SOIL FUNGAL BIODIVERSITY FOR PLANT AND SOIL HEALTH

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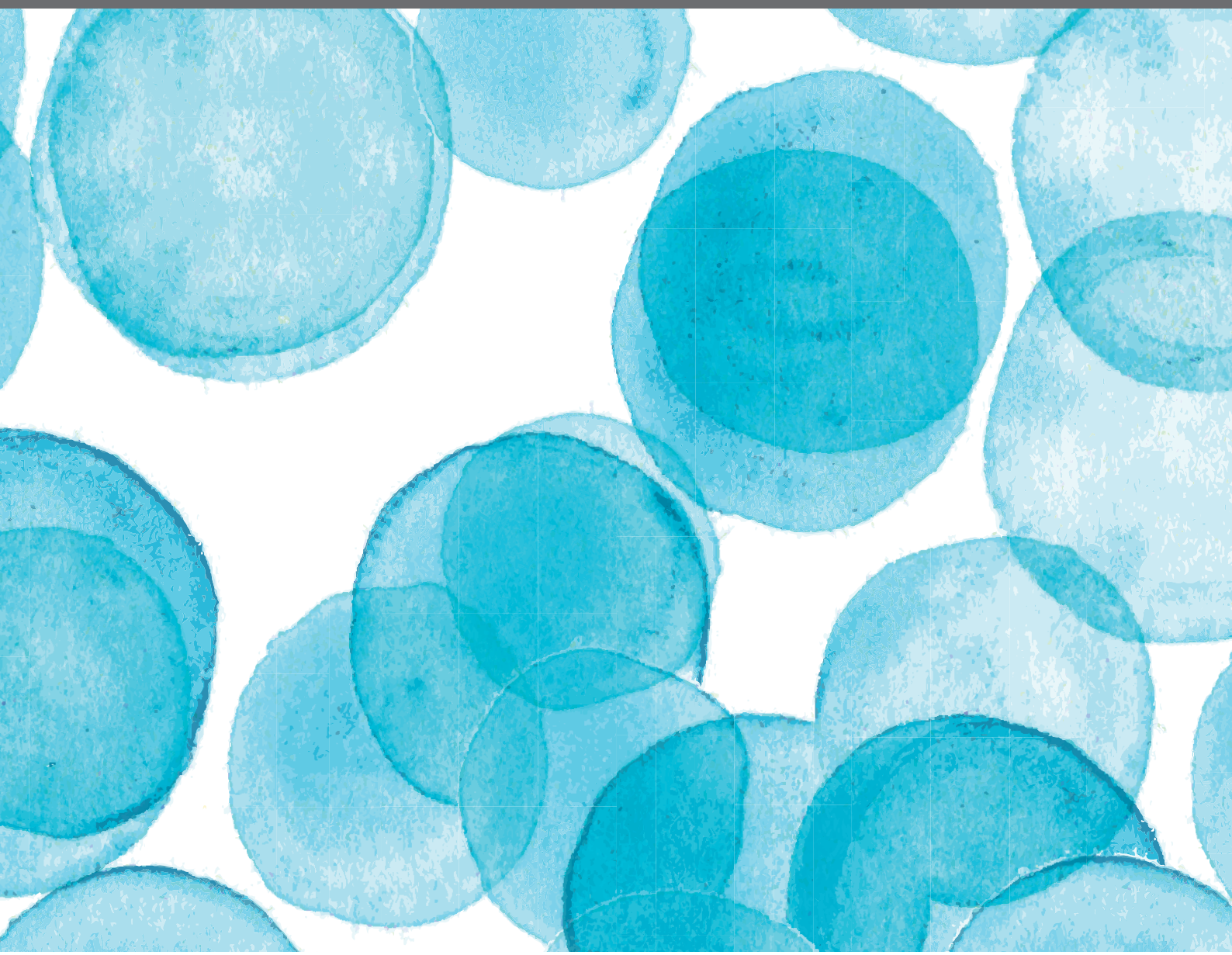

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SOIL FUNGAL BIODIVERSITY FOR PLANT AND SOIL HEALTH

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Fungi represent a large portion of the biodiversity on Earth and they are key players in soils where they provide numerous ecosystem functions. Soil fungi have pivotal ecological roles influencing plant health as symbionts, pathogens or decomposers. Soil fungal biodiversity is increasingly recognized as providing benefits to soil health as they facilitate if not control numerous ecosystem processes. Continued research on the identity, abundance and distribution of soil fungi, their various roles in context with the differentiation of the soil fungal community are thus fundamental to better understand the dimensions of fungal biodiversity, its impact on plant health as well as the prevention of fungal diseases.

This Research Topic aims at collecting contributions that provide taxonomic, physiological and ecological characterizations of soil fungal communities that will aid in the understanding of their biology, their interrelationships as well as the mechanisms that underpin the various ecosystem functions they provide in the soil environment.

This Research Topic focusing on environmental mycology encourages in particular to report sensitive, accurate and fast methods for the detection, identification and distribution of fungi, including metagenomics, metatranscriptomics and metabolomics approaches, as they increasingly reveal the impact of fungal biodiversity for soil and plant health.

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Fungal Biodiversity and Their Role in Soil Health

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Soil health, and the closely related terms of soil quality and fertility, is considered as one of the most important characteristics of soil ecosystems. The integrated approach to soil health assumes that soil is a living system and soil health results from the interaction between different processes and properties, with a strong effect on the activity of soil microbiota. All soils can be described using physical, chemical, and biological properties, but adaptation to environmental changes, driven by the processes of natural selection, are unique to the latter one. This mini review focuses on fungal biodiversity and its role in the health of managed soils as well as on the current methods used in soil mycobiome identification and utilization next generation sequencing (NGS) approaches. The authors separately focus on agriculture and horticulture as well as grassland and forest ecosystems. Moreover, this mini review describes the effect of land-use on the biodiversity and succession of fungi. In conclusion, the authors recommend a shift from cataloging fungal species in different soil ecosystems toward a more global analysis based on functions and interactions between organisms.

