing gradient gel electrophoresis profiles from bulk soil samples used in the GGT1 bioassay. Prin 1 represents 20.6% (A) and 16.0% (B) of the variation, and Prin 2, 17.5% (A) and 16.1% (B). ba = soil grown to barley (triangle), Bs = soil grown to Brussels sprouts (squares), m = soil grown to the mix crop (circles), Ab = location Achterberg, and Wh = location Wageningen Hoog.

plots clustered together more than the crop treatments designated to those plots.

Effect of mixed cropping on microbial activity. There were no significant differences for microbial respiration measured in soils used for the bioassays FOL2 and GGT2 between single and mixed cropped soils (data not shown). No significant correlation was found between microbial respiration of the sampled soils and final disease severity.

Fig. 2. Percentage disease plotted against the percentage Shannon-Weaver index **A**, 16S-rDNA and **B**, 18S-rDNA from the GGT1 bioassay. Percentages were calculated for both Shannon-Weaver indices and disease scores as fractions of the highest value within one location $\times 100$. Corresponding R^2 values are 0.31 ($P = 0.06$) (A) and 0.42 ($P = 0.02$) (B).

Relationships between disease suppressiveness, microbial diversity, and microbial activity. In the GGT1 (Fig. 2) and RS1 bioassays, relative disease score and relative bacterial (16S-rDNA) SW indices were negatively correlated (Table 5). Slopes were negative but not significant in the bioassays FOL1, FOL2, and GGT2. The fungal (18S-rDNA) SW indices were positively correlated with disease scores in the FOL1 and in the GGT1 bioassays (Table 5), whereas they were negatively correlated with disease score in the GGT2 bioassay. Microbial respiration was not significantly correlated with the disease scores or SW indices in both bioassays.

When relative disease scores and relative SW scores from the crops were pooled, disease scores were correlated to SW indices in the Brussels sprouts–barley experiments. A negative correlation between 16S-rDNA diversity and disease for location AB (slope = -1.72 , $P = 0.0032$) was found; however, for the WH location, there was no significant correlation. A positive correlation was detected between 18S-rDNA SW indices and disease (slope = 1.48 , $P = 0.0135$) for location WH but the relation was not significant for the AB location.

Overall regression analysis for the Brussels sprouts–barley system indicated a negative correlation between 16S-rDNA SW indices and disease scores (slope = -0.96 , $P = 0.005$) and a positive correlation between 18S-rDNA SW indices and disease scores (slope = 0.68 , $P = 0.007$). In the triticale–white clover bioassays no correlations were significant.

Effect of mixed cropping on available nitrogen. Available nitrogen content of the soils used in the bioassays was generally low. Nitrate concentration ranged from 7.7 to 41.7 mg of NO_3 kg^{-1} of soil, and ammonium concentration ranged from 0.5 to 3.48 mg of NH_4 kg^{-1} of soil. No differences were observed among ammonium concentrations in soils from the different locations grown to various crops. Nitrate content of soils sampled at location AB for the FOL1 bioassay was significantly higher in soils cropped to Brussels sprouts than in soils with barley alone or with the mixed crop ($P = 0.0014$ and 0.0032 , respectively). Nitrate concentration in soils collected for the other bioassays or from the WH location did not differ among the crops grown in the Brussels sprouts–barley systems. Also, soils from the triticale–white clover system did not differ in available nitrogen concentrations among the three crops. No significant correlations were observed between nitrate or ammonium contents and relative disease scores (as fractions of the highest values per bioassay per soil type) in either cropping system (data not shown).

DISCUSSION

In this study, no significant effects of mixed cropping on general disease suppressiveness of three different soils to three different plant pathogens were found. Except in one bioassay (FOL1), an adverse effect of mixed cropping on disease suppression to *F. oxysporum* was found. For Fusarium wilt, soils cropped with

TABLE 5. Simple linear regression analysis of relative disease severity (as a fraction of the highest value per test^a) and relative 16S-rDNA or 18S-rDNA diversity indices per bioassay

Bioassay ^b	16S-rDNA				18S-rDNA			
	Intercept	Slope	R^2	$P < r$ (slope)	Intercept	Slope	R^2	$P < r$ (slope)
FOL1	1.65	-0.30	0.04	0.53	0.37	0.66	0.31	0.06
FOL2	1.62	-0.41	0.04	0.63	-0.50	0.42	0.06	0.54
GGT1	3.06	-1.44	0.31	0.06	-0.58	1.31	0.42	0.02
GGT2	2.44	-0.96	0.22	0.21	3.86	-1.89	0.70	0.01
RS1	3.44	-1.60	0.54	0.01	0.86	0.27	0.04	0.54
RS2 ^c	0.47	0.36	0.73	0.35	1.45	-0.34	0.09	0.81

^a Fractions were square root arcsine-transformed prior to statistical analysis.

^b Bioassays with soils from different locations and crops were performed with *Rhizoctonia solani* on carrots (RS), *Fusarium oxysporum* f. sp. *lini* on flax (FOL), and *Gaeumannomyces graminis* var. *tritici* on barley or triticale (GGT).

