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Impact of a resistance gene against a fungal pathogen on the plant host residue microbiome: The case of the *Leptosphaeria maculans–Brassica napus* pathosystem

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Abstract

Oilseed rape residues are a crucial determinant of stem canker epidemiology as they support the sexual reproduction of the fungal pathogen *Leptosphaeria maculans*. The aim of this study was to characterize the impact of a resistance gene against *L. maculans* infection on residue microbial communities and to identify microorganisms interacting with this pathogen during residue degradation. We used near-isogenic lines to obtain healthy and infected host plants. The microbiome associated with the two types of plant residues was characterized by metabarcoding. A combination of linear discriminant analysis and ecological network analysis was used to compare the microbial communities and to identify microorganisms interacting with *L. maculans*. Fungal community structure differed between the two lines at harvest, but not subsequently, suggesting that the presence/absence of the resistance gene influences the microbiome at the base of the stem whilst the plant is alive, but that this does not necessarily lead to differential colonization of the residues by fungi. Direct interactions with other members of the community involved many fungal and bacterial amplicon sequence variants (ASVs). *L. maculans* appeared to play a minor role in networks, whereas one ASV affiliated to *Plenodomus biglobosus* (synonym *Leptosphaeria biglobosa*) from the *Leptosphaeria* species complex may be considered a keystone taxon in the networks at harvest. This approach could be used to identify and promote microorganisms with beneficial effects against residue-borne pathogens and, more broadly, to decipher the complex interactions between multispecies pathosystems and other microbial components in crop residues.

KEYWORDS

ecological network analysis, metabarcoding, microbial communities, microbiome, oilseed rape residues, pathobiome, stem canker

Frédéric Suffert and Valérie Laval contributed equally to this work.

1 | INTRODUCTION

Plants support a large number of microorganisms, and the assembly and structuring of this microbial community is dependent on many factors, such as the type of plant, the organ considered (Comby *et al*., 2016; Grudzinska-Sterno *et al*., 2016), and its age (Wagner *et al*., 2016). Many of these microorganisms are considered beneficial (e.g., plant growth-promoting bacteria), whereas others are pathogenic and decrease the yield and quality of agricultural produce. The pathobiome is the subset of microbial communities consisting of a pathogen and the organisms that can influence that pathogen or be influenced by it (Vayssier-Taussat *et al*., 2014; Jakuschkin *et al*., 2016). The role of the microbiota in the plant's response to a disease or in the pathogenicity of a fungal pathogen is currently being studied in various pathosystems, but remains poorly understood (Cho and Blaser, 2012; Jakuschkin *et al*., 2016; Lebreton *et al*., 2019).

Stem canker is a widespread disease of oilseed rape (*Brassica napus*), the main causal agent of which is the ascomycete *Leptosphaeria maculans*. This fungus has a complex life cycle. It enters the leaves through stomata (Hammond and Lewis, 1987), leading to the development of leaf spots (Travadon *et al*., 2007). The fungus then progresses through the xylem, from the leaf spots to the base of the petiole and into the stem (Hammond *et al*., 1985; Hammond and Lewis, 1987). In Europe, *L. maculans* begins its necrotrophic phase in late spring, causing crown canker (or stem canker) at the stem base (West *et al*., 2001), leading to stem breakage yield losses. The fungus continues its life cycle as a saprophyte on the infected stem bases left on the soil at harvest, on which it reproduces sexually and survives the intercropping period, sometimes over a period of several years (Petrie and Lewis, 1985; Baird *et al*., 1999; West *et al*., 2001; Fitt *et al*., 2006). Control strategies are mostly based on improving plant immunity by combining quantitative, polygenic resistance sources and major resistance genes (*Rlm* genes) matching fungal effectors acting as avirulence (*AvrLm*) determinants (Delourme *et al*., 2006).

Another *Leptosphaeria* species, *Leptosphaeria biglobosa*, has a similar life cycle to *L. maculans* and colonizes oilseed rape plant tissues in a similar manner. This fungus is present in all countries except China (Liu *et al*., 2014), but its agronomic impact is considered negligible relative to that of *L. maculans*. *L. biglobosa* was recently renamed *Plenodomus biglobosus* (De Gruyter *et al*., 2013), but this nomenclature has not yet been adopted as the standard among plant pathologists working on oilseed rape stem canker (Dutreux *et al*., 2018). For the sake of consistency, we use *P. biglobosus* here because the genus name *Plenodomus* is derived from the taxonomic assignment of metabarcoding sequences.

Crop residues are an essential ecological niche for both *L. maculans* and *P. biglobosus*, and the severity of stem canker disease is generally correlated with the number of ascospores ejected by the residues of the previous crop (McGee and Emmett, 1977; Petrie, 1995). Residues also constitute a compartment rich in microorganisms, in which transfers occur between organisms from the plant and microorganisms from the soil (Kerdraon *et al*., 2019a). These microorganisms may interact with *L. maculans* or be influenced by its presence, as recently established for *Fusarium* spp. in maize (Cobo-Díaz *et al*., 2019) and *Zymoseptoria tritici* in wheat (Kerdraon *et al*., 2019b). Culture-dependent approaches have shown that some fungal species (e.g., *Alternaria* spp., *Trichoderma* spp., *Chaetomium* sp., *Gliocladium* sp.) are present together with *L. maculans* on buried oilseed rape residues (Naseri *et al*., 2008). The characterization of interactions between pathogens and other microorganisms can open up new opportunities for managing residue-borne diseases (Poudel *et al*., 2016).

An initial description of the microbiota associated with *L. maculans* during its saprophytic/reproduction stage on oilseed rape residues was obtained by culture-independent approaches (Kerdraon *et al*., 2019c). *L. maculans* and *P. biglobosus* were both found to be highly represented in the metabarcoding data set. However, questions remain about the influence of these species on the fungal and bacterial communities and the effect of *L. maculans* on the composition and evolution of the residue microbiome. We addressed these questions with a pair of near-isogenic oilseed rape lines with the same genetic background, differing by the presence or absence of the *Rlm11* resistance gene: Darmor and Darmor-*Rlm11*. The major gene *Rlm11* was introgressed from *Brassica rapa* into the French cultivar Darmor following an interspecific cross and a series of backcrosses to Darmor (Balesdent *et al*., 2013). Population surveys showed that more than 95% of French *L. maculans* isolates were avirulent toward *Rlm11* (Balesdent *et al*., 2013). In addition, Darmor is characterized by a high level of quantitative, polygenic resistance. This context constituted the perfect framework for a metabarcoding approach to investigate, under field conditions, how the presence of one efficient major resistance gene, and hence the lack or low levels of *L. maculans*, affected the plant residue microbiome, including other plant pathogens of oilseed rape.

2 | RESULTS

2.1 | Overall diversity

The impact of the *Rlm11* resistance gene on the oilseed rape residue microbiome was assessed by analysing the composition of fungal and bacterial communities of 120 residue samples (60 from Darmor and 60 from Darmor-*Rlm11* residues) obtained during four different sampling periods (July, i.e., just after harvest; October, i.e., just after sowing of the subsequent wheat crop; December; and February). Metabarcoding analysis identified 610 bacterial amplicon sequence variants (ASVs) and 335 fungal ASVs. Fungal diversity (Shannon index) and observed richness were higher in Darmor-*Rlm11* residues than in Darmor residues in July, but did not differ significantly between these two types of residue on subsequent sampling dates, over which it remained relatively constant (Figures S1 and S2). Bacterial diversity, observed richness, and Pielou's evenness increased from July to October for both cultivars, and then slightly decreased from December to February. Fungal diversity remained constant throughout the experiment for both cultivars.