Keywords: soil health, soil ecosystem, microbial communities, fungal diversity, fungal functions, fungal plant pathogens, soil biology, soil mycobiome

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FUNGI IN SOILS

Fungi are very successful inhabitants of soil, due to their high plasticity and their capacity to adopt various forms in response to adverse or unfavorable conditions (Sun et al., 2005). Due to their ability to produce a wide variety of extracellular enzymes, they are able to break down all kinds of organic matter, decomposing soil components and thereby regulating the balance of carbon and nutrients (Žifčáková et al., 2016). Fungi convert dead organic matter into biomass, carbon dioxide, and organic acids (**Figure 1**). Many species of fungi possess the ability to act as an effective biosorbent of toxic metals such as cadmium, copper, mercury, lead, and zinc, by accumulating them in their fruiting bodies. Though these elements may inhibit their growth and affect their reproduction (Baldrian, 2003). The diversity and activity of fungi is regulated by various biotic (plants and other organisms) and abiotic (soil pH, moisture, salinity, structure, and temperature) factors (López-Bucio et al., 2015; Rouphael et al., 2015). Fungi can be found in almost every environment and can live in wide range of pH and temperature (Frąc et al., 2015).

Soil fungi can be classified into three functional groups including: (1) biological controllers, (2) ecosystem regulators, and (3) species participating in organic matter decomposition and compound transformations (Swift, 2005; Gardi and Jeffery, 2009). Ecosystem regulators are responsible for soil structure formation and modification of habitats for other organisms by regulating the dynamics of physiological processes in the soil environment. Biological controllers

can regulate diseases, pests, and the growth of other organisms (Bagyaraj and Ashwin, 2017). For example, the mycorrhizal fungi improve plant growth by increasing the uptake of nutrients and protect them against pathogens (Bagyaraj and Ashwin, 2017).

Fungal populations are strongly influenced by the diversity and composition of the plant community and in return affect plant growth through mutualism, pathogenicity and their effect on nutrient availability and cycling (Wardle, 2002; Wagg et al., 2014; Hannula et al., 2017). Moreover, fungi participate in nitrogen fixation, hormone production, biological control against root pathogens and protection against drought (Jayne and Quigley, 2014; Baum et al., 2015; El-Komy et al., 2015). They also play an important role in stabilization of soil organic matter and decomposition of residues (Treseder and Lennon, 2015).

METHODS AND RECENT ACHIEVEMENTS IN STUDIES OF SOIL-BORNE FUNGI

The advent of next generation sequencing (NGS) has facilitated a sea-change in the analysis of soil and plant-associated fungal communities. Standardized pipelines for preparing rhizosphere soil samples for Illumina sequencing are widely available (Lindahl et al., 2013; Schöler et al., 2017) and in a relatively short time following sampling, files with millions of sequences can be generated. Important points to consider when preparing samples for NGS are: sufficient biological replication (Prosser, 2010), sufficient sequencing depth (Smith and Peay, 2014; Weiss et al., 2015), adequate coverage of target organisms (i.e., primer selection and DNA extraction), and the avoidance of contamination and bias (Salter et al., 2014; Schöler et al., 2017). There are multiple pipelines for the analysis of fungal NGS data available (Bálint et al., 2014; Gweon et al., 2015), so it is not data-analysis that is problematic, but the interpretation of results.

Alpha-diversity, representing either the number of species or diversity indices that account for evenness, was proposed as an indicator for robust, healthy soil (Ferris and Tuomisto, 2015). However, it is open to question whether absolute diversity or functional diversity should be emphasized (Wagg et al., 2014; Ferris and Tuomisto, 2015). Usually the second step in analysis is to look at beta-diversity to see the effects of treatment/manipulation on the fungal community. Recently, it has been shown that fungal biodiversity in soils is strongly affected by plant community (Yang et al., 2017), soil moisture (Bagyaraj and Ashwin, 2017), and the intensity of agricultural practices (Thomson et al., 2015). Studies identified key fungal species affected by soil treatments, but it is unknown if results obtained from studies conducted on one particular soil and ecosystem can be used to infer trends and identify key fungal groups at a global or continental scale (Tedersoo et al., 2014; Delgado-Baquerizo et al., 2017).

Unlike the analysis of bacteria and archaea, where 16S rRNA is used as a barcode, fungi are usually identified based on the sequence of the Internal Transcribed Spacer (ITS) region allowing identification up to species level (Schoch et al., 2012; Porras-Alfaro et al., 2014). Although in some cases, such as for soil-dwelling Fusaria, sequencing of additional genes, such as β-tubulin gene (β-*Tub*), and aminoadipate reductase gene (*LYS2*) was proposed to obtain the correct taxonomic identification at the species level (Watanabe et al., 2011), whereas others have suggested the use of Translation Elongation Factor (Geiser et al., 2004). Many researchers have in-house databases for functional classification of fungi but recently online resources for functional annotation of fungi have been made publicly available (Nguyen et al., 2016).

