Winter rye cover cropping changes squash (*Cucurbita pepo*) phyllosphere microbiota and reduces *Pseudomonas syringae* symptoms

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Abstract

Cover cropping is a soil conservation practice that may reduce the impacts of the economically important pathogen *Pseudomonas syringae* on crops including squash (*Cucurbita pepo*). To date, no studies have directly quantified the effect of rye cover crops on *P. syringae* populations, nor on the bacterial community of squash leaves. In this work, we tested the hypothesis that the protective effects of cover cropping on squash may be mediated by cover cropping effects on the plant’s microbiota that in turn protects against *P. syringae*. Using combined 16S sequencing and culture-based approaches, we showed that rye cover cropping protects squash against *P. syringae*, by decreasing pathogen population size on squash leaves and increasing fruit health and marketability at harvest. We also found evidence of a strong effect of rye cover crops on
bacterial communities of the squash phyllosphere. Those findings were more striking early in the growing season. Finally, we identified numerous phyllosphere bacteria belonging to the genera *Sphingomonas*, *Methylobacterium* and *Pseudomonas* that were promoted by rye cover crops. Overall, our findings suggest cover cropping is effective for the sustainable management of *P. syringae* on squash and may provide a reservoir of potential microbial biocontrol agents colonizing the phyllosphere.

**Introduction**

Cover cropping, or the growth of a plant to cover the soil for environmental benefits rather than for its harvest, is an increasingly popular option available to farmers to address the environmental and human health challenges associated with agricultural intensification (Fawell and Nieuwenhuijzen, 2003). Cover cropping allows equivalent yield (LU et al., 2000) or an increase in yield (Fawcett et al., 2015, 2017; Stirzaker and White, 1995), weed control (Teasdale, 1996), nematode control (Hooks et al., 2010), and reduces soil erosion (Dabney et al., 2001; Stirzaker and White, 1995). Winter cover crops used in northern countries are a promising avenue to reduce soil erosion and depletion by covering the soil during the winter (Dabney et al., 2001). Cover crops can also improve water quality by reducing herbicide runoffs (Hall et al., 1984), and improve soil condition by reducing temperature variations and water loss (Teasdale and Mohler, 1993). Many studies have demonstrated the benefits of cover cropping, although there is controversy about potential negative effects of cover cropping such as the potential transfer of pathogens from cover crop to crop (Bakker et al., 2016). Cover cropping is known to
shape the soil microbiome (Hartman et al., 2018), but to date no study has quantified cover crop
effects on aboveground microbial communities.

The phyllosphere microbiome, the microbial communities of aboveground plant parts,
particularly leaves, are composed of a broad range of microorganisms such as bacteria, viruses,
fungi and archaea (Lindow and Brandl, 2003). The microbiota on the aboveground parts of plants
can improve plant fitness and biomass, primarily by reducing pathogen symptoms thanks to
direct competition or associated with plant volatile compound (Abanda-Nkpwatt et al., 2006;
Ritpitakphong et al., 2016). Microorganisms are also important pathogens of the phyllosphere:
20–30% of crop production losses worldwide are due to various pests and pathogens (Savary et
al., 2019), and microbial pathogens are estimated to account for 16% of potential losses (Oerke,
2006). Pseudomonas syringae, one of the most widely studied bacterial plant pathogens, can
infect a wide range of host plants including many economically important crops; it begins life as
a leaf epiphyte, colonizes the host apoplast through wounds and stomates, and then damage
fruits (reviewed in (Hirano and Upper, 2000; Xin et al., 2018). Long-term intensive and frequent
monocropping favours emergence of local pathogenic P. syringae reservoirs (Lindemann et al.,
1984). Efforts have been made to biologically control this pathogen on leaves using microbial
competition; Lindemann and Suslow (1987) used a competition population of P. syringae
disarmed with an ice nucleation mutation to prevent pathogen-related frost damage on
strawberry plants and Innerebner et al. (2011) used several Sphingomonas species on leaf
surfaces to protect Arabidopsis thaliana plants from P. syringae. Moreover, many studies of plant
induced systemic resistance (ISR) have noted potential leaf control of P. syringae based on the
microbe-associated molecular patterns (MAMPs) mechanism and, interestingly, via microbial
competitors located in the root-associated microbiome (reviewed in Pieterse et al., 2014; Van Wees et al., 2008). Finally, prior colonization of the plant phyllosphere by the beneficial bacterium is a well-known strategy to pre-emptively prevent the growth of the pathogen (reviewed in (Andrews, 1992).

Despite the potential for microbiota-based biological control of *P. syringae*, management of this pathogen is primarily done through copper application, which has led to the development of resistance (Bender and Cooksey, 1986). Past studies have suggested that cover crops may provide soil borne biological control of pathogens (Abawi and Widmer, 2000; Collins et al., 2006), and we have found that rye cover crops helped to reduce *P. syringae* symptoms incidence on squash leaves (Toussaint et al., personal communication). The mechanism of this protective effect of rye cover crops against squash bacterial leaf spot is not known, but we hypothesize that it may be mediated by cover cropping effects on the plant’s microbiota that in turn protects against *P. syringae*.

In this study we used sequence and culture-based approaches to quantify the effects of different cover cropping approaches on bacterial communities on squash leaves infected by *P. syringae*. We first evaluated if cover crops could help to reduce *P. syringae* populations on squash leaf surfaces by direct measurement of pathogen abundance on leaves. We also considered the fruit’s health and marketability at harvest in such cropping practices. We then estimated the effects of different cover cropping methods on phyllosphere bacterial communities by quantifying leaf microbiome diversity and composition using a bacterial metabarcoding approach. Finally, we identified the bacterial taxa that were most strongly influenced by cover cropping practices.
**Material & Methods**