2.2 | Microbial community structure

The Bray–Curtis index (beta diversity analysis) was used to characterize divergence in community structure according to the presence of *Rlm11* and sampling date. The presence of *Rlm11* significantly modified the structure of the bacterial (PERMANOVA, $R^2 = 0.022$, $p = .001$) and fungal (PERMANOVA, $R^2 = 0.042$, $p = .001$) communities. For fungi, the difference between Darmor and Darmor-*Rlm11* was significant only in July (Table 1, Figure 1). Beta diversity analysis highlighted changes in the community over time, for both fungi (PERMANOVA, $R^2 = 0.178$, $p = .001$) and bacteria (PERMANOVA, $R^2 = 0.37$, $p = .001$). The strong divergence observed between July and the other sampling dates for both bacterial and fungal communities (Table 1) is consistent with the rapid degradation of residues after the summer (loss of almost 50% in terms of weight; Figure S3). Some genera, such as *Cryptococcus*, *Alternaria*, *Sphingobacterium*, and *Rhodococcus*, decreased in abundance or disappeared over time, whereas others, such as *Torula*, *Schizotecium*, *Sphingomonas*, and *Sphingopyxis*, emerged over time (Figure 1).

2.3 | Disease assessment and presence of *L. maculans* and *P. biglobosus* on residues

The G2 score, characterizing stem canker severity from 0 (no disease) to 9 (all plants lodged), was obtained for the 60 oilseed rape residue samples collected in June and highlighted the difference in susceptibility to *L. maculans* between Darmor (G2 = 3.78) and Darmor-*Rlm11* (G2 = 2.24). Some Darmor-*Rlm11* plants had stem canker symptoms, but the presence of *Rlm11* reduced stem necrosis due to *L. maculans* (Figure 2). Consistently, *Leptosphaeria* (probably *L. maculans*) was detected less frequently and less strongly by metabarcoding in Darmor-*Rlm11* residue samples than in Darmor residue samples. Moreover, the relative abundance (RA) of reads associated with *Leptosphaeria* decreased during residue degradation for Darmor (from 15.07 ± 14.39 in July to 3.95 ± 4.63 in February) but was lower and remained constant for Darmor-*Rlm11* (3.85 ± 6.65). The RA of ASVs affiliated to *Plenodomus* (probably *P. biglobosus*) increased over time for Darmor (from 37.10 ± 11.67 to 53.18 ± 12.62) and Darmor-*Rlm11* (from 38.47 ± 11.80 to 55.32 ± 11.66) (Figure 3c). The good relationship between the amount of *P. biglobosus* and *L. maculans* DNA quantified by quantitative PCR (qPCR) in 33 residue samples and the corresponding sequence read numbers, as established by Spearman's rank correlation test ($\rho = 0.93$, $p < .0001$ for *L. maculans*; $\rho = 0.60$, $p < .001$ for *P. biglobosus*; Figure S4), justified the quantitative interpretation of metabarcoding data.

2.4 | Impact of the presence of *Rlm11* on microbial community composition

Linear discriminant analysis (LDA) was used to characterize the ASVs affected by the presence of *Rlm11*. The RA of 29 fungal ASVs (16 genera, including unclassified) was affected by the presence/absence of *Rlm11* (Figure 3). In July, fungal communities differed significantly between Darmor and Darmor-*Rlm11* (Table 1) due to a higher RA of ASVs affiliated to *Alternaria*, *Cladosporium*, *Filobasidium*, and *Plenodomus* in Darmor-*Rlm11* residues, and a higher RA of ASVs affiliated to *Leptosphaeria* and *Pyrenopeziza* in Darmor residues. The communities were not significantly different overall in October, December, and February (Table 1), but some ASVs displayed significantly different abundances between Darmor and Darmor-*Rlm11*. For example, one ASV (the second most abundant, with 4.5% of reads) affiliated to *Plenodomus* was favoured by the degradation of Darmor-*Rlm11* residues. The genus *Hydropisphaera*, which colonized residues after July (Figure 1), was more abundant in Darmor-*Rlm11* than in Darmor residues (Figure 3). Surprisingly, the genus *Pyrenopeziza*, including *Pyrenopeziza brassicae*, the causal agent of light leaf spot disease in oilseed rape, which reproduces on oilseed rape residues (Gilles *et al.*, 2001), was more abundant in Darmor than in Darmor-*Rlm11* in July.

The RA of 93 bacterial ASVs (43 genera, including unclassified) was affected by the presence/absence of *Rlm11* (Figure 3). Bacterial

TABLE 1 Divergences of fungal (below diagonal) and bacterial (above diagonal) assembly structures between the oilseed rape residues from Darmor and Darmor-*Rlm11*, assessed by pairwise adonis analysis

		Darmor-*Rlm11*				Darmor			
		Jul	Oct	Dec	Feb	Jul	Oct	Dec	Feb
Darmor-*Rlm11*	Jul.	–	0.335**	0.385**	0.459**	0.078	0.345**	0.364**	0.457**
	Oct.	0.201**	–	0.104**	0.329**	0.286**	0.105**	0.132**	0.333**
	Dec.	0.277**	0.041	–	0.212**	0.346**	0.137**	0.056	0.217**
	Feb.	0.376**	0.096**	0.044	–	0.446**	0.339**	0.217**	0.069**
Darmor	Jul.	0.142**	0.202**	0.243**	0.337**	–	0.276**	0.316**	0.435**
	Oct.	0.223**	0.087	0.084	0.155**	0.124	–	0.083	0.324**
	Dec.	0.254**	0.083	0.048	0.089	0.171**	0.041	–	0.195**
	Feb.	0.352**	0.113**	0.064	0.064	0.283**	0.095	0.057	–

The significance of divergences was assessed by calculating a *q* value.
**$q < 0.01$.