Species level identification is, however only the first step from 'What is there?' toward the question 'What role does it play?' (**Figure 2**). Identification neither implies the microorganisms are alive and active (Blagodatskaya and Kuzyakov, 2013) nor does it describe their function (Prosser, 2015). To unravel the function of the community, either (shotgun) metagenomics (Uroz et al., 2013; Hannula and van Veen, 2016; Castañeda and Barbosa, 2017), metatranscriptomics (Damon et al., 2012; Turner et al., 2013; Hesse et al., 2015) or time-intensive culture based methods combined with functional tests must be used (Behnke-Borowczyk et al., 2012; Gałązka et al., 2017; Wolińska et al., 2017). A fast, but coarse alternative to molecular methods is MicroResp (Creamer et al., 2016) or community level physiological profiles (CLPP) approach (Frąc et al., 2017), which gives an indication on substrate use of the total microbial community. This method, however, does not identify the species responsible for the process. Increasingly, especially in studies where plant community is included alongside NGS approaches, microorganisms are isolated from the soils and plant roots for further functional testing.

To construct a more complete picture of a soil fungi community their interactions with other organisms must be taken into consideration. Strong linkage was proved between functional soil biodiversity and the function of the soil ecosystem (Wagg et al., 2014; Delgado-Baquerizo et al., 2017; Morrien et al., 2017). Fungi interact with other soil organisms and thus changes in the fungal community have the potential to affect the function of the whole soil ecosystem (Yang et al., 2017). Analysis of the interactions in the soil can be achieved through indirect estimation of species interactions using co-occurrence networks (Creamer et al., 2016; Morrien et al., 2017) or directly by using isotope tracers (Hannula et al., 2017) and/or gut content analysis (Kurakov et al., 2016).

FUNGAL BIODIVERSITY AND THEIR FUNCTIONS IN SOIL HEALTH OF AGRICULTURAL AND HORTICULTURAL ECOSYSTEMS

The term 'soil health' is widely used in reference to sustainable agriculture (Kibblewhite et al., 2008; Cardoso et al., 2013), especially in the context of soil as a dynamic, living organism functioning holistically rather than as an inert substrate (Doran and Jones, 1996). Therefore, in this article we prefer to use the terminology of soil health, rather than soil quality, which is defined as the capacity of the soil to maintain environmental quality, sustain biological productivity, and promote animal, human, and plant health (Doran and Parkin, 1994). In

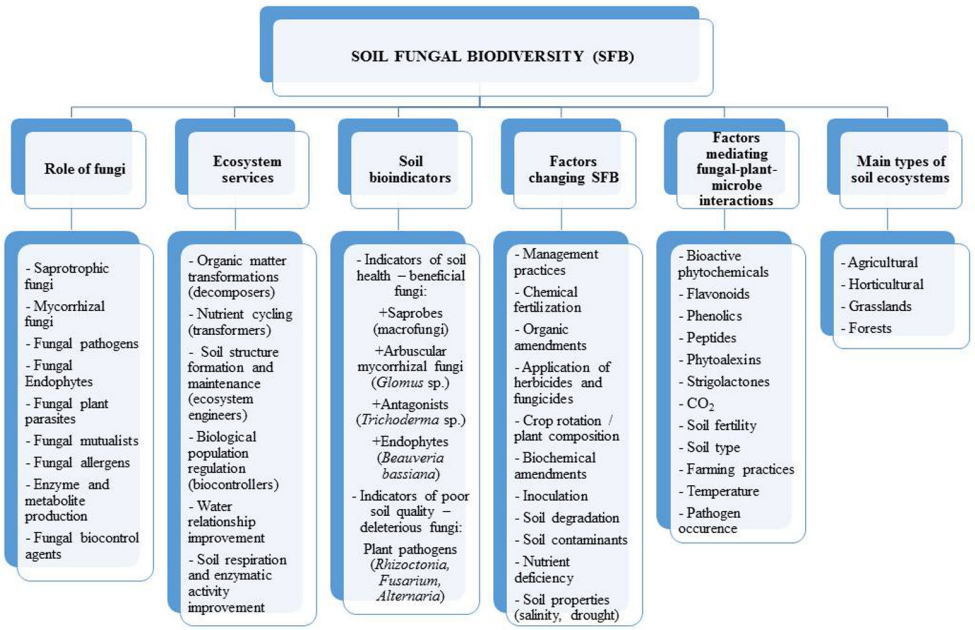

FIGURE 1 | Aspects of soil fungal biodiversity.

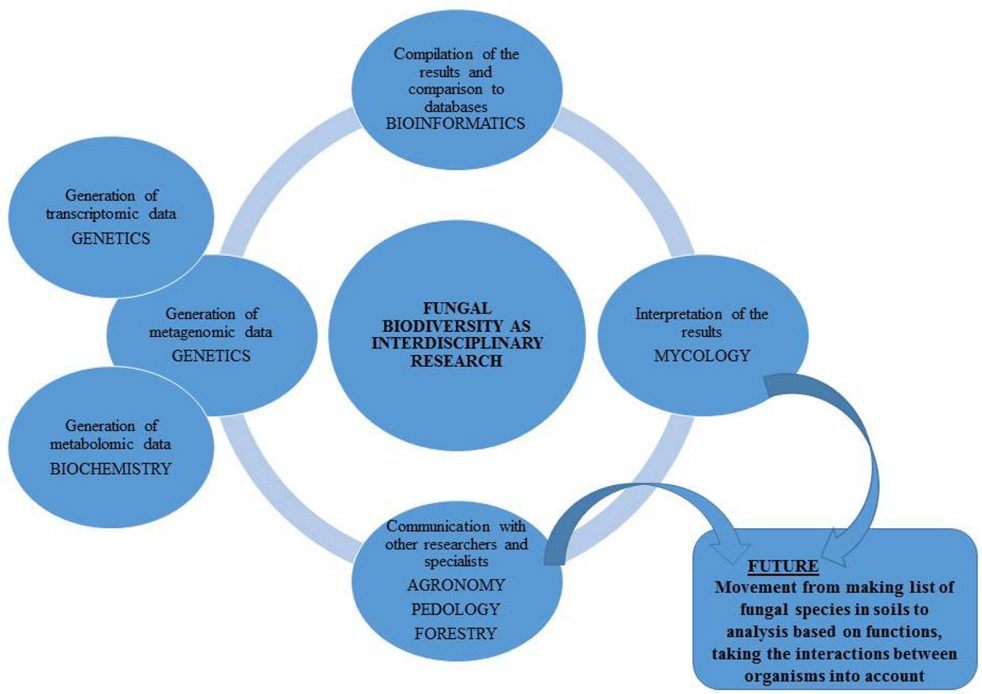

FIGURE 2 | Soil fungal biodiversity as an interdisciplinary research.