**Experimental design and field treatment**

All samples in this study were collected from the Agriculture and Agri-Food Canada L’Acadie Experimental Farm at Saint-Jean-sur-Richelieu, Quebec, Canada (45°17'48.7"N 73°20'14.8"W). The experiment comprised 6 replicates of 4 cover cropping treatments in a fully randomized block design, with a total of 24 plots containing 3 raised beds with a single line of squash each (see Supplemental Figure 1). The cover cropping treatments were Rye (*Secale cereale*) Cover Crop (RCC), Chemically Terminated Rye Cover Crop (CT-RCC), Plastic Cover (PC) and Bare Soil (BS). Specifically, for 2016 and 2017 growing season, the RCC treatment consisted of fall rye (cv Gauthier) seeded at a rate of 250kg/ha on September 14 2015 and September 16 2016 and rolled to the ground the following spring by crimping rye with a 3-sectional roller crimper (I&J Mfg., PA, USA) on June 13 2016 and 2017 (see Supplemental Figure 2 for crimping process); the CT-RCC treatment was the same as RCC except rye was killed with a herbicide (glyphosate (Roundup, WeatherMaxMD, Bayer, Canada) at a rate of 2.16 kg a.e. ha\(^{-1}\)) before the rye crimping (on June 10 2016 and June 8 2017); the PC treatment consisted of the application of an agricultural plastic mulch over each raised bed within plots; and the BS treatment was a bare soil mound lane that did not receive any cover cropping treatment. All the soil was covered by rye and most of the cover crop material remained on the soil during the growing season and, except for a change in color, no significant decay was observed (see Supplemental Figure 2). Squash seeding was performed on June 15 2016 and 2017; mechanically with a seeder for the bare soil and both rye treatments, or manually for the plastic cover (see Supplemental Figure 2). To monitor treatment effects on pathogen populations, we inoculated squash seeds with a rifampicin-resistant *P.*
syringae strain prior to direct seeding into raised beds. While plants are frequently naturally
inoculated by seedborne or soil pathogen reservoirs, we needed to ensure potential infection to
be able to quantify the effects of the cover cropping treatments. Strain pathogenicity was
confirmed by hypersensitive reaction (HR) testing in tobacco leaves and seed inoculation was
validated by growing on-field negative control plants at the border of each treatment plot.
Control plants were asymptomatic during the entire growing season.

Microbial collection, DNA extraction and sequencing

Microbial communities of the phyllosphere were collected from squash at three different times
each growing season in 2016 (July 12, August 1, and September 1), and 2017 (June 12, July 31,
and September 5), defined as Early, Mid, and Late season. Each sample consisted of a mix of
young and old leaves harvested from the squash canopy by clipping an average of 16.8±8.3 g and
20.7±4.8 g of leaves for years 2016 and 2017 respectively (see Supplemental Table 1) from an
individual plant into sterile sample bags (SCR-7012-ID, Innovation Diagnostics Inc., Blainville,
Canada) with surface-sterilized shears. Replicate samples (3 per plot) were collected for a total
of 72 samples per sampling date (3 samples x 4 treatments x 6 replicates). Microbial cells were
then gathered by washing each leaf sample using with 110 ml of saline buffer [0.85% NaCl] and
using a homogeniser blender (Stomacher® 400, Seward, UK) for 30 sec at 250 rpm.

A volume of 1ml of wash solution from each sample was placed on King’s B (KB) medium with
cycloheximide (50mg/l) (C7698, Sigma Aldrich, Oakville, CA) and rifampicin (50mg/l) (R3501,
Sigma-Aldrich, Oakville, CA), allowing us to estimate P. syringae population size by counting
colony forming units (CFU) after 4 days of growth at 28°C.
The remaining 100 ml wash solution was divided into two 50 ml Falcon tubes; one was centrifuged at 11,500 × g for 20 min and the other at 4,500 × g for 20 min. The aqueous phase was removed from both tubes and the pellet in the Falcon tube centrifuged at 4,500 × g was frozen at -80°C. The DNA of the remaining pellet was extracted using MoBio PowerSoil DNA extraction kits (CA-11011-418, VWR, Mont-Royal, CA) and stored at -20°C for future processing. Amplicon libraries were prepared for Illumina sequencing using PCR targeting the V5–V6 region of the bacterial 16S rRNA gene using cyanobacteria-excluding primers [16S primers 799F-1115R] (Chelius and Triplett, 2001; Redford et al., 2010) to exclude chloroplast DNA (for a 16S amplicon structure overview, see Supplemental Figure 3). The 25 μL PCR reactions consisted of 5 μL 5x HF buffer (Thermo Scientific, Waltham, MA, USA), 0.75 μL DMSO, 0.5 μL dNTPs (10 mM each), 0.25 μL Phusion Hot Start II polymerase (Thermo Scientific), 1 μL each primer (5 μM), 1 μL of genomic DNA, and 15.5 μL molecular-grade water (IDT, Coralville, IA, USA). We included a negative control (1 μl of sterile water; IDT, Coralville, IA, USA) as well as a positive control (1 μl of P. syringae DNA) in each 96 well library plate. Libraries were checked on Agarose gels (2%), normalized with SequaPrep kit (A1051001, Life Technologies, Burlington, CA) on a Gilson robot (Middleton WI, USA) and sequenced on a MiSeq (Illumina, San Diego, CA, USA). For each year, samples were randomly assigned between two sequencing runs, representing a total of 4 runs.

Sequence analysis

Sequencing adaptors were removed with the bbduk tool from bbmap (38.86, https://sourceforge.net/projects/bbmap/), with the following parameters: ktrim=r k=23 mink=11 hdist=1 tpe tbo (Bushnell, 2014). Sequences were thereafter demultiplexed allowing one mismatch on the barcode sequence with deMulMe (https://github.com/RemiMaglione/).
Sequence barcodes were removed with cutadapt 2.10 in paired-end mode (Martin, 2011). In total we obtained 11,929,677 and 11,816,138 demultiplexed paired-end sequences, for 2016 and 2017 respectively. All subsequent data processing and computations were done with DADA2 1.12.1 (Callahan et al., 2016) in R 3.6.0 (RC Team, 2013) and graphs were produced using the ggplot2 3.2.1 package (Wickham, 2016). Sequences were trimmed and quality filtered with `filterAndTrim` with default parameters except: trimLeft = c(19, 26), truncLen = c(230, 210), maxEE=c(2,3); `trimleft` was set to remove PCR primers used for library preparation, `truncLen` and `maxEE` were set to yield filtered sequences with a quality around a phred score of 30. Amplicon sequence variants (ASVs) were constructed from filtered sequences with the following set of built-in DADA2 functions and their default parameters except as mentioned: `dada` in pseudo-pooling mode, `mergePairs` with minOverlap = 30, `collapseNoMismatch` with minOverlap = 240, `removeBimeraDenovo` with method="pooled". ASVs were then taxonomically annotated by `assignTaxonomy` with the SILVA version 128 database (Quast et al., 2013; Yilmaz et al., 2014). For 2016 and 2017 data, the DADA2 pipeline yielded a mean of 10,484,056 filtered paired-end sequences used to identify 7,604 ASVs, which represent an average of 29,652 sequences and 165 ASVs per sample.