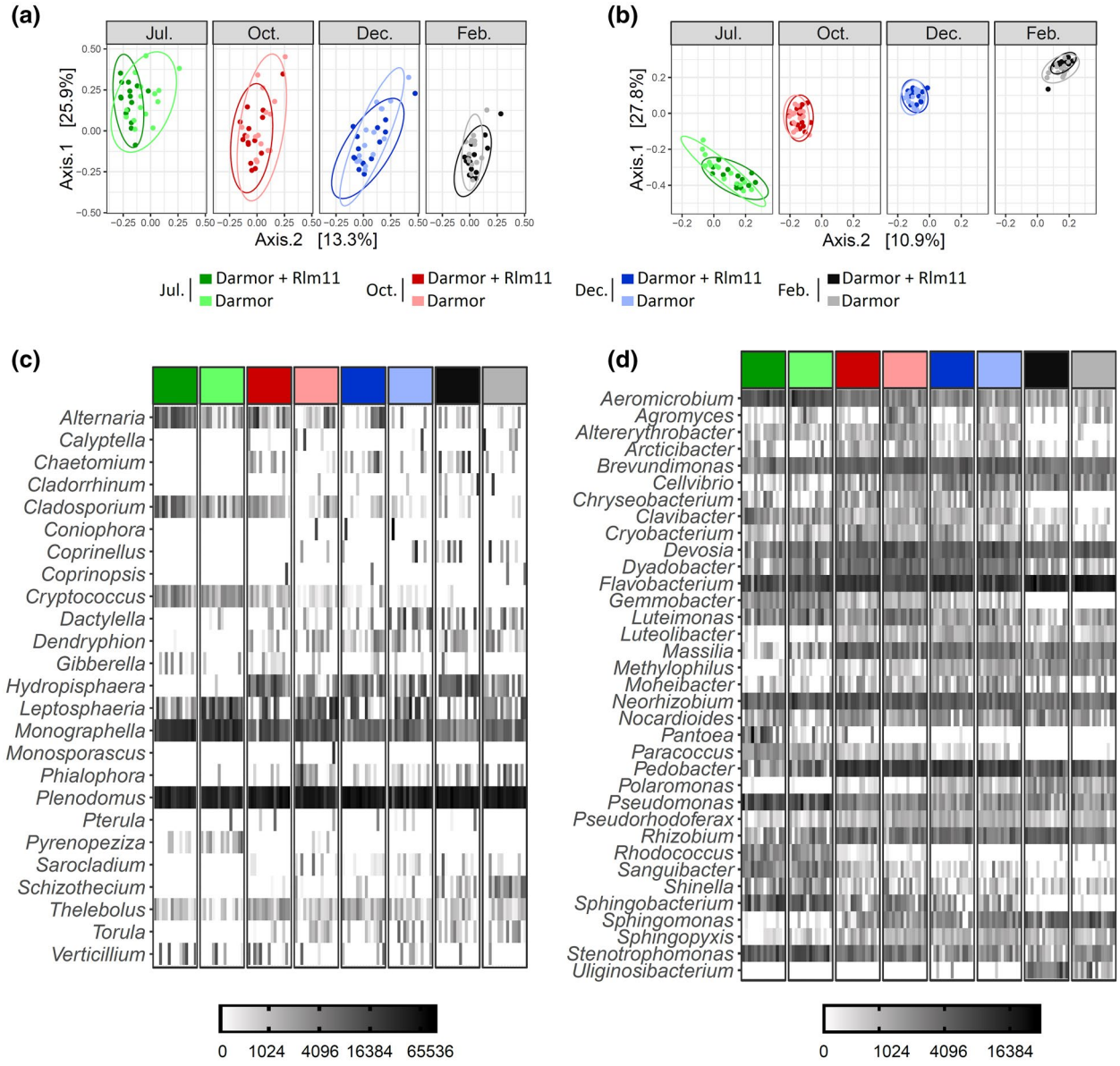

FIGURE 1 Effect of the presence of the *Rlm11* resistance gene and of sampling date (July, October, December, and February) on fungal (a, c) and bacterial (b, d) communities originating from 120 samples of oilseed rape residues. (a) and (b) Visualization of compositional distances between samples through multidimensional scaling (MDS) based on the Bray–Curtis dissimilarity matrix. MDS analysis was performed on all samples together and was faceted according to the sampling date. Each data point corresponds to one sample of oilseed rape residues. The colours of the points distinguish between sampling dates (July, green; October, red; December, blue; February, grey) and cultivar (Darmor, light hues; Darmor-*Rlm11*, dark hues). (c) and (d) Diversity and predominance of the 25 most abundant (25/62, excluding unclassified genera) fungal genera (a) and the 35 most abundant (35/134, excluding unclassified genera) bacterial genera (b) distributed in all samples, distinguishing between the different experimental conditions. The colours used to distinguish samples are as in (a) and (b)

community structure differed significantly between the two types of residues in October and February (Table 1). The bacteria colonizing residues were affected by the presence of *Rlm11*. For example, ASVs affiliated to *Sphingomonas* preferentially colonized Darmor residues in October and *Uliginosibacterium* preferentially colonized Darmor-*Rlm11* residues in February. No significant difference in microbial communities was found between the two types of residues in July, but 28 bacteria had different abundances in Darmor and Darmor-*Rlm11*, including *Pantoea*, which was more abundant in Darmor-*Rlm11* residue samples in July.

2.5 | Direct impact of *L. maculans* on microorganisms

2.5.1 | Dynamics of ecological interaction networks associated with oilseed rape residues

Ecological network analysis (ENA) combining bacterial and fungal data sets were performed to characterize the interactions between *Leptosphaeria* (probably *L. maculans*) and the other members of the bacterial and fungal communities at each of the four

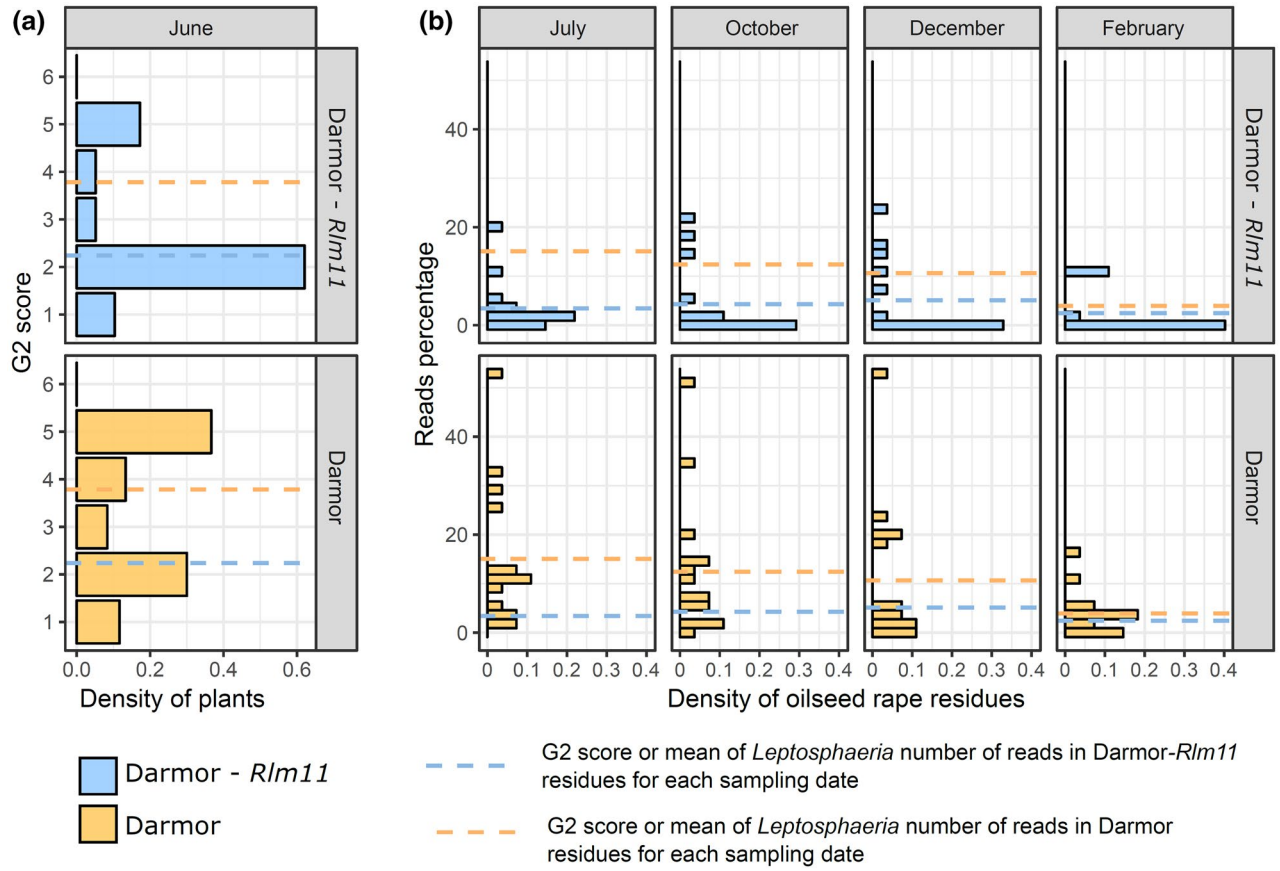

FIGURE 2 Influence of the presence of the *Rlm11* resistance gene and sampling date (July, October, December, and February) on (a) the stem canker G2 score (proxy for disease severity, estimated for 60 plants of Darmor and Darmor-*Rlm11*) and (b) the percentage of reads corresponding to *Leptosphaeria maculans*. (a) Percentage of plants with each G2 score for the two cultivars. The dashed lines correspond to the G2 score for each of the two cultivars (Darmor, yellow; Darmor-*Rlm11*, blue). (b) Percentage of reads affiliated to *L. maculans* in the 15 samples for each condition. The dashed lines correspond to the mean read percentage for each of the two cultivars at each sampling date (Darmor, yellow; Darmor-*Rlm11*, blue)

sampling dates (Figure 4). The mean number of nodes in networks (138.8 ± 22.1) increased during residue degradation. On average, 11.8 ± 5.5 nodes were isolated. Mean node degree was 2.49 ± 0.34. *Leptosphaeria* appeared to play a weak role in networks, with only a few degrees (2 to 5 only) and a low ranking for betweenness centrality (Figure 4). By contrast, one ASV affiliated to *Plenodomus* (probably *P. biglobosus*, the other oilseed rape pathogen associated with *L. maculans*) may be considered a keystone taxon in the July network following the definition proposed by Banerjee *et al.* (2018).