recent years the potential application of cultivating soil fungal biodiversity to improve soil quality and increase productivity of agricultural ecosystems has been highlighted as a new and very promising development in plant productivity (Bagyaraj and Ashwin, 2017), which may come be called 'the 2nd Green Revolution.' The implementation of such solutions may offer an alternative to the current overuse of fertilizers toward more sophisticated manipulations of plant productivity. Fungi

participate in decomposition of organic matter and deliver nutrients for plant growth. Their role is very important in plant protection against pathogenic microorganisms as biological agents, which influences soil health (Frąc et al., 2015). The assessment of fungal biodiversity as quality indicators cannot be limited only to the determination of biodiversity indexes, but also should include a structure analysis of fungal population in order to determine the functions they play in affecting soil quality and plant health. The use of different kinds of organic manure has a strong influence on soil health, through indirect effects (i.e., via changes in physicochemical characteristics) and a direct effect on soil fungal communities. Soil management is fundamental to all agricultural systems, and the reduction of soil degradation is a priority to sustain future production. This effect can be only achieved by taking soil fungal biodiversity into account. All cultural practices, such as the use of cover and rotational crops, composts and tillage systems, besides their known effects on soil-borne pathogens (Abawi and Widmer, 2000), are likely to affect also the other groups of soil fungi, especially beneficial fungal populations. It has long been known that the suppressiveness of soils can be enhanced by adding biopolymers such as chitin and its derivatives (i.e., chitosan). This suppressiveness is related to a change in the activity and structure of soil microorganisms (Cretoiu et al., 2013). Therefore, we should utilize our knowledge on the interactions between different fungal groups and their ecology in the management of agricultural systems. It is worth mentioning that chitin addition to the soil increases bacteria and fungi that can degrade pathogenic fungal cell walls and can thus, increase the soil suppressiveness against plant pathogens. This might be a good alternative to fungicides that kill all fungi, including beneficial ones. Different tillage treatments can also impact soil fungi by soil disturbances that affect the functioning of fungal communities. Reduced tillage decreases the breakdown of hyphae causing fungal populations to remain more stable, retaining more nutrients and providing more suppressive effects against pathogenic microorganisms (Goss and deVarennes, 2002). Understanding and selecting the appropriate cultural practices, increasing fungal biodiversity, can prevent or decrease damage of root diseases and play a crucial role in the maintenance of soil quality and health. It should be taken into account that fungal diversity determines plant biodiversity, ecosystem variability, and productivity (van der Heijden et al., 1998; Wagg et al., 2014).

Arbuscular mycorrhizal fungi (AMF) are the most important class of beneficial microorganisms in agri- and horticultural soils (Smith and Read, 2008, **Table 1**). Significant increases in the yield of crop plants following inoculation with AMF have been observed in numerous experiments (Thilagar and Bagyaraj, 2015; Bagyaraj and Ashwin, 2017). The key effects of AMF symbiosis include: improvement of rooting and plant establishment, stimulation of nutrient cycling, improvement of soil structure, enhancement of plant tolerance to stresses, increased uptake of low mobility ions, and enhancement of plant community diversity (Azcón-Aguilar and Barea, 1997). The diseases of crop plants can be controlled by some antagonistic fungi such as *Glomus* sp. or *Trichoderma* sp. suppressing fungal pathogens (Dawidziuk et al., 2016). Species of *Trichoderma* (*T. asperellum*, *T. atroviride*, *T. harzianum*, *T. virens*, and *T. viride*) are frequently used in biocontrol and are known as biostimulants for horticultural crops (López-Bucio et al., 2015). Other positive effects of fungi on soil quality and plant health include inoculation by microbial consortia of AMF together with plant growth promoting rhizobacteria (PGPR) and others such as N-fixing and P-solubilizing microorganisms (Bagyaraj and Ashwin, 2017). A synergistic, favorable impact of AMFs and PGPRs on horticultural plant growth and soil microbial diversity and activity has been reported (Azcón-Aguilar and Barea, 1997; De Coninck et al., 2015).

Besides beneficial fungi, agri- and horticultural ecosystems contain also plant pathogens. The major groups of soil-borne root pathogenic fungi and oomycetes constitute of genera *Fusarium* (Michielse and Rep, 2009), *Verticillium* (Klosterman et al., 2009), *Rhizoctonia* (Gonzalez et al., 2011), *Pythium, Phytophthora* (van West et al., 2003) and many others, of global and local importance. The soil fungal diversity and methods of increasing it, particularly the populations of beneficial fungi within ecosystems should be used in practice for more sustainable plant production, decrease of chemical applications and protection of the soil environment.