A preliminary evaluation of control samples was performed by comparing the composition of control and phyllosphere samples using a principal component analysis ordination of a distance matrix obtained with centred log ratio (clr) transformation of the original community matrix (Gloor, 2016). Since negative control samples would be lost by excluding samples with very few sequences, the clr transformation allowed us to keep all the samples while identifying outlier samples. Since the positive and negative control samples were distinct compositionally from the
Phyllosphere samples (see Supplemental Figure 4), they were removed from all further analyses. Positive control samples were examined and determined to be dominated by ASVs corresponding to the expected mock community species composition.

Data analysis

*P. syringae* abundance analysis on squash leaves

*P. syringae* count differences between treatments were evaluated with a linear mixed model of treatment effects on *P. syringae* abundance, for 2016 and 2017 data. Effects of experimental blocks were integrated as a random effect. Effect of treatment was estimated with a TukeyHSD post-hoc test performed on the above-mentioned model.

Squash fruit health and marketability at harvest

We quantified squash fruit health and marketability by harvesting fruit within a 10m x 10m area within each plot. Fruit health and marketability was determined with 4 categories of *P. syringae* symptoms based on the visually estimated proportion of fruit affected by the considered symptoms: *P. syringae* symptoms outside of the fruits, *P. syringae* symptoms that penetrate the fruits, *P. syringae* symptoms that left a scar at the surface of the fruits and *P. syringae* symptoms that generate squash rot. Marketability was assessed based on these categories of *P. syringae* symptoms, where more than 1% of fruit affected in at least one category prevents marketability. This cut-off was chosen to address the actual market plasticity, where squash fruits with low symptoms can still be sold. Healthy fruit was defined as a squash fruit with no *P. syringae* symptoms. Thus, marketability and fruit health were binomially distributed and their differences among treatments were evaluated with generalized mixed linear model, where blocking effect...
was integrated as random variable, for both years. Effects of treatment on fruit health and marketability was estimated with a TukeyHSD post-hoc test (using the *glht* function of *multcomp* R package) performed on the above-mentioned model.

Effect of cover cropping treatments on bacterial community diversity

Diversity analyses were performed using the R package phyloseq 1.30.0 (McMurdie and Holmes, 2013), picante 1.8.1 (Kembel et al., 2010), and vegan 2.5-6 (Oksanen et al., 2007). To evaluate the effect of treatments on community diversity on squash leaves, samples were randomly rarefied to 5000 sequences per sample: this threshold was chosen to preserve the maximum number of samples with a sufficient quantity of ASVs to capture the majority of the diversity in each sample (see Supplemental Figures 5 and 6). For all diversity analyses, rarefactions and their subsequent analyses were repeated 1000 times but no qualitative differences were observed between iterations, and so we report here the results of a single random rarefaction of the data.

The uniformity of relative abundance distributions of ASVs (alpha diversity) was assessed with the Shannon index (Haegeman et al., 2013). The effect of treatment on alpha diversity was evaluated with a post-hoc test (TukeyHSD) of a linear model (alpha diversity as a function of treatment). Variation in bacterial community structure among samples was quantified with the Bray-Curtis index (Bray and Curtis, 1957). Major gradients in community composition were evaluated with nonmetric multidimensional scaling (NMDS) ordination of weighted Bray-Curtis distances among samples. We partitioned the variance in phyllosphere bacterial community structure explained by sampling date and treatment using permutational ANOVA analysis of the variance in Bray-Curtis dissimilarities, and linear mixed models of sample scores on the NMDS ordination axes. We evaluated whether treatments were associated with compositionally distinct
groups of samples with a least squares comparison (emmeans v1.4.8 R package; Lenth et al., 2018) of a linear mixed model with NMDS axis scores as a function of treatment (fixed effect) with experimental block as random effect.

**Differential abundance analysis of ASVs**

Differential abundance analysis of ASVs among treatments were performed with DeSeq2 3.11 (Love et al., 2014). The ASV matrix was filtered using the CoDaSeq R package 0.99.4 (Gloor, 2016), with the `codaSeq.filter` function with the following parameters: min.reads=1000 (minimum reads per sample), min.prop=0.00001 (minimum proportional abundance of a read in any sample), min.occurrence=0.005 (minimum fraction of non-zero reads for each variable in all samples). Since DeSeq2 takes non-zero positive integers as input, we transformed the abundance matrix to pseudocounts by adding 1 to each cell in the matrix prior to analysis (Nearing et al., 2021). DeSeq2 analysis was executed with parameters recommended for single-cell analysis that better fit data with a zero-inflated negative binomial distribution such as our community matrix. We tested for differential abundance by contrasting ASV abundances across all six possible treatment comparisons: Rye Cover Crop versus Chemically Terminated-Rye Cover Crop, Rye Cover Crop versus Plastic Cover, Rye Cover Crop versus Bare Soil, Chemically Terminated-Rye Cover Crop versus Plastic Cover, Chemically Terminated-Rye Cover Crop versus Bare Soil and Plastic Cover versus Bare Soil. We used the following model: `design = ~ block + treatment` and the blocking random variable was controlled through the `reduced` parameter. Only contrasts with *adjusted P*-value*≤*0.01 and *log*$_2$-*fold-change* ≥ 1 were considered to be significantly differentially abundant. Taxonomic annotations were set at the genus level for the differential abundance analysis of
Results

Cover cropping reduced *P. syringae* abundance on squash leaves and improved fruit health and marketability.