2.5.2 | Subnetworks highlighting direct interactions between *L. maculans* and other species

The combination of ENA and LDA highlighted interactions between the ASVs affected by the presence/absence of *Rlm11*. These ASVs were mostly connected. *Leptosphaeria* interacted with as many ASVs promoted or inhibited by the presence of *Rlm11* as with ASVs not affected by the presence of this gene (Figure 5). ASVs interacting with *Leptosphaeria* but not affected by the presence of *Rlm11* had RA values that changed with the presence of *Leptosphaeria*, regardless of the cultivar. During residue degradation, *Leptosphaeria* interacted positively with ASVs affiliated to *Cryptococcus* (July), *Nocardioides* (October), *Monographella* (in October and February), *Altererythrobacter*, *Sphingomonas* (December), and *Acidovorax* (February), and interacted negatively with *Alternaria* (July and February), *Massilia*, *Sanguibacter*, and *Chaetomium* (October).

3 | DISCUSSION

The use of two near-isogenic lines appeared to be a relevant strategy to characterize the effect of *L. maculans* on the microbiome of oilseed rape residues. Indeed, oilseed rape residues with and without *L. maculans* infection are difficult to obtain in controlled conditions, in contrast to other pathosystems, such as *Z. tritici* on wheat residues (Kerdraon *et al.*, 2019b) or *Plasmodiophora brassicae* on *B. napus* (Lebreton *et al.*, 2019). There is also, generally, a large difference between the microbial communities present on plants in field conditions and those obtained in greenhouses (Ritpitakphong

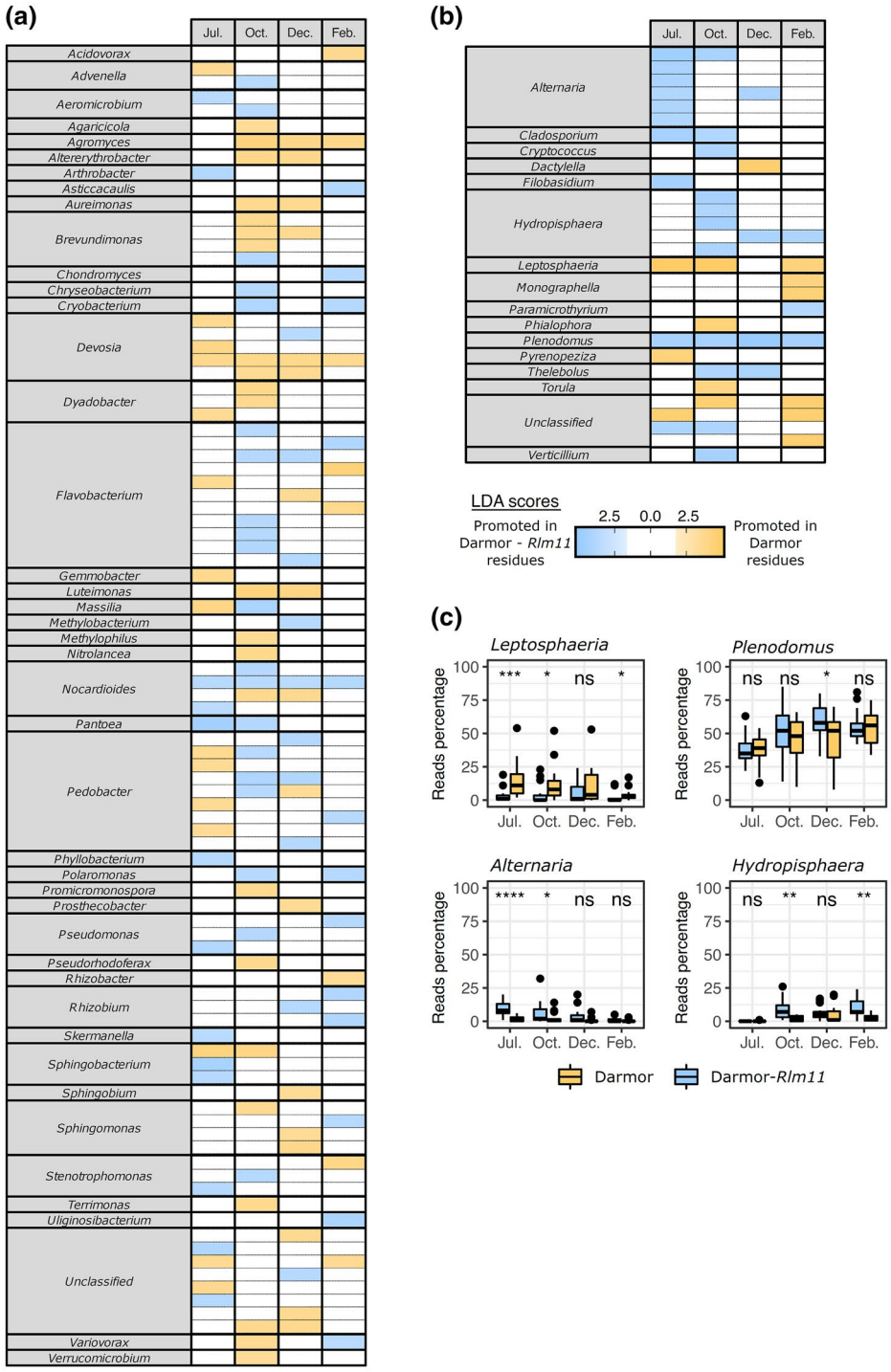

FIGURE 3 Impact of the presence of the *Rlm11* resistance gene on microbial communities in oilseed rape residues. (a) and (b) Significant differences in the predominance of bacterial (a) and fungal (b) amplicon sequence variants (ASVs) between the Darmor samples (orange) and the Darmor-*Rlm11* samples (blue) obtained in linear discriminant analysis (LDA). Only ASVs with $p < .05$ for the Kruskal–Wallis test and LDA scores >2 were retained for the plot. (c) Relative abundances of *Leptosphaeria*, *Plenodomus*, *Alternaria*, and *Hydropisphaera* by host cultivar (Darmor and Darmor-*Rlm11*) and sampling date (July, October, December, and February). All ASVs were grouped by genus (*Alternaria* 8 ASVs, *Hydropisphaera* 8 ASVs, *Leptosphaeria* 13 ASVs, *Plenodomus* 81 ASVs)

et al., 2016). The use of fungicides, another plausible technical solution for achieving healthy conditions in the field, would have affected nontargeted endophytic or pathogenic fungal communities (Karlsson *et al*., 2014; Knorr *et al*., 2019). While host genetics has been shown to influence the plant and root microbiome strongly (Sapkota *et al*., 2015; Wagner *et al*., 2016; Rybakova *et al*., 2017), the use of near-isogenic lines differing only by the presence or absence of a specific resistance gene has limited the genotype effect (Newton *et al*., 2010). This strategy was effective as it made it possible to achieve different levels of *L. maculans* on the two types of