FUNGAL BIODIVERSITY AND THEIR FUNCTIONS IN SOIL HEALTH OF GRASSLAND ECOSYSTEMS

Soil microorganisms, including fungi are an important component of grassland ecosystems due to their biochemical activity and engagement in nutrient cycling (Dengler et al., 2014). Grasslands provide many forms of ecosystem services including: supporting, provisioning, regulatory, and cultural services. Importantly, the role of biodiversity has been established as fundamental in ensuring the performance of ecosystem functioning. Grazing activities influence soil fungal community structure by changing edaphic conditions and the vegetation biodiversity in plant communities (Yang et al., 2017). It has been proven that moderate grazing sustains plants diversity while heavy grazing results in species loss (Joubert et al., 2017). Furthermore, plant-fungal interactions can inhibit biodiversity in grasslands due to the production of different root exudates such as enzymes, organic compounds, and polysaccharides (Huhe et al., 2017).

Plant pathogenic fungi also have a large impact on plant diversity in grasslands by limiting the abundance of their hosts, affecting biomass production. The study by Allan et al. (2010) suggests that fungal pathogens could affect nutrient cycling in grasslands reducing the abundance of dominant grasses and enhancing the growth of legumes. Soil fungal communities in grasslands can also be influenced by human activities and the components of long-term fertilization and other treatments (Cassman et al., 2016). Unlike in agricultural soils, where ascomycetes dominate, in grasslands, basidiomycetes are major decomposers of dead organic matter (Deacon et al., 2006).

TABLE 1 | Fungal community composition in different soil ecosystems and their function.

Ecosystem	Fungal composition	Fungal function/reaction	Reference
Agricultural	*Agaricales* *Hypocreales*	Increase in drought-affected soils	Bastida et al., 2017
	Sordariales *Capnodiales* *Eurotiales*	Reduction in soils affected by drought	
	Mortierella *Fusarium* *Gibberella*	The dominant fungi in NPK treated soils	Ding et al., 2017
	Mortierella *Fusarium* *Schizothecium*	The dominant fungi in manure treated soils	
	Ascomycota *Sordariomycetes* *Eurotiomycetes* *Dothideomycetes*	Key decomposers in agricultural soils. Increase after nitrogen fertilization	Žifčáková et al., 2016
	Leotiomycetes *Helotiales*	Decline in N and P treated soils	
	Cyphellophora *Penicillium* *Chloridium* *Trichoderma* *Acremonium*	Increase in fertilized soils	
	Exophiala *Clonostachys* *Sarocladium* *Schizothecium* *Magnaporthe* *Phaeosphaeriopsis*	Decrease in N and P fertilized soils	
Horticultural	*Glomus* *Gigaspora* *Scutellospora* *Acaulospora* *Entrophospora*	Arbuscular mycorrhizal fungi (AMF) which improve plant growth by increasing phosphorus and the uptake of other nutrients	Smith and Read, 2008; Bagyaraj and Ashwin, 2017
	Trichoderma such as: *T. asperellum* *T. atroviride* *T. harzianum* *T. virens* *T. viride*	Biostimulants and biocontrol agents, suppressing fungal pathogens like *Fusarium* sp., *Colletotrichum* sp., or *Rhizoctonia* sp.	López-Bucio et al., 2015
Grasslands	*Hygrocybe* *Camarophyllopsis* *Dermoloma* *Entoloma* Clavariaceae Geoglossaceae	Saprotrophs, decomposers	Arnolds, 2001; Öster, 2006; Hannula et al., 2017
	Glomus	Play a dominant role under different conditions of grasslands as AMF	Johnson et al., 2003; Santos-González, 2007
	Agaricomycotina *Saccharomyceta* *Paraglomerales* *Chytridiales* *Archaeosporales* *Lobulomycetales* *Rhizophydiales* *Glomerales*	AMF, pathogens or decomposers	Cassman et al., 2016
	Acaulospora *Glomus* *Scutellospora*	AMF important for soil and plant health, the species richness decrease in severely degraded grasslands	Cai et al., 2014
	Ascomycota Glomeromycota	Increase in Ascomycota and decrease in Glomeromycota after N and P addition into the soil. Glomeromycota phylum is composed almost entirely of AMF	Leff et al., 2015

(Continued)

TABLE 1 | Continued

Ecosystem	Fungal composition	Fungal function/reaction	Reference
	Hypocreales Pezizales Dothideales (*Aureobasidium*)	Most of them is known as plant pathogens	Porras-Alfaro et al., 2011
	Pleosporales such as: *Alternaria* *Cochliobolus/Bipolaris* *Phaeosphaeria* *Leptosphaeria* *Phoma*		
	Saprotrophic fungi	Decomposers which convert organic matter and produce enzymes	Lucas et al., 2007; Porras-Alfaro et al., 2011
Forests	*Armillaria* *Phellinus* *Cronartium* *Arceuthobium*	Pathogen of trees	Winder and Shamoun, 2006; Wakelin et al., 2014
	Clonostachys candelabrum *Geomyces pannorum* *Penicillium adametzii* *P. commune* *P. daleae,* *P. janczewskii* *Trichoderma*	Antagonistic microbes suppressing soil-borne plant pathogens	Pal et al., 2006; Małecka et al., 2015
	Basidiomycota Ascomycota	Ectomycorrhizal mutualists which protect plant families such as Pinaceae, Fabaceae, Betulaceae, and Fagaceae	Rillig and Mummey, 2006; Phosri et al., 2012
	Macrofungi	Biosorbents of toxic metals and compounds	Baldrian, 2003
	Tremellomycetes Dothideomycetes	The dominant fungal class in forest soil	Liu et al., 2015

FUNGAL BIODIVERSITY AND THEIR FUNCTIONS IN SOIL HEALTH OF FOREST ECOSYSTEMS

Knowledge of the soil chemical and physical properties has always been of interest to foresters to evaluate the capacity of sites and to increase forest productivity (Schoenholtz et al., 2000). Forest soils (including humus, litter, and coarse woody debris) are an important reservoir of microorganisms and soil biota that in turn influence carbon storage, soil structure, fertility, productivity, and plant/tree growth.

