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
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 step 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, poligenic 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, poligenic 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 S2 and S3). 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 S4).

Some genera, such as Cryptococcus, Alternaria, and Rhodococcus, decreased in abundance or disappeared over time, whereas others, such as Torula, Schizotectum, 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 ($p = 0.93$, $p < .0001$ for L. maculans; $p = 0.60$, $p < .001$ for P. biglobosus; Figure S5), 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   |                         | Darmor-Rlm11   |                         |
|----------------|----------------|-------------------------|----------|-------------------------|----------------|-------------------------|
|                | Jul  | Oct  | Dec  | Feb  | Jul  | Oct  | Dec  | Feb  | Jul  | Oct  | Dec  | Feb  | Jul  | Oct  | Dec  | Feb  |
| Darmor-Rlm11   |      |      |      |      |      |      |      |      | 0.286 | 0.105 | 0.132 | 0.333 | 0.346 | 0.137 | 0.056 | 0.217 |
|                | 0.211 | 0.041 |      |      | 0.212 | 0.212 | 0.446 | 0.217 | 0.339 | 0.217 | 0.217 | 0.069 | 0.446 | 0.339 | 0.217 | 0.069 |
| Darmor         | 0.142 | 0.202 | 0.243 | 0.337 |      |      |      |      |      |      |      |      |      |      |      |      |
|                | 0.223 | 0.087 | 0.084 | 0.155 | 0.124 | 0.276 | 0.316 | 0.435 | 0.124 | 0.276 | 0.316 | 0.435 | 0.124 | 0.276 | 0.316 | 0.435 |
|                | 0.254 | 0.083 | 0.048 | 0.089 | 0.171 | 0.041 |      |      | 0.171 | 0.041 |      |      |      |      |      |      |
|                | 0.352 | 0.113 | 0.064 | 0.064 | 0.283 | 0.095 | 0.057 |      | 0.283 | 0.095 | 0.057 |      |      |      |      |      |

The significance of divergences was assessed by calculating a q value.

**q < 0.01.
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 \textit{Rlm11}. For example, ASVs affiliated to \textit{Sphingomonas} preferentially colonized Darmor residues in October and \textit{Uliginosibacterium} preferentially colonized Darmor-\textit{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-\textit{Rlm11}, including \textit{Pantoea}, which was more abundant in Darmor-\textit{Rlm11} residue samples in July.

### 2.5 Direct impact of \textit{L. maculans} on microorganisms

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

Ecological interaction networks (ENA) combining bacterial and fungal data sets were built to characterize the interactions between \textit{Leptosphaeria} (probably \textit{L. maculans}) and the other members of the bacterial and fungal communities at each of the four...
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 taxa 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...
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

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**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 analyses (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).
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 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. Isolab) 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), $Leptosphaeria$, 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)
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, comm. pers.). 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 genera 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 (Nocardioles, 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 pathogens 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 x 8 m microplots on the Grignon experimental domain (Yvelines, France; 48°51′N, 1°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 m². 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 a 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.
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′-TACCAAGTTCAGCTCGGCC-3′) were designed based on the partial sequence of the EF1 alpha gene. For the quantification of *L. biglobosa* (LB assay), the forward primer LB_ACT_F1 (5′-TTTGAAGGGTGGCATCCA-3′), reverse primer LB_ACT_R2 (5′-CACCAGACTTGTGTCTTTGTC-3′), and probe LB_P (5′-AGATGGTGGCCGTAGAGGTCGTTCT-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 v.1.8 (Callahan) 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 S6) 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 S1).

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 Picolou’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 phylseq 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 linear differential analysis (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, ecological interaction analysis (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).

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-Rm11 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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