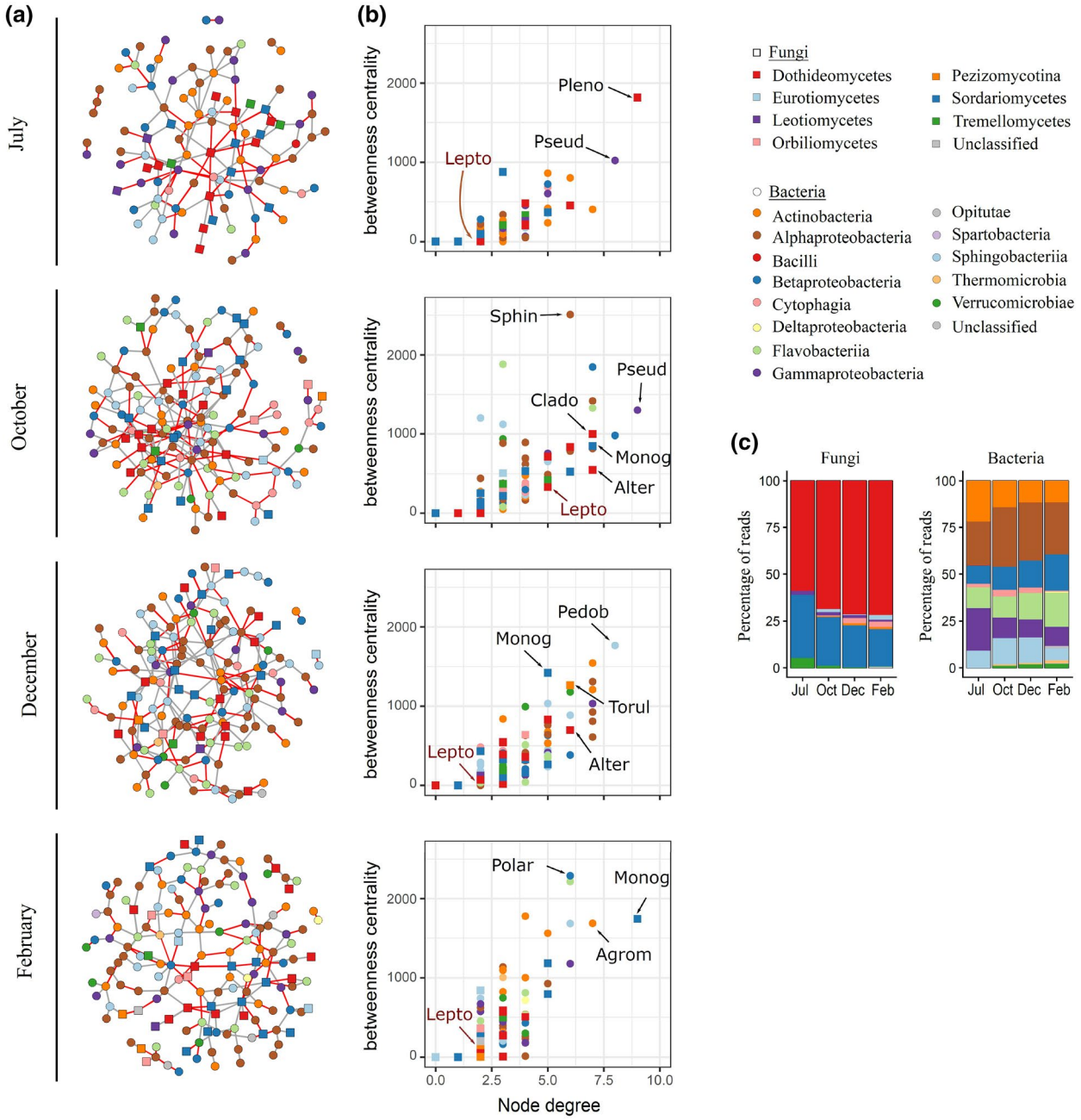

FIGURE 4 Temporal dynamics of co-occurrence networks. (a) Networks based on bacterial and fungal amplicon sequence variants (ASVs) combined. In all networks, circles and squares correspond to bacterial and fungal ASVs, respectively, with colours representing the class. Isolated nodes are not indicated. Edges represent positive (grey) or negative (red) interactions. (b) Betweenness centrality and degree of each ASV in the networks. The place of *Leptosphaeria maculans* in the networks is indicated. Colour and shape are as in (a). The genera of the fungal and bacterial ASVs with the highest degree and centrality have been added: *Agrom(yces)*, *Alter(naria)*, *Clado(sporium)*, *Lepto(sphaeria)*, *Monog(raphella)*, *Pedob(acter)*, *Pleno(domus)*, *Polar(omonas)*, *Pseud(omonas)*, *Sphin(gopyxis)*, *Torul(a)*. (c) Percentage of reads associated with fungal and bacterial classes for each network. Isolated nodes are indicated. Colours are as in (a)

residues, mimicking infected and healthy conditions, and could be adapted to achieve similar objectives with other pathosystems having a complex biological cycle. However, it was not possible with this strategy to distinguish between the effects of the presence/absence of *Rlm11* on the residue microbiome and those of the presence/absence of *L. maculans*, which was itself strongly affected by *Rlm11*. To our knowledge, this study is the first to investigate the effect of a fungal plant pathogen on microbial communities on near-isogenic lines (Newton *et al*., 2010). In addition, very few studies have focused on the microorganisms associated with *L. maculans* during the necrotrophic and saprophytic stages of the life cycle of this pathogen (Naseri *et al*., 2008; Kerdraon *et al*., 2019c).

The proportion of *L. maculans*-infected samples was smaller for Darmor-*Rlm11* residue samples than for Darmor residue samples. The detection of *L. maculans* in a few plants assumed to be resistant cannot be explained by contamination with plants lacking *Rlm11*, as