We found that *P. syringae* was less abundant on the leaves of squash grown with rye cover crops (Figure 1) and harvested squash fruits were more marketable and healthier with rye cover crop treatments (Table 1). In 2016, *P. syringae* population size was significantly lower for the Rye Cover Crop treatment compared to Plastic Cover and Bare Soil treatments during the *Early* season (Tukey HSD post-hoc on linear model; Figure 1). There were no significant differences among treatments during *Mid* and *Late* season sampling in 2016. On the other hand, in 2017, *P. syringae* population size was significantly lower during the *Early* season for both rye cover crop treatments (Rye Cover Crop and Chemically-Terminated Rye Cover Crop) compared to Plastic Cover and Bare Soil treatments (Tukey HSD post-hoc test on linear mixed model; Figure 1). No *P. syringae* colonies were retrieved at *Mid* season on the squash leaves grown with rye cover crops, and pathogen populations were lower for Chemically-Terminated Rye Cover Crop as compared to Plastic Cover and Bare Soil treatments. Finally, pathogen viable cells counts were significantly lower in Rye Cover Crop as compared with Bare Soil at *Late* season sampling. Taken together, *P. syringae* populations showed the greatest reduction in the rye cover crop treatments early in the growing season. This result was consistent during both years (see Supplemental Tables 2 and 3).

Moreover, fruits health and marketability were significantly different across all years of harvest (p<0.05, Tukey HSD). Indeed, marketability with Rye Cover Crop was significantly different from
Bare soil and Plastic Cover. Average marketability with Rye Cover Crops was 13% higher in 2016 and 6.2% higher in 2017 as compared to Bare Soil, and 8% higher in 2016 and 4.3% higher in 2017 as compared to Plastic Cover (Table 1). Fruit health increased 14% in 2016 with Rye Cover Crops treatment as compared to Bare Soil and 13% in 2017 with Chemically-Terminated Rye Cover Crop treatment as compared to Bare Soil (Table 1). No further significant differences among treatments were observed for fruit health and marketability in both years of harvest.

Phyllosphere microbial communities differed between sampling dates and treatments. Cover cropping treatments influenced bacterial community composition on squash leaves. Treatments also affected bacterial diversity and richness (see Supplemental Analysis 1 with Supplemental Figures 7 & 8). A nonmetric multidimensional scaling ordination of the overall community distance matrix suggests that squash phyllosphere samples clustered by sampling dates in both years (see Supplemental Figure 9); sampling date accounted for 28% ($R^2 = 0.28$, $p<0.001$) and 11% ($R^2 = 0.11$, $p<0.001$) of community compositional variation between samples for 2016 and 2017 respectively (PERMANOVA on Bray-Curtis distances among samples). Because there was an interaction between sampling date and treatments (PERMANOVA on Bray-Curtis distances among samples; sampling date * cover crop treatment interaction $P<0.001$ both years), and sampling date accounted for the majority of the effect, we thus analyzed the effect of treatments on communities separately for each date in order to summarize these complex effects.
Cover cropping treatments influence squash phyllosphere community diversity and composition

Bacterial community alpha diversity was significantly different among treatments for several sampling dates (linear model; Shannon index vs. cover cropping treatment, P<0.05; Figure 2). Bacterial community alpha diversity was higher for both rye treatments as compared to bare soil and plastic treatments in 2016 in the *Early* season sampling (Tukey HSD on linear model, P<0.05; Figure 2). No further differences were observed between treatments at the other sampling dates in 2016. In 2017, although both rye cover cropping practices resulted in significantly lower alpha diversity in *Mid* season sampling as compared to Bare Soil, their alpha diversity was higher as compared to plastic treatments in *Early* season sampling. No further differences were observed between treatments in late season sampling of 2017. Taken together alpha diversity increased early in the growing season for both rye cover cropping treatments as compared to bare soil and plastic treatments of 2016 or to the plastic treatment of 2017.

Community composition varied among cover cropping treatments for each sampling date of 2016 and 2017 (PERMANOVA on Bray-Curtis distances for each sampling date; cover cropping effect P<0.001). Moreover, distances between treatment clusters visible in the ordination (Figure 3) suggests that treatment effects were more important for *Early* season sampling as compared to the other sampling dates. Differences in community composition among treatments were more pronounced in *Early* (PERMANOVA on Bray-Curtis distances; effect of cover cropping treatment $P_{2016 \& 2017}<0.001$; $R^2_{2016} = 0.24$, $R^2_{2017} = 0.31$) rather than *Mid* ($P_{2016 \& 2017}<0.001$; $R^2_{2016} = 0.14$, $R^2_{2017} = 0.30$) and *Late* season sampling ($P_{2016 \& 2017}<0.001$; $R^2_{2016} = 0.07$, $R^2_{2017} = 0.16$) (Figure 3).
Ordination of samples based on community composition also indicated that samples clustered into two compositionally distinct groups: Rye and Chemically-Terminated and Rye Cover Cropping, versus Plastic and Bare Soil (Figure 3). At each sampling date, sample scores on the first axis of the ordination differed significantly among treatments (linear mixed model with NMDS axis scores as a function of treatment (fixed effect) with experimental block as random effect). Rye and Chemically-Terminated Rye were different from the Plastic and Bare Soil, but not different from each other (least squares comparisons on the above linear mixed model; Supplemental Figure 10). Moreover, these two compositionally distinct groups (Rye and Chemically-Terminated Rye versus Plastic and Bare Soil) are more different during the Early season as compared to every other sampling date: these community compositional differences remained throughout the growing season, although they progressively decreased in magnitude (least squares comparisons intervals between these two groups are closer throughout the growing season on the first axis; Supplemental Figure 10). However, plastic and bare soil remained different in Mid season in 2017 and were often separated along the second axis of the NMDS ordination (Figure 3; estimated marginal means test of ordination axis scores; Supplemental Figure 10).