Ectomycorrhizal associations are created by a specific group of plant families that includes the Pinaceae, Fabaceae, Betulaceae, and Fagaceae (Phosri et al., 2012). The results of research obtained by Högberg and Högberg (2002), indicate a significant contribution by ectomycorrhizal mycelium to forest soil microbial biomass and by ectomycorrhizal roots to the production of extractable dissolved organic carbon, which is a carbon source for other microbes.

During the processes of thinning, the transfer of nutrients from aboveground biomass to forest soil takes place (Tian et al., 2010). A higher concentration of nutrients comes from the green litter of thinned trees than litter returned to the forest floor after senescence (Girisha et al., 2003) or from the woody residue left on the ground after harvesting (Cookson et al., 2008). Consequently, the quality and quantity of organic substrates presented to the soil fungal community by thinned and non-thinned forests may vary to a great extent. The community of soil microorganisms depends highly on organic matter as it provides a suitable environment and energy sources for them that are critical to maintain the nutritional quality and water-retaining capacity of forest soils (Jiménez-Morillo et al., 2016). Soil organic matter is of key relevance in maintaining soil resistance and stability, although it is uncertain how deterioration of soil properties and changes in fungal communities affect the functional stability of soils. Degradation of soil properties followed by deforestation may lead to decreases in soil fungal diversity and functional stability (Chaer et al., 2009).

CONCLUDING REMARKS

Soil health conditions have a tremendous impact on environmental sustainability including sustainability in agriculture, horticulture, and forestry. Moreover, soil health is directly connected with the production of healthy food which impacts public and animal health. More research is required to find the best way to maintain fungal biodiversity in soil, taking into consideration fungal functions and ecosystem services, including disease control, contamination detection,

and bioremediation. Having the right tools, and being able to both identify species and characterize their role in the environment is important. The ability to compare functional structures between ecosystems and predict responses to environmental changes and interventions would be a useful advance.

AUTHOR CONTRIBUTIONS

MF, SH, MB, and MJ: wrote, drafted, read, corrected, improved, revised, and accepted the last version of manuscript.

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Metabolic and Genetic Properties of *Petriella setifera* Precultured on Waste

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Although fungi that belong to *Petriella* genus are considered to be favorable agents in the process of microbial decomposition or as plant endophytes, they may simultaneously become plant pests. Hence, nutrition factors are supposed to play an important role. Therefore, it was hypothesized that *Petriella setifera* compost isolates, precultured on three different waste-based media containing oak sawdust, beet pulp (BP) and wheat bran (WB) will subsequently reveal different metabolic properties and shifts in genetic fingerprinting. In fact, the aim was to measure the influence of selected waste on the properties of *P. setifera.* The metabolic potential was evaluated by the ability of five *P. setifera* strains to decompose oak sawdust, BP and WB following the MT2 plate® method and the catabolic abilities of the fungus to utilize the carbon compounds located on filamentous fungi (FF) plates®. Genetic diversity was evaluated using Amplified Fragment Length Polymorphism analysis performed both on DNA sequences and on transcript-derived fragments. *P. setifera* isolates were found to be more suitable for decomposing waste materials rich in protein, N, P, K and easily accessible sugars (as found in WB and BP), than those rich in lignocellulose (oak sawdust). Surprisingly, among the different waste media, lignocellulose-rich sawdust-based culture chiefly triggered changes in the metabolic and genetic features of *P. setifera*. Most particularly, it contributed to improvements in the ability of the fungus to utilize waste-substrates in MT2 plate® and two times increase the ability to catabolize carbon compounds located in FF plates®. Expressive metabolic properties resulting from being grown in sawdust-based substrate were in accordance with differing genotype profiles but not transcriptome. Intraspecific differences among *P. setifera* isolates are described.

Keywords: *Petriella setifera*, genetic and catabolic diversity, lignocellulose utilization, waste debris

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INTRODUCTION

Fungi represent a large share of the total biodiversity on Earth and they are regarded as key players in performing numerous ecosystem functions, especially in soil. It has been established that there is a link between the soil fungal community structure and function and to extent to which the soil is intensively managed (Knoblauch et al., 2017). It is also widely acknowledged that organic waste, applied as biofertilizers or exogenous organic matter (EOM) is superior to chemical fertilizers at

improving the biological quality of soil (Li R. et al., 2017). A wide range of biofertilizers have been proposed over the last couple of decades. These were, for example, mainly manure (Chen and Jiang, 2014), composts or biochar (Liu et al., 2012), waste from biogas plants (Minale and Worku, 2014), municipal sewage sludge (Tontti et al., 2017), dairy sewage sludge (Frąc et al., 2012), algae or zeolite (Türkmen and Kütük, 2017), bone meal (Chen et al., 2011), agricultural or food wastes (Zhang et al., 2011). It has been described previously that the microbial community structure of soil after long-term organic fertilization reveals important associations between nutrients and specific taxa involved in nutrient transformation which occurs in soil (Li F. et al., 2017). Land application is the best recycling option since most organic wastes contain valuable nutrients and organic matter which may be used to improve soil fertility.