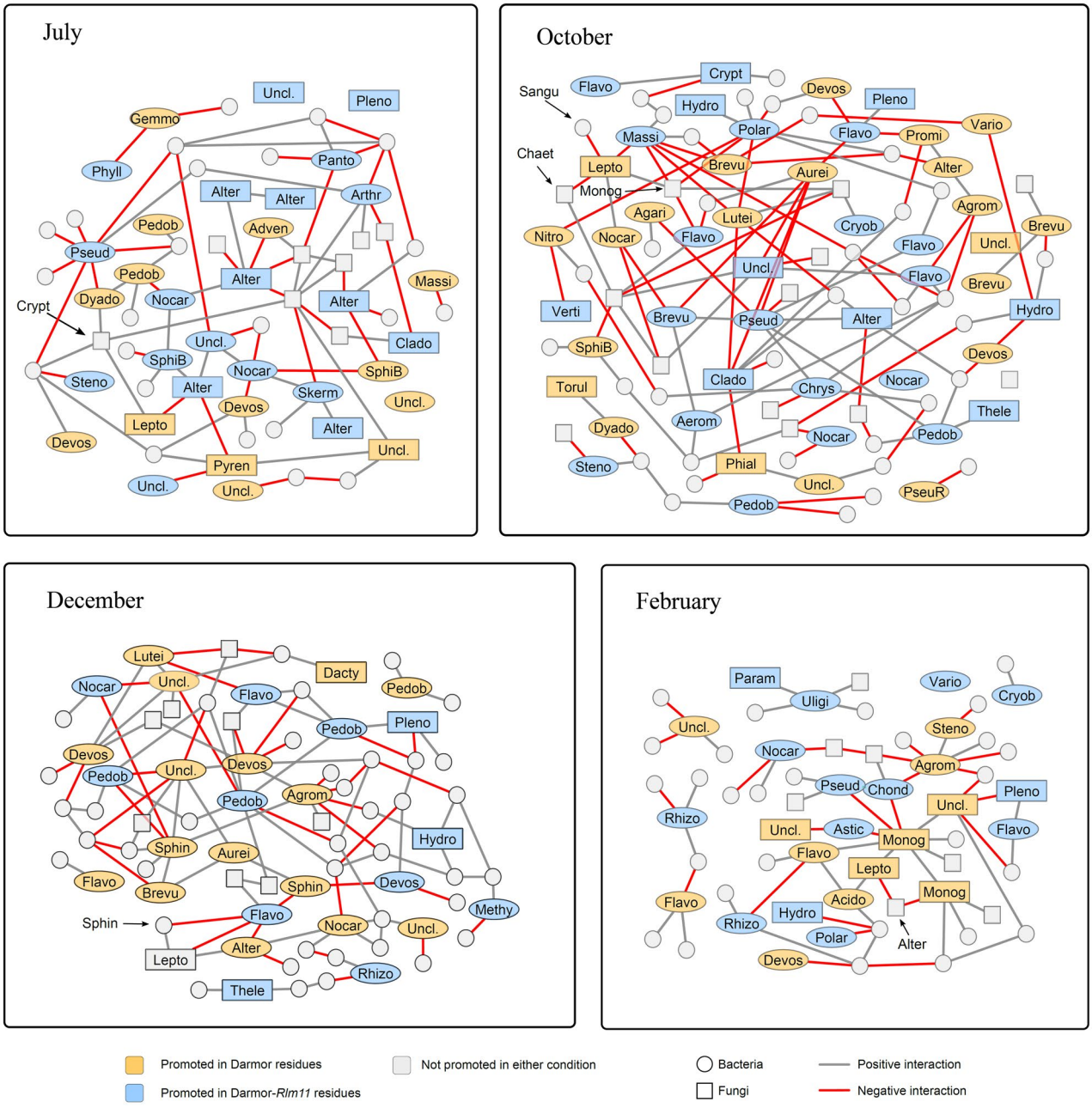

FIGURE 5 Subnetworks combining linear discriminant analysis (LDA; see Figure 3) and ecological interaction networks (see Figure 4), focusing on bacterial and fungal amplicon sequence variants (ASVs) identified as differential in LDA and their first adjacent nodes. Node colour indicates the results of LefSe differential analysis (Segata*et al*., 2011) between Darmor (yellow) and Darmor-*Rlm11* (blue) treatments. Only genera with $p < .05$ for the Kruskal–Wallis test and LDA scores >2 were retained for the plot. Edges represent positive (grey) or negative (red) interactions. Differential ASVs are plotted with genus name abbreviations: *Acido(vorax)*, *Adven(ella)*, *Aerom(icrobium)*, *Agari(cicola)*, *Agrom(yces)*, *Alter(naria)*, *Alter(erythrobacter)*, *Arthr(obacter)*, *Astic(cacaulis)*, *Aurei(monas)*, *Brevu(ndimonas)*, *Chond(romyces)*, *Chrys(eobacterium)*, *Clado(sporium)*, *Cryob(acterium)*, *Crypt(ococcus)*, *Dacty(lella)*, *Devos(ia)*, *Dyado(bacter)*, *Flavo(bacterium)*, *Gemmo(bacter)*, *Hydro(pisphaera)*, *Lepto(sphaeria)*, *Lutei(monas)*, *Massi(lia)*, *Methy(lobacterium)*, *Monog(raphella)*, *Nitro(lancea)*, *Nocar(dioides)*, *Panto(ea)*, *Param(icrothyrium)*, *Pedob(acter)*, *Phial(ophora)*, *Phyll(obacterium)*, *Pleno(domus)*, *Polar(omonas)*, *Promi(cromonospora)*, *Pseud(omonas)*, *PseuR(=Pseudorhodoferax)*, *Pyren(opeziza)*, *Rhizo(bium)*, *Skerm(anella)*, *SphiB(=Sphingobacterium)*, *Sphin(gomonas)*, *Steno(trophomonas)*, *Thele(bolus)*, *Torul(a)*, *Uligi(nosibacterium)*, *Uncl.(assified)*, *Vario(vorax)*, *Verti(cillium)*

the purity of the Darmor-*Rlm11* seed lot was established before the experiment. The frequency of *avrLm11* (virulent) isolates in the local population was tested by pathotyping a population of *L. maculans* collected from the residues used to reinforce the inoculum. None of the 92 isolates tested was virulent on Darmor-*Rlm11*, suggesting that the proportion of virulent isolates in the field ranged from 0% to 3.9% (Daudin *et al*., 2001). This is consistent with previous epidemiological surveys indicating that less than 4% of *L. maculans*

isolates are virulent against *Rlm11* in French pathogen populations (Balesdent *et al*., 2013). These elements suggest that rare virulent isolates may lead to the infection of Darmor-*Rlm11* in field conditions and account for the lower levels of *L. maculans* colonization on Darmor-*Rlm11* residues than on Darmor residues, as established by metabarcoding and qPCR.

Fungal community structure clearly differed between Darmor and Darmor-*Rlm11* in July, but not on subsequent sampling dates. This suggests that the presence/absence of the *Rlm11* gene, or the whole introgression, influences the microbiome of the stem base whilst the plant is alive, but does not lead to differential colonization of the residues by fungi, partly from the bulk soil, later on. Bacterial community structure was slightly affected by the presence of *Rlm11*. In July, the divergences between communities observed included an under-representation of *Pyrenopeziza* (probably *P. brassicae*) in Darmor-*Rlm11*. Darmor is a cultivar known to be susceptible to the fungal pathogen *P. brassicae*, which is prevalent mostly in western France and the north of the UK (Gilles *et al*., 2000). Several hypotheses can be put forward to account for this unexpected difference between the two near-isogenic lines: (a) the presence of *L. maculans* facilitates *P. brassicae* infection; (b) the resistance of Darmor-*Rlm11* to *L. maculans* isolates carrying the avirulence gene *AvrLm11* gene indirectly prevents the early infection of *B. napus* leaves with *P. brassicae*, with a significant impact on the development of polycyclic light leaf spot epidemics; and (c) the introgression of *Rlm11* from *Brassica rapa* to *B. napus* was accompanied by the introgression of a locus conferring resistance to *P. brassicae* or replacing a susceptibility locus initially present in cultivar Darmor (Régine Delourme, INRA IGEPP, personal communication). These three hypotheses should be tested in future experiments.

All the fungal genera and most of the most abundant bacterial genera identified here were also detected on oilseed rape residues in a previous study (Kerdraon *et al*., 2019c). The immediate proximity of the experimental plots of the two studies may explain these similarities. As previously established, bacterial and fungal communities changed with the degradation of residues over the interepidemic period. For example, *Chaetomium*, detected on the residues between July and October, has been shown to colonize oilseed rape residues 2 months after deposition on the ground (Naseri *et al*., 2008; Kerdraon *et al*., 2019c). *Alternaria* spp. accounted for a large percentage of the microbes isolated from infected oilseed rape residues in a previous study (Naseri *et al*., 2008; Kerdraon *et al*., 2019c). Similarly, *Alternaria* ASVs had a high RA in this study. The genus *Monographella*, detected on the residues in this study, was previously isolated from the rhizosphere of senescent oilseed rape plants and has been described as an antagonist of *Verticillium* (Berg *et al*., 2005), but no significant direct interactions between *Verticillium* and *Monographella* were detected by ENA in this study. The existence, or lack of existence, of interaction between species or genus as revealed by ENA can help to identify potential biocontrol agents, but a subsequent validation based on functional testing is required.