*Sphingomonas* and *Methylobacterium* were more abundant with cover crops treatments.

An analysis of differential abundance of ASVs among treatments and sampling dates identified several ASVs that were more abundant in certain treatments and at certain times. As mentioned previously, the abundance of *P. syringae* and the squash phyllosphere communities were influenced by the sampling date, thus the cover crop effect on taxa abundance was analyzed
separately for each sampling date. To identify ASVs that were strongly associated with different cover cropping systems we took the top differentially abundant ASVs with the highest log$_2$-fold change in abundance for each treatment comparison (2017: Figure 4, 2016: Supplemental Figure 11). Different cover cropping treatments had several differentially abundant ASVs with log$_2$-fold changes in abundance between treatments ranging from -10.2 to 9.7. Overall, the contrasts of Rye versus Chemically-Terminated Rye, and Plastic versus Bare Soil consistently had few and weakly differentially abundant ASVs. Conversely, contrasts of both rye treatments versus Plastic and Bare soil exhibited more and strongly differentially abundant ASVs. In 2016, ASVs that were significantly more abundant for Rye and Chemically-Terminated Rye Cover Crop treatments included those annotated at the genus level as *Rhizobium*, *Pseudomonas* and *Saccharibacillus* during the *Early* season, and *Chryseobacterium* and *Sphingomonas* during the *Mid* season. Conversely, ASVs that were significantly more abundant in Bare Soil and Plastic Cover treatments included those annotated as *Pseudarthrobacter* during the *Early* season, *Exiguobacterium* and *Pseudarthrobacter* during the *Mid* season, and *Deinococcus* during the *Late* season. In 2017, ASVs that were significantly more abundant for Rye and Chemically-Terminated Rye Cover Crop treatments included those annotated as the genera *Sphingomonas*, *Methylobacterium* or *Hymenobactrium* during the *Early* season, *Sphingomonas*, *Methylobacterium*, *Aureimonas* and *Microbacterium* during the *Mid* season, and *Chryseobacterium* and *Rhizobacterium* during the *Late* season. On the other hand, ASVs that were significantly more abundant in Bare Soil and Plastic Cover treatments included *Massila* and *Exiguobacterium* at *Early* season, *Massila*, *Exiguobacterium*, *Hymenobacter*, *Deinococcus* and *Pseudarthrobacter* at *Mid* season and *Deinococcus* and *Microbacterium* at *Late* season.
Discussion

Rye cover cropping reduced the abundance of *P. syringae* on squash leaves, improved the health and marketability of fruit, and shaped phyllosphere bacterial community composition and diversity. The greatest effect of cover cropping on both the phyllosphere community and *P. syringae* abundance was observed early in the growing season. *P. syringae* begins life on leaves as an epiphyte but then must colonize host tissue through stomata or wounds (Misas-Villamil et al., 2013). Disease severity could be lowered if the early establishment and survival of *P. syringae* is compromised.

We found that both Rye and Chemically-Terminated Rye Cover Cropping treatments induced a strong shift in the squash phyllosphere microbiota, leading to a distinct community composition in comparison with Bare Soil and Plastic Cover treatments. The largest difference in leaf bacterial community composition associated with rye cover crops was observed early in the growing season. Our results are consistent with previous reports of homogenisation of phyllosphere community structure over time; early in the growing season, the leaf microbiota was more diverse and colonization from the soil is likely a strong driver of the phyllosphere microbiome (Copeland et al., 2015). Moreover, shifts in microbial community composition are likely driven by changes in environmental conditions as well as shifts in sources of bacterial migration to the phyllosphere. Cover cropping can directly modify soil abiotic properties such as temperature and moisture and chemical properties (Villamil et al., 2006). Cover cropping also likely influences bacterial dispersal sources both by promoting colonization by bacteria living on the cover crops themselves, as well as through their effects on dispersal from different potential sources such as soils (Bodenhausen et al., 2013), water splash (Butterworth and McCartney, 1991) and insects.
(Manirajan et al., 2016; Vega and Herrera, 2013). Taken together, we hypothesize that such local environmental shifts modify bacterial migration to the phyllosphere early in the growing season. We further speculate that early shift in squash phyllosphere may intervene by mechanical transfer from the cover crop, when the young plant goes through the rye mulch.

In addition to the effects of cover cropping on phyllosphere microbial communities, cover cropping treatments also likely influenced environmental conditions, which may explain part of their protective effects against *P. syringae*. Cover crops influence humidity and temperature (Teasdale and Mohler, 1993). Moreover, dispersal of epiphytic bacteria is a function of humidity (Lindemann and Upper, 1985), and the structure of phyllosphere bacterial communities is significantly influenced by soil temperature (Ren et al., 2015). We observed that soil moisture and temperature varied among cover cropping treatments; soil moisture was higher under rye cover crops and plastic cover relative to bare soils, and temperatures were elevated under plastic cover relative to other treatments (results not shown). Rye is also known to have allelopathic properties (Schulz et al., 2013), which can influence soil microbiota (Hu et al., 2018). Moreover, rye degradation can lead to decreases in soil pH (Abdollahi and Munkholm, 2014) and improve weed control (Barnes and Putnam, 1983). During this experiment, no weeds were found for the plastic cover treatment, a few grew in both rye cover treatments, while many were found in the bare soil treatment (all weeds were manually removed on a routine basis during the growing season). All of these effects of cover cropping on the abiotic and biotic environment could influence early pathogen development and interact with shifts in phyllosphere microbiota to modulate the potential protective effects of cover crops. Furthermore, since *P. syringae* may likely be seed borne (reviewed in Hirano and Upper, 2000), we inoculated seeds with this
pathogen at the time of planting, but in real situations, the effect of cover crops on pathogen populations will also be a function of temporal variation in seed and soil pathogen and microbiota reservoirs.