However, adding organic waste to soil is not only useful due to the organic matter and nutrients introduced; also additional fungal species may be included with biofertilizers (Frąc et al., 2014). Whenever the indigenous microbial fungal species of the organic waste meet the appropriate conditions for growth and development, they may have pivotal ecological roles influencing plant health as symbionts or decomposers. On the other hand they may act as plant pathogens (Frąc et al., 2014), produce toxins, or even cause animal or human mycoses (Pascual et al., 2008). To the best of our knowledge the mycological compositions of biofertilizers are rather poorly described compared to the soil after its incorporation. However, biofertilizers may influence soil fungal biodiversity. Biofertilizers are more often analyzed to discover their physicochemical properties. This is primarily to prove their expected positive impact on soil or to evaluate the potential risks associated with pollution from heavy metals or toxic organic compounds. If the microbial composition is being taken into consideration, then the waste is characterized by the presence of pathogenic bacteria, viruses and parasites (Pascual et al., 2008), but very rarely fungi. In order to prevent the spread of pathogenic microorganisms, EU Directive 86/278/EEC asserted that some biofertilizers, e.g., sludge should be appropriately treated to satisfy specific microbial standards before it is applied to land. The EU has also specified use restrictions according to the type of treatment applied.

However, as the ascospores produced by fungi, belonging to Ascomycota are resistant to many chemicals and physical treatment, they can survive sanitization or disinfection applied prior to agricultural disposal and in consequence they can develop in soil (Frąc et al., 2015). Therefore, it seems reasonable to make the mycological characterization of biofertilizers more widespread and to reinforce restrictions in order to diminish the risks to human health, farm animals and wildlife from contaminated plants. It is not only important to name the taxa, but also to describe in detail the metabolic properties of ecotypes of genera inhabiting diverse biofertilizers.

This may be useful since more attention has been paid recently to the possibility of using filamentous fungi (FF) in the degradation and detoxification of waste incorporated into the soil (Mannan et al., 2005; Rahman et al., 2016). The great diversity of fungi occurring in soil and in various types of waste may be the source of isolates with diverse biotechnological properties, used in new technologies and in the acquisition of natural products (e.g., enzymes). To speed up the process of waste degradation and reduce the risk associated with the presence of potentially pathogenic fungi in these wastes it is possible to use selected strains of microorganisms to facilitate waste-based biomass decomposition, especially when it is applied onto the soil. It is therefore essential to provide research concerning the characterization of fungal strains isolated from waste (Frąc, 2012).

The species of *Petriella setifera* (Alf. Schmidt) Curzi, belonging to the Ascomycota phylum, Microascaceae family, is often found in the soil enriched with manure or composts (Danon et al., 2010; Lackner et al., 2014) this is interesting in two contexts: waste degradation and pathogenesis, however, to date, it has been rather poorly described. *P. setifera* is classified as a soft-rot-causing fungi. These are known to secrete cellulase from their hyphae, this is an enzyme that breaks down cellulose and hemicellulose in wood (Janusz et al., 2013), however, as was demonstrated previously, *P. setifera* also demonstrates the ability to degrade lignin, and therefore it may be regarded as a brown rot fungi (Mathieu et al., 2013). Although these fungi are important ecological agents in the process of nutrient recycling by microbial decomposition, they are classified as pests in their role as destructive agents of wood rot. The genus of *Petriella* was also described as a pathogen of oak twigs sessile and the bark of Scots pine (Kwaśna et al., 2005), and also a root endophyte of *Salvia miltiorrhiza* (Lou et al., 2013). Fungi do live as parasites, but if the plant dies, whether as a consequence of the fungal infection or not, the fungus continues to degrade the biomass without further need for parasitic activity (Agrios, 2005). *Petriella*, being a facultative parasite, usually conforms to this saprophytic activity but may resort to parasitic action, but does not absolutely rely on any host for the completion of its life cycle.

Finding *P. setifera* strains in industrial compost and knowing the fact that this genus is simultaneously a saprophyte and a parasite, we assumed that *P. setifera* may have a strong tendency to live or to degrade not only oak but also wheat or sugar beet. The fact is the environmental factors, among others the nutritional constraints, alter the catabolic and genetic properties of microorganisms. These ecological principles shape and drive the long-term dynamics and evolution of microbial ecosystems (Zampieri and Sauer, 2016). However, for the facultative parasite, the short-term nutritional history may alter its further pathogenic and saprophytic activity, or as for *P. setifera* also endophytic activity, manifested by shifts in their catabolic and genetic features. In this context, it was hypothesized that *P. setifera* compost isolate, precultured on three different wastes [oak sawdust, beet pulp (BP) and wheat bran (WB)] would reveal different metabolic properties and genetic fingerprints. These results might be useful in the prediction of possible pathogenesis/degradation of different plants. Therefore, the aim of this study was to evaluate the influence of waste on *P. setifera* metabolic and genetic profile.

MATERIALS AND METHODS

Waste Material

The three following waste materials were considered: oak sawdust (SD), dried BP and durum WB. The wastes were powdered using a ball mill Retsch MM 400, 30 Hz for 15 min. The chemical analysis of wastes such as total solids (TS), volatile solids (VS), crude ash (CA), crude fat (CF), total nitrogen (N_{tot}), crude protein (CP), non-fiber carbohydrates (NFC) and crude fiber fraction: neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were conducted as described by Oleszek and Krzemińska (2016). Briefly, TS, VS, and CA were investigated with a muffle furnace after drying at 105 and 550°C, respectively. CF was determined by extraction with hexane. Total nitrogen was analyzed using the Kjeldahl method, and then N_{NO3} and N_{NH4} were subtracted, leaving the organic nitrogen value (N_{org}). CP was calculated by multiplying N_{org} by a coefficient of 6.25. NDF, ADF, and ADL were evaluated with van Soest and Wine's method (Van Soest, 1963). Based on NDF, ADF, and ADL results, the cellulose (CEL) and hemicellulose (HCEL) contents were calculated by subtracting ADF from NDF and ADL from ADF, respectively. NFC were estimated by using the following formula:

$$NFC = 100\% - (CP + CF + NDF + CA)$$

where NFC is NFC (% TS), CP is crude protein (% TS), CF is crude fat (% TS), NDF is neutral detergent fiber (% TS), and CA is crude ash (% TS), where TS is TSs.