The dual approach based on LDA and ENA used here detected an effect of the presence of *L. maculans* on the whole residue microbiome. Based on a similar study focusing on wheat residues hosting *Z. tritici* (Kerdraon *et al*., 2019b), LDA and ENA were also expected to facilitate the identification of beneficial microorganisms against residue-borne pathogens, such as *L. maculans*. The stem canker caused by *L. maculans* is usually controlled by the deployment of resistant cultivars, so few studies have focused on interactions between *L. maculans* and potential biocontrol agents. We detected a few direct interactions between *L. maculans* and other microorganisms in the residue microbial community. These rare, but significant, interactions concerned both fungi (*Cryptococcus* in July, *Alternaria* in July and February, *Monographella* in October and February, *Chaetomium* in October) and bacteria (*Nocardioides*, *Sanguibacter*, and *Massilia* in October; *Altererythrobacter* and *Sphingomonas* in December; and *Acidovorax* in February). Interestingly, *Chaetomium* was previously shown to be a biocontrol agent against several pathogenic microorganisms, including *Sclerotinia sclerotiorum* in oilseed rape (Zhao *et al*., 2017). These differential interactions may also be related to some of the properties of *L. maculans*, which produces phytotoxins such as sirodesmin PL (Rouxel *et al*., 1988) that were shown to have in vitro inhibitory effects on some fungi and bacteria, while *P. biglobosus* does not produce this toxin (Pedras and Biesenthal, 2000; Elliott *et al*., 2007). However, fungi usually produce a number of toxic metabolites and, apart from the lack of production of sirodesmin PL by *P. biglobosus*, both species produce a complex set of secondary metabolites whose toxic effects have not been analysed yet (Grandaubert *et al*., 2014).

This study revealed a key role of *P. biglobosus* in stem residue communities. This fungal species has received much less attention than *L. maculans*, to which it is related. These two pathogenic species belong to the same species complex, but little is known about their effective interaction in plants. The species follow the same infectious cycle, but differ slightly in terms of their ecological niches on oilseed rape, with *L. maculans* colonizing the stem base, whereas *P. biglobosus* is restricted to upper parts of the stem (Fitt *et al*., 2006a). In Europe *P. biglobosus* is considered to be much less important than *L. maculans*. A key finding of the metabarcoding analysis was the higher RA for *Plenodomus* than for *Leptosphaeria*. By contrast, other data acquired in the same experimental area and the same cropping season (2016–2017) (Kerdraon *et al*., 2019c) highlighted similar RA values for *Leptosphaeria* (*L. maculans*) and *Plenodomus* (*P. biglobosus*) on residues of cv. Alpaga, which is susceptible to *L. maculans*. The lower RA of *Leptosphaeria* on Darmor than on Alpaga can be attributed to the high level of quantitative resistance of Darmor to *L. maculans*. Thus, when Darmor or Darmor-*Rlm11* stems are infected with low levels of *L. maculans*, *P. biglobosus* freely colonizes the stem base. The high RA of *Plenodomus* in Darmor also suggests that the high level of quantitative resistance of Darmor is not effective against *P. biglobosus*. The hypothesis that *P. biglobosus* colonizes the stem base niche in the absence of *L. maculans* or in the presence of low-level *L. maculans* colonization is consistent with the results of ENA, which detected no direct interactions between *Leptosphaeria* and *Plenodomus*. This should be also connected to the results of field studies from Australia whereby *P. biglobosus* has been

shown to colonize the stems of *Brassica juncea* plants, a species resistant to most *L. maculans* isolates (Van de Wouw et al., 2008; Elliott *et al*., 2011).

Overall, the data presented here clearly suggest that the impact of *P. biglobosus* on the development of stem canker symptoms and yield losses may have been underestimated, at least in some parts of Europe, and should therefore be reevaluated. The most abundant ASV from *Plenodomus* (93.8% of the ASVs for this genus) had a central position in the July networks, in terms of degree and betweenness centrality, thus highlighting the importance of this species in the living plant. By contrast, the noncentral position of *Leptosphaeria* in networks suggests a weak impact on the residue microbial community as a whole, and vice versa, despite the role of this fungus as the causal agent of the disease. These observations may reflect the high level of quantitative resistance to *L. maculans* in Darmor, clearly illustrated by the low RA of *Leptosphaeria* ASVs even in the absence of the *L. maculans* resistance gene *Rlm11*.

This study provides an original example of research revealing alterations to the crop residue microbiome induced by the creative modulation of pathogen levels with a resistance gene, with high-throughput DNA sequencing techniques. The dual approach based on LDA and ENA revealed that the pathogen studied here (*L. maculans*), although prominent, appeared to play a weak role in ecological interaction networks, whereas another initially neglected pathogen (*P. biglobosus*) was found to be a keystone taxon in the networks at harvest. As previously shown in studies of wheat residues hosting *Z. tritici* (Kerdraon *et al*., 2019b), this approach can help to identify beneficial microorganisms against residue-borne pathogens. More broadly, it can be used to decipher the role of complex interactions between multispecies pathosystems and other microbial components of crop residues in the shaping of a plant-protective microbiome.

4 | EXPERIMENTAL PROCEDURES

4.1 | Oilseed rape field assay

The two cultivars Darmor (INRA-SERASEM, 1984) and its derived isogenic line Darmor-*Rlm11* were sown in 1.75 × 8 m microplots on the Grignon experimental domain (Yvelines, France; 48°51′N, 01°58′E). The isogenic line Darmor-*Rlm11* was generated by introgression of the resistant gene *Rlm11* from *B. rapa* in *B. napus* as described by Balesdent *et al*. (2013). Before sowing, Darmor-*Rlm11* plants were checked as previously described (Balesdent *et al*., 2013) to confirm the purity of the seed lot. All 167 plants tested possessed *Rlm11*. Seeds were sown in September 2016 with an INOTEC single-seed speeder, at a density of 60 plants per m^2. Oilseed rape residues of a susceptible cultivar (cv. Alpaga) from the previous growing season (2015/2016) were placed on the ground 1 month later (17 October 2016), with the stubble from 50 plants added per plot to increase inoculum pressure (Brun *et al*., 2010). We checked for the presence of typical leaf lesions on plants three times between 14 November and 2 December 2016. The first lesions appeared on the leaves in early December, but disease pressure was too low for a quantitative assessment of the primary inoculum at the rosette stage. One month before full maturity (June), 60 plants of each plant genotype were collected from each plot to establish the G2 score (Aubertot *et al*., 2004) characterizing stem canker severity. Briefly, each plant was cut at the collar and disease severity (proportion of tissue necrotic due to *L. maculans*) was estimated and converted into a score from 1 (no necrosis) to 6 (100% of the section necrotic). The G2 score, ranging from 0 (no disease) to 9 (all plants lodged), was then calculated for each plot, as described by Aubertot *et al*. (2004).

4.2 | Preparation of oilseed rape residues

After harvest (July), 60 plants of Darmor and 60 of Darmor-*Rlm11* were collected. The plants were washed and portions of the stem were cut from 3 cm below and 6 cm above the crown to serve as residues. We first used residues from 15 Darmor and 15 Darmor-*Rlm11* plants to characterize the microbial communities present at harvest. We then weighed the residues from the remaining 45 plants of each cultivar and placed them in nylon bags (residues from one plant per bag). In late July, the bags were placed on the ground in a neighbouring plot in the same experimental area (wheat–oilseed rape rotation, Kerdraon *et al*., 2019c) at 15 sampling points 20 m apart, with three Darmor and three Darmor-*Rlm11* bags per sampling point.

The impact of season on the fungal and bacterial communities of the residues was assessed by collecting the residues from one Darmor and one Darmor-*Rlm11* bag on each of three dates (October, December, and February) from each sampling point (15 residue replicates per sampling date and per cultivar). The residues were rinsed with water, dried in air, and weighed to assess degradation. Residues were then crushed individually in liquid nitrogen with a zirconium oxide blender in an MM 400 mixer mill (Retsch) for 60 s at 30 Hz.