Previous studies have reported a protective effect against pathogens by phyllosphere microbial diversity per se (Keesing et al., 2010), for example where increasing diversity of Sphingomonas genus on leaves increased protection against P. syringae (Innerebner et al., 2011). We did not find strong evidence for an effect of alpha diversity on its own to explain the protective effect of cover cropping against P. syringae; there were no overall differences in alpha diversity of phyllosphere bacteria among cover cropping treatments, although rye cover cropping did increase diversity in the early season. To properly test for a protective effect of phyllosphere diversity against P. syringae, future studies that directly manipulate diversity while keeping other factors constant will be required, but our results suggest that it was the composition of bacterial communities and not the diversity of the community per se that could explain the protective effects of cover cropping treatments.

Our results support the hypothesis that rye cover crops could protect against P. syringae by promoting the establishment of potential competitors or plant growth promoting bacteria (PGPB) on the leaf surface. This included several ASVs belonging to the genus Sphingomonas, which were more abundant with rye cover cropping, especially early in the growing season. Previous studies have demonstrated that Sphingomonas strains protect Arabidopsis against P. syringae in a controlled environment (Innerebner et al., 2011). Our findings provide field-based evidence suggesting that the Sphingomonas clade is a potential competitor, given the increased abundance of this genus in the protective rye cover crop treatments. We also found many other
taxa preferentially associated with squash under rye cover cropping treatments, including ASVs belonging to the genus *Methylobacterium* that is a phyllosphere-associated clade (Delmotte et al., 2009) known to be an important PGPB in agriculture (Madhaiyan et al., 2006), and non-pathogenic ASVs belonging to the genus *Pseudomonas*, which has been shown to be an antagonist of the pathogens *Erwinia* (Cabrefiga Olamendi et al., 2007), Tobacco Necrosis Virus (Maurhofer et al., 1998), and *Botrytis cinerea* (De Meyer and Höfte, 1997). Thus, rye cover crops appear to favor the establishment of potentially plant beneficial bacteria in the phyllosphere. While our results need to be followed up with more experimental tests to quantify the potential benefits of these genera, the potentially beneficial bacterial ASVs associated with rye cover crops that we have identified are already candidates for exploration of microbiome engineering approaches to directly inoculate protective bacterial strains to protect crops against pathogens (Quiza et al., 2015).

### Availability of data and materials

The demultiplexed sequence data have been deposited as sequences read archive under the BioProject: [PRJNA705113](https://www.ncbi.nlm.nih.gov/proj/:PRJNA705113). The scripts used to perform analyses for the current study are available in a GitHub repository: [https://github.com/RemiMaglione/Science-Communication/tree/main/Article/cover-crop-squash-phyllosphere-microbiota-2021](https://github.com/RemiMaglione/Science-Communication/tree/main/Article/cover-crop-squash-phyllosphere-microbiota-2021)

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Funding

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Competing interests

The authors declare no competing interests for this work.


Figures and Tables

Table 1: Proportion of squash fruit (mean ± standard deviation) with no *P. syringae* symptoms and marketable fruits with no damage for the two-growing seasons 2016 and 2017. Differences among treatments were tested using Tukey’s honestly significant difference (HSD) test, based on a generalized mixed model with General Linear Hypotheses provided by glht function of multcomp package. Within each year, treatments that do not share a letter were significantly different according to the Tukey HSD test (p<0.05).

<table>
<thead>
<tr>
<th>Year</th>
<th>Plastic Cover</th>
<th>Rye Cover Crop</th>
<th>Chemically-Terminated Rye Cover Crop</th>
<th>Bare Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proportion of squash fruit without <em>P. syringae</em> symptoms (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2016</td>
<td>56.8 ± 6.7 ab</td>
<td>63.2 ± 7.7 a</td>
<td>59.7 ± 10.4 ab\textsuperscript{1}</td>
<td>49.2 ± 9.3 b</td>
</tr>
<tr>
<td>2017</td>
<td>74.0 ± 13.0 ab</td>
<td>73.2 ± 7.8 ab</td>
<td>78.7 ± 12.1 a</td>
<td>65.7 ± 7.7 b</td>
</tr>
<tr>
<td></td>
<td>Proportion of marketable squash fruit with no damages (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2016</td>
<td>79.5 ± 5.4 a</td>
<td>87.5 ± 5.8 b</td>
<td>81.3 ± 3.4 ab</td>
<td>74.5 ± 7.3 a</td>
</tr>
<tr>
<td>2017</td>
<td>91.2 ± 7.2 b</td>
<td>95.5 ± 3.4 a</td>
<td>94.2 ± 3.0 ab\textsuperscript{2}</td>
<td>89.3 ± 3.0 b</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Difference between CT-RCC and Bare soil was marginally significant (p=0.057). \textsuperscript{2}Difference between CT-RCC and Bare soil was marginally significant (p=0.064).
Figure 1: *P. syringae* populations on squash leaves for different cover cropping practices during 2016 and 2017. Squash pathogen population sizes were estimated based on CFU count from bacterial culture of each leaves sample retrieved from 4 cropping treatments: Bare Soil (BS), Plastic Cover (PC), Rye Cover Crop (RCC) and Chemically Terminated Rye Cover Crop (CT-RCC). Different letters represent significantly different treatments (p<0.05) from a post-hoc test (TukeyHSD) of a linear model (*P. syringae* as a function of treatment) in 2016 or a linear mixed model (*P. syringae* as a function of treatment (fixed effect) and block (random effect) in 2017.
Figure 2: Alpha diversity (Shannon index) of phyllosphere bacterial communities for each treatment and each sampling date during the growing season of years 2016 and 2017. Horizontal red line represents the mean. Blue: PC (Plastic Cover), green: RCC (Rye Cover Crop), red: CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil). Different letter represents significantly different treatments (p<0.05) from a post-hoc test (TukeyHSD) of a linear model (alpha diversity as a function of treatment).
Figure 3: Non-metric multidimensional scaling (NMDS) ordination of bacterial community composition in squash phyllosphere samples from different cover cropping treatments in 2016 and 2017. Each point represents a phyllosphere community; symbol size indicates the abundance of *P. syringae* colony forming units (log10(CFUs)) in that sample; colors indicate the cover cropping treatment: blue: PC (Plastic Cover), green: RCC (Rye Cover Crop), red: CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil).
Figure 4: Log$_2$-fold change (LFC) heatmap of most differentially abundant ASV from DeSeq2 analysis for each sampling date of the 2017 samples. For each panel, the left track is the phylogenetic tree from PyNAST alignment of ASVs sequence while right track is the corresponding taxonomic genus name. Each heatmap column is a different contrast between two
treatments mentioned in header as followed: the upper name is the “tested” treatment whereas the lower name is the “control” treatment meaning that a positive LFC value represents an ASV more abundant for the tested treatment. Grey color represents no LFC for the ASV. Each number on the bottom LFC colour scale represents a level of LFC. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil).
Supplemental Table 1: Mean weight (g) of squash leaves samples for each sampling date during years 2016 and 2017. The mean weights (g) are reported with their standard deviations, from the weight distribution of the pooled samples.