^c Three data points only.

barley had the highest level of disease suppressiveness compared with soils cropped with Brussels sprouts and the mixed crop. This result was supported by the results from the GGT1 bioassay, in which a weak but similar trend for suppressiveness against take-all disease of barley was noted. Relative disease scores were significantly different among crops when bioassays were analyzed together. Again, soils collected from barley plots had the lowest relative disease severity compared with soils from plots grown to Brussels sprouts or the mixed crop. In the triticale–white clover system, differences among disease scores in any of the bioassays were too small to be significant. Collectively, these results indicate that mixed cropping does not increase the general disease suppressiveness of soils, at least not for the cropping systems and soilborne pathogens tested in this study. In our experiments, we observed a trend toward higher levels of disease suppression in soils grown to barley, but not to triticale. This was true for bioassays with both dicotyledonous and monocotyledonous test plants. This suggests that the lower disease severity in soils grown first to a barley crop was not simply a rotation effect.

Three pathogens were tested in our bioassays because the mechanisms of attack and suppression differ for these fungi, so that results could be generalized with respect to disease suppression induced by mixed crops. *R. solani* is primarily affected by general microbial antagonism (40). Suppression of *F. oxysporum* has mainly been attributed to the activity of nonpathogenic *F. oxysporum* species and *Pseudomonas* spp., which act primarily by competition and induced systemic resistance (47). *G. graminis* var. *tritici* is sensitive to general and specific microbial activity in the soil and its full pathogenic potential is not reached in natural soils because of competition and antibiosis (14,47). In our experiments, no relationship was found between soil microbial activity and disease severity of any of the tested pathogens including *G. graminis* var. *tritici*. Besides general suppression, specific disease suppression of take-all by *P. fluorescens* has been described many times (47). From soils used in both systems, *P. fluorescens* colonies were enumerated, but no relation was found between colony numbers and take-all severity (data not shown). Qualitative differences and more specifically the effects of mixed cropping on specific antagonists, including antibiotic-producing *Pseudomonas* spp., are currently under investigation. Pathogen behavior can also be affected directly or indirectly by the nitrogen form and availability. This has been described for *R. solani* (42) and *G. graminis* var. *tritici* (23,34,39). However, in our study no relationship was found between available nitrogen content in soil and disease severity.

Because plants have a profound effect on soil microbial communities and their processes (24,46), increased root diversity and density by mixed cropping were thought to result in increased microbial activity and diversity. However, SW diversity indices based on 16S- and 18S-rDNA were hardly affected by the crops that had been grown. Because crop-induced microbial shifts are mainly present in the rhizosphere (15,26,46,51), the effects of sampling and mixing bulk and rhizosphere soil probably diluted or masked these shifts, so that specific rhizosphere effects were not discernable and influences on general disease suppression were relatively small or not detectable. However, disease suppressiveness of soils has been demonstrated in bioassays using field-collected bulk soil, even if the site of suppression appeared to be in the rhizosphere as in the case of suppression of *G. graminis* var. *tritici* by *P. fluorescens* (32). Furthermore, differences in disease scores may have been the result of small population changes not detectable by PCR-DGGE profiles (7,12,30) generated by the general primers we used.

Similarity coefficients of DGGE-gels of bacterial communities from soils grown to Brussels sprouts were not different from those grown to barley or the mixed crop. Nevertheless, barley or the mix with barley resulted in slightly higher SW indices than Brussels sprouts. This may have been due to the release of iso-

thiocyanates (ITCs) by this crop (6). Brussels sprouts are known to produce ITCs that are released during decomposition of residues and are known to exhibit fumigating effects (5,10,37) affecting microbial communities including soilborne plant pathogens (1,35). Consequently, they may also affect beneficial organisms, which may adversely affect disease suppressiveness of the soil. In two of our bioassays, the relative SW index based on 16S-rDNA was negatively correlated with relative disease severity, and in three bioassays, this correlation was insignificant. This indicates that disease suppression is to some extent related to bacterial diversity. Similarly, a negative correlation between actinomycete diversity and disease severity was found for corky root of tomato (49). No consistent relationship was found between fungal diversity based on 18S-rDNA and disease severity in our bioassays.

In several studies, total microbial activity affected disease suppression (19) significantly more than the effects of individual species or select microbial groups (3). However, in our study there were no correlations between respiration measurements, diversity indices, and disease scores, and there were no significant differences among the soils grown to different crops. Similarly, Wardle et al. (46) showed that removal of a plant species did not result in a decrease in microbial respiration except when all plants were removed. These findings support our observations that crop mixtures do not have a substantial influence on bulk soil microbial communities and their ecological processes.

We conclude that mixed cropping does not increase the disease suppressiveness of the soil or the diversity of the soil microbial community. Effects of growing crops on a soil remain apparently limited to the rhizospheres. Cross-over effects of mixed crops, due to root–root interactions, might be small but have not yet been thoroughly investigated. It should be emphasized that our study did not consider direct effects of mixed cropping on disease incidence in the respective crops in the mix, but focused primarily on the effects of mixed cropping on general suppressiveness of bulk soils. It would therefore be too simple to state that mixed cropping has no advantages in suppression of soilborne diseases compared with single crops. On the contrary, mixed cropping has shown promising results by reducing disease severity of soilborne pathogens within the field where the crops are grown (1,2,8,45,50). Besides reduction in disease severity, other factors may be important for deciding to grow crop mixtures, for example, reduced damage by airborne pathogens (29), weed suppression, pest suppression (12,43), and possibly increased productivity.

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