4.3 | DNA extraction, PCR, and Illumina sequencing

Total DNA was extracted with the DNeasy Plant Mini kit (Qiagen), according to the manufacturer's instructions, with minor modification as described by Kerdraon *et al*. (2019b). The internal transcribed spacer 1 (ITS1) genomic region and the v4 region of the 16S rRNA gene were amplified for the analysis of fungal and bacterial community profiles, respectively. Two rounds of amplifications were performed following the standard operating procedures for building amplicon libraries. The first round of amplifications (30 cycles of PCR) was performed with the ITS1F/ITS2 (Buée *et al*., 2009) and 515f/806r (Caporaso *et al*., 2011) primers and the Type-it Microsatellite PCR kit, as described by Kerdraon *et al*. (2019b). The amplicons were then sent to an external sequencing platform (Genomics and Transcriptomics GeT core facility, Genotoul, Toulouse, France) for the second round of amplification (12 cycles) performed with primers containing Illumina adapters and indices

(Kerdraon *et al*., 2019b). Libraries were sequenced in one run, with MiSeq reagent kit v. 3 (600 cycles).

4.4 | Quantification of *L. maculans* and *P. biglobosus* by quantitative real-time PCR

We first checked whether, under the conditions of our study, there was a quantitative relationship between biomass and the number of sequencing reads generated by metabarcoding analysis (Lamb *et al*., 2019). We quantified the DNA of *P. biglobosus* and *L. maculans* by qPCR in 33 of the residue samples used for our metabarcoding analysis (same DNA used for both experiments). For the quantification of *L. maculans* (LM assay), the forward primer LM_EF1-F5 (5′-TGGACACTTCTTCTTGACAA-3′), reverse primer LM_EF1-F5 (5′-TGGACACTTCTTCTTGACAA-3′), and probe LM_P (5′-TACCACGTTCACGCTCGGCC-3′) were designed based on the partial sequence of the EF1α gene. For the quantification of *L. biglobosa* (LB assay), the forward primer LB_ACT_F1 (5′-TTGAGAGCGGTGGCATCCA-3′), reverse primer LB_ACT_R2 (5′-CACCAGACTGTGTCTTTGTC-3′), and probe LB_P (5′-ACGATGTTGCCGTAGAGGTCTTTC-3′) were designed based on the partial sequence of the actin gene. For each assay (LM or LB) the reaction mixture contained 5 μl of sample DNA, 12.5 μl of 2 × qPCR MasterMix Plus without UNG (Eurogentec), 100 mM probe, and 300 mM forward and reverse primers, in a total volume of 25 μl. The quantification cycle (Cq) values were acquired for each sample with the CFX96 Real Time PCR Detection System (Bio-Rad) under the following cycling conditions: 10 min at 95°C for initial denaturation; 40 cycles of amplification consisting of heating at 95°C for 15 s and 1 min at the annealing temperature of 60°C for LM assay and 62°C for LB assay. For the standard calibration curve, reference DNA concentrations from 5 to 0.005 ng were analysed in triplicate. A standard curve was generated for each PCR plate. The fluorescence threshold was automatically calculated with Bio-Rad CFX Manager software. The results of the qPCR experiments were compared with the numbers of reads obtained for *Leptosphaeria* or *Plenodomus*. A Spearman's rank correlation test was performed with the R package "car" to investigate the relationship between the two variables (Fox and Weisberg, 2018).

4.5 | Sequence processing

Primer sequences were removed with Cutadapt v. 1.8 (Martin, 2011). Fastq files were processed with DADA2 v. 1.8.0 (Callahan *et al*., 2016) as recommended in the DADA2 Pipeline Tutorial (1.8) (Callahan, 2020) using the following parameters: truncLen = c(200, 150), maxN = 0, maxEE = c(1, 1), and truncQ = 5. A mock sample composed of DNA from known microorganisms was included in the sequencing run (Figure S5) to establish a detection threshold for spurious haplotypes. At a threshold of ≤3‰ of the library size, ASVs were considered spurious and were removed from the sample. Taxonomic affiliations of ASVs were performed with a naive Bayesian classifier (Wang *et al*., 2007) implemented in DADA2. ASVs derived from 16S rRNA gene and ITS1 reads were classified at a minimum bootstrapping threshold of 50 with the RDP trainset 14 (Cole *et al*., 2009) and the UNITE7.1 database (Abarenkov *et al*., 2010), respectively. We used the default parameter of the "assignTaxonomy" function with a minimum bootstrapping threshold of 50. ASVs classified as chloroplasts (for bacteria) or unclassified at the phylum level were also removed from each sample. After quality filtering, the median read numbers were 27,122 and 26,436 for 16S rRNA gene and ITS1 sequences, respectively (Figure S6).

4.6 | Microbial community analysis

Normalization by proportion was performed to standardize total library size for each sample. The effects of season (sampling date) and the presence of *Rlm11* on alpha diversity were assessed with the Shannon index, complemented with the observed richness (number of ASVs) and the Pieoule's index (taxa evenness). The phylogenetic diversity was not estimated because of the polymorphism in size of ITS1 that hampered sequence alignment. The divergences in microbial community composition, illustrated by MDS (ape package v. 5.2; Paradis and Schliep, 2019), were assessed with the Bray–Curtis dissimilarity matrix, calculated with the phyloseq v. 1.24.2 package (McMurdie and Holmes, 2013). The effects of season and of the presence of *Rlm11* on microbial community structure were investigated by performing PERMANOVA with the "margin" option (ADONIS2 function, "vegan" package; Oksanen *et al*., 2017). The "PairwiseAdonis" function was used to characterize the divergence between conditions in post hoc tests (Martinez Arbizu, 2019).

4.7 | Influence of the *Rlm11* gene and the presence of *L. maculans* on the residue microbiome

The impact of the presence of *Rlm11* on the microbial communities was investigated by performing a LDA for each sampling date with LefSe (Segata *et al*., 2011) implemented in Galaxy (http://huttenhower.org/galaxy). LefSe uses the Kruskal–Wallis rank-sum test with a normalized relative abundance matrix to detect features with significantly different abundances between assigned taxa and performs LDA to estimate the effect size of each feature. The alpha risk for the Kruskal–Wallis test was set at 0.05 and the effect-size threshold was set at 2.0.

For the identification of interactions between *L. maculans* and other organisms, ENA was performed for each time point for both cultivars (Darmor and Darmor-*Rlm11* samples considered together). Networks were computed with SPIEC-EASI (Kurtz *et al*., 2015) from combined bacterial and fungal data sets (Tipton *et al*., 2018) for each sampling date (30 samples per network). Network sensitivity was increased by removing rare ASVs (Berry and Widder, 2014) by defining

a minimum threshold of six occurrences. The Meinshausen and Bühlmann (MB) method was used as graphical inference model for the four networks (Kurtz *et al*., 2015). The StARS variability threshold was set at 0.05. The betweenness centralities of each node and node degree were estimated with the igraph v. 1.2.2 package (Csardi and Nepusz, 2006).

The two analyses—LDA and ENA—were combined by constructing subnetworks focusing on the interactions between ASVs identified as differential in Darmor relative to Darmor-*Rlm11* by the LDA approach. The differential ASVs highlighted by LDA and their adjacent nodes were used for the analysis of subnetworks constructed with Cytoscape v. 3.6.1 (Shannon *et al*., 2003). The scripts for network construction and analysis are available from GitHub.

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AUTHORS' CONTRIBUTIONS

L.K., V.L., F.S., M.H.B., and M.B. conceived the study, participated in its design, and wrote the manuscript. L.K. conducted the experiments and analysed the data. F.S. and V.L. supervised the project. All the authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The raw sequencing data are available from the European Nucleotide Archive at https://www.ebi.ac.uk/ena under study accession number PRJEB35369 (samples ERS4024677 to ERS4024484). We provide the command-line script for data analysis and all necessary input files via GitHub (https://github.com/LydieKerdraon/2019_OilseedRape).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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