<table>
<thead>
<tr>
<th>Season</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>16.91±5.34</td>
<td>20.38±4.50</td>
</tr>
<tr>
<td>Mid</td>
<td>11.49±10.86</td>
<td>19.21±3.74</td>
</tr>
<tr>
<td>Late</td>
<td>21.62±4.14</td>
<td>22.44±5.54</td>
</tr>
<tr>
<td>All</td>
<td>16.81±8.32</td>
<td>20.67±4.82</td>
</tr>
</tbody>
</table>
Supplemental Table 2: Mean counts (± standard deviation) of *P. syringae* colony forming units (CFUs) recovered from squash leaves grown in different cover cropping practices. Means are reported as log_{10}-tranformed number of colonies (± standard deviation) for the four cropping practices at the three seasons of sampling each year.

<table>
<thead>
<tr>
<th>Year/Season</th>
<th>Rye Cover Crop Mean CFUs (log10) ± standard deviation</th>
<th>Chemically-Terminated Rye Cover Crop</th>
<th>Plastic Cover</th>
<th>Bare Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2016</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>4.06 ± 2.33</td>
<td>4.46 ± 1.97</td>
<td>5.49 ± 0.43</td>
<td>5.69 ± 0.45</td>
</tr>
<tr>
<td>Mid</td>
<td>4.38 ± 2.1</td>
<td>4.17 ± 1.99</td>
<td>5.29 ± 1.49</td>
<td>5.33 ± 1.42</td>
</tr>
<tr>
<td>Late</td>
<td>4.6 ± 0.5</td>
<td>4.75 ± 0.67</td>
<td>4.29 ± 0.74</td>
<td>4.64 ± 0.52</td>
</tr>
<tr>
<td><strong>2017</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>0.19 ± 0.81</td>
<td>0.4 ± 1.18</td>
<td>1.98 ± 2.65</td>
<td>4.19 ± 2.2</td>
</tr>
<tr>
<td>Mid</td>
<td>0 ± 0</td>
<td>1.44 ± 2.43</td>
<td>2.93 ± 2.5</td>
<td>3.58 ± 2.38</td>
</tr>
<tr>
<td>Late</td>
<td>0.93 ± 1.81</td>
<td>2.43 ± 2.63</td>
<td>2.4 ± 2.62</td>
<td>3.59 ± 2.1</td>
</tr>
</tbody>
</table>
Supplemental Table 3: Ratios of transformed *P. syringae* CFUs counts (recovered from squash leaves) between different cover cropping practices at each sampling date during 2016 and 2017. Ratios of significantly different treatments according to Tukey HSD; *: p<0.05; **p<0.01; ***p<0.001. *italic value*: no *P. syringae* CFU were recovered at mid season in 2017 for RCC treatments. CFU counts were transformed using the formula $10^{\text{mean}(\log_{10}(\text{CFU count}))}$ in order to be able to calculate ratios for CFU counts of zero.

<table>
<thead>
<tr>
<th></th>
<th>2016 CT-RCC/RCC</th>
<th>2016 PC/RCC</th>
<th>2016 BS/RCC</th>
<th>2016 PC/CT-RCC</th>
<th>2016 BS/CT-RCC</th>
<th>2016 BS/PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early</td>
<td>2.51</td>
<td>26.9*</td>
<td>42.08*</td>
<td>10.74</td>
<td>16.8</td>
<td>1.56</td>
</tr>
<tr>
<td>Mid</td>
<td>0.61</td>
<td>8.05</td>
<td>8.99</td>
<td>13.19</td>
<td>14.74</td>
<td>1.12</td>
</tr>
<tr>
<td>Late</td>
<td>1.4</td>
<td>0.48</td>
<td>1.08</td>
<td>0.35</td>
<td>0.78</td>
<td>2.25</td>
</tr>
<tr>
<td></td>
<td>2017 CT-RCC/RCC</td>
<td>2017 PC/RCC</td>
<td>2017 BS/RCC</td>
<td>2017 PC/CT-RCC</td>
<td>2017 BS/CT-RCC</td>
<td>2017 BS/PC</td>
</tr>
<tr>
<td>Early</td>
<td>1.62</td>
<td>60.71*</td>
<td>9871.12***</td>
<td>37.43*</td>
<td>6086.54***</td>
<td>162.59**</td>
</tr>
<tr>
<td>Mid</td>
<td>27.27</td>
<td>858.06***</td>
<td>3796.51***</td>
<td>31.47</td>
<td>139.22**</td>
<td>4.42</td>
</tr>
<tr>
<td>Late</td>
<td>31.56</td>
<td>29.48</td>
<td>459.35**</td>
<td>0.93</td>
<td>14.56</td>
<td>15.58</td>
</tr>
</tbody>
</table>
Supplemental Figure 1: Aerial photo of the experimental field with cover cropping treatments indicated for each experimental plot. Each experimental plot is 12m long by 6m wide. Plots within the same column are separated by 8-10m between plots of the same row. Each column defines a block of four randomized treatments. Each treatment was applied on 3 raised-bed lines of squash within each experimental plot. Treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and yellow: BS (Bare Soil).
Supplemental Figure 2: Photos of cover crop treatments. Left-upper panel present the process of rolling the rye on the ground with the roller crimper. The right-upper panel highlights the squash seeding process directly through the rye cover crop. The left- and right-bottom panels show squash growing in cover crop treatments at mid season in 2018 (July 18 and July 31 respectively).
Supplemental Figure 3: Structural overview of 16S amplicons. Each amplicon owns a dual indexing (combination of Barcode#1 and #2) for each sample. Barcode lengths are variable between samples.
Supplemental Figure 4: Principal component analysis ordination among phyllosphere samples and control samples for the growing season 2016 and 2017. Ellipses represent standard deviation around samples from each category. Control samples, including positive controls Mock_Control (known mix of 5 bacterial isolates) and Pseudo_Control (P. syringae isolate) and negative control PCR_Control (water), are distinct compositionally from the phyllosphere samples. Corresponding sampling dates are as follows: Early=Date 1, Mid=Date 2, Late=Date 3.
Supplemental Figure 5: Rarefaction curves (ASVs versus number of sequences/sample) for each squash sample of growing season 2016 and 2017. Each label represents a different sample. The flat section of each curve indicates that enough sequences has been reached to capture the majority of ASVs for a given sample.
Supplemental Figure 6: Plot of rarefaction levels effects on PERMANOVA results and sample number. 100 iteration of rarefaction levels, from 1000 to 10000 random sampling per sample, were tested to evaluate their effects on: $R^2$ (A), the coefficient of determination representing the proportion of the variable variation explained by the model, and the $p$-value (B), of the PERMANOVA (per sampling date effect of treatment on community structure), and remaining sample number after rarefaction (C). The vertical dotted line represents the chosen rarefaction level for our study. Each data point represent a given value ($R^2$, $p$-value or remaining sample) for each rarefaction level at each season sampling (Early, Mid or Late). If a sample had fewer sequences than the rarefaction level, it was removed by the pipeline.
Supplemental Analysis 1: Effect of treatments on overall community diversity

Cover cropping treatments influenced the diversity of the bacterial community on squash leaves as measured by the Shannon index of rarefied bacterial communities (supplemental Figure 6). During the growing season, the community diversity was marginally significantly higher for the rye treatment in 2016 (TukeyHSD post-hoc test performed on linear model of community diversity * treatment; p=0.0565) and significantly higher for the bare soil (TukeyHSD post-hoc test performed on linear mixed model of community diversity ~ treatment with block as the random effect p= 0.0332) in 2017 as compare to the other treatments whereas overall treatment effect was marginally significant (liner model of community diversity ~ treatment; 2016: p=0.086, 2017: p= 0.053). Moreover, community diversity was significantly different between sampling dates (linear model of community diversity ~ sampling date; 2016, p> 0.001, 2017: p= 0.003). While this diversity was lower for sampling date 2 as compare to sampling date 1 (TukeyHSD post-hoc test performed on the above-mentioned linear model; p= 0.031) and date 3 (p= 0.003) in year 2017 (no significant differences between date 1 and 3), it was higher for both sampling dates 2 (TukeyHSD post-hoc test performed on the above-mentioned linear mixed model; p= 0.045) and 3 (p< 0.001) than the sampling date 1 in year 2016 (no significant difference between date 2 and 3).

The total richness of ASVs in bacterial communities also differed among treatments and dates (supplemental Figure 7). No significant ASVs number differences between treatments were observed during the 2016 overall growing season (liner model of community richness ~ treatment; p=0.201), however it was statistically different in 2017 (p< 0.001) with more ASVs in
the bare soil (p< 0.001) as compared to other treatments. Unlike the alpha diversity of 2016, the
ASVs numbers were different between sampling dates (linear model of community richness ~
sampling date; p= 0.009) and significantly lower at date 2 (TukeyHSD post-hoc test performed on
the above-mentioned linear model; p= 0.009). On the contrary, as the overall alpha diversity, the
ASVs number was still different in the growing season in 2017, with less ASVs at sampling date 2
as compared to dates 1 (p< 0.001) and 3 (p= 0.067).
Supplemental Figure 7: Alpha diversity (Shannon index) for each treatment and sampling dates (Early, Mid or Late season) during the growing season of years 2016 and 2017. Horizontal red lines represent the mean value. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil).
Supplemental Figure 8: Violin plot of ASV richness at each treatment and sampling date during the growing season of years 2016 and 2017. Horizontal red line represents the distribution mean. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil).
Supplemental Figure 9: Non-metric multidimensional scaling (NMDS) ordination of squash phyllosphere community during the growing season of 2016 and 2017 at the 3 sampling dates. Each point represents a phyllosphere community. Corresponding season sampling dates are as follows: Early=Date 1, Mid=Date 2, Late=Date 3.
Supplemental Figure 10: Least square means plot of linear mixed model comparison between treatment and the 2 NMDS axis scores, at each sampling date of the growing season 2016 and 2017. Least squares comparisons have been performed with the emmeans v1.4.8 R package (Lenth et al., 2018) on a linear mixed model with NMDS axis scores as a function of treatment.
(fixed effect) with experimental block as random effect. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated Rye Cover Crop) and BS (Bare Soil). Two overlapping horizontal bars indicating that the 2 treatments are not significantly different. The more distant the 2 bars, the greater the differences between the 2 associated treatments.
Supplemental Figure 11: Log2-fold change (LFC) heatmap of most differentially abundant ASV from DeSeq2 analysis for each sampling date of the 2016 samples. For each panel, left track is the phylogenetic tree from PyNAST alignment of ASVs sequence while right track is the corresponding taxonomic name at the Genus rank. Each heatmap column is a different contrast.
between two treatments mentioned in the header as follows: above name is the “tested”
treatment whereas the below one is the “control” treatment meaning that positive an LFC value
represent an ASV more abundant for the tested treatment. Grey color represents no LFC for the
ASV. Tested treatment: PC (Plastic Cover), RCC (Rye Cover Crop), CT-RCC (Chemically Terminated
Rye Cover Crop) and BS (Bare Soil).