The phosphorus level (P) was determined calorimetrically and potassium (K) was estimated by flame photometry according to the Spurway method (Spurway and Lawton, 1949). N_{org}, N_{NO3}, N_{NH4}, P, and K analyses were determined at District Chemical and Agricultural Station in Rzeszów. The results were obtained as mean values. All chemical analyses of tested waste were performed in triplicates.

Petriella setifera Isolates

The five fungal strains G11/16, G14/16, G16/16, G17/16, G18/16 were selected from among the fungal collection of the Laboratory of Molecular and Environmental Microbiology, Institute of Agrophysics Polish Academy of Sciences (Lublin, Poland). These were isolated from industrial composts using a serial dilution method on Bengal Rose LAB-AGAR medium (BIOCORP, Poland) and identified as *P. setifera* using two approaches. These were based on the D2 domain of Large-Subunit ribosomal DNA (D2 LSU rDNA) and Internal Transcribed Spacer 1 rRNA (ITS1) sequencing (Thermo Fisher Scientific, United States). Nucleotide sequences of the strains were deposited in the National Centre for Biotechnology Information (NCBI) under the following accession numbers: KX639331, KX639334, KX639335, KX639336, KX639337, respectively, following D2 LSU rDNA sequencing, and: MG594608, MG594609, MG594610, MG594611, MG594612, following ITS1sequencing. The industrial compost consisted of sewage sludge from the treatment of wastewater, sawdust, biodegradable waste from gardens and from parks, soil, the extracts of medicinal plants, and lime sludge. The concentration of carbon, nitrogen and phosphorus in the compost were 17.9, 2.3, and 0.75%, respectively, and pH was 5.3.

Waste Decomposition Using MT2 Plates®

Petriella setifera isolates were evaluated in four replicates against waste substrate decomposing abilities based on the growth intensity on powdered waste-based substrate, such as oak sawdust, BP, WB (prepared as described above), using MT2 plates® manufactured by Biolog®. To prepare an inoculum, each isolate was cultivated on Potato Dextrose Agar medium (PDA) (Oxoid Ltd., England) with a 3% addition of oak sawdust (SDM), beet pulp (BPM) or, wheat bran (WBM), and control medium (CLM) without any additives, at 27°C in the dark, for 25 days including 7 days with white light exposure for spore formation. 100 µl of the mycelium water suspension was added to wells on the MT2 plate, where previously 50 µl of 1% SD, BP or WB water solution was placed, following the modified procedures of Kadali et al. (2012), Taha et al. (2015) and Frąc et al. (2016), in four replicates. The inoculated microplates were incubated at 27°C for 10 days. The optical density (OD) at 750 nm was determined every 24 h using a microplate reader.

The Catabolic Profile of *Petriella* Fungi Using FF Plates®

The catabolic profiles of *P. setifera* isolates were generated from FF plates® based on the growth intensity of the organism on 95 low-molecular-weight carbon sources. The inoculation procedure was based on the FF plate® method according to the manufacturer's protocol modified by Frąc et al. (2012). The inoculation procedure was performed as for the MT2 plate analyses. After the homogenization of the mycelium suspension in inoculating fluid (FF-IF, Biolog®) the transmittance was adjusted to 75% using a turbidimeter (Biolog®). 100 µl of the mycelium suspension was added to each well and microplates were incubated at 27°C for 10 days. The OD at 750 nm was determined using a microplate reader every 24 h, in four replicates. Functional diversity was determined by the number of different substrates utilized by the individual isolates and expressed as substrate Richness (R), and Average Well-Density Development (AWDD) index calculated as following Average Well-Colour Development (AWCD) (Frąc et al., 2012), based on OD readings.

Genetic Diversity Based on AFLP and cAFLP

From each of the five strains cultured on SD, BP, and WB, 200 mg of fungal mycelium was taken and sterilely transferred into 2 ml tubes containing 250 mg of glass beads of 1.45 mm diameter. Then, 500 mg of glass beads of 3.15 mm diameter and they were homogenized with FastPrep-24 homogenizer (MP Bio, United States) for 20 s at 4 m/s. The DNA was extracted in accordance to EURx GeneMATRIX Plant and Fungi DNA Purification Kit (EURx, Poland) protocol. The quantity and purity of extracted DNA were evaluated with NanoDrop-2000 Spectrophotometer (Thermo Scientific, United States).

The AFLP reactions were performed with the use of *Pst*I and *Mse*I restriction enzymes. The results of the analysis were visualized by capillary electrophoresis with an Applied Biosystems 3130 Genetic Analyser (Applied Biosystems, United States). The sequences of adapters (5′–3′) and primers used in this study are denoted: *Mse*I_AF GAC GAT GAG TCC TGA G; *Mse*I_AR TAC TCA GGA CTC AT; *Pst*I_AF CTC GTA GAC TGC GTA CAT GCA; *Pst*I_AR TGT ACG CAG TCT AC; 6-FAM-*Pst*I+ACA *FAM- GAC TGC GTA CAT GCA GAC A; *Mse*I+CA GAT GAG TCC TGA GTA ACA. The AFLP reactions were performed in three biological replications for each isolate. The double-stranded *Pst*I and *Mse*I oligonucleotide adapters were formed in a final volume of 2 μl by incubating 0.5 μl of 10 μM *Pst*I_AF, 0.5 μl of 10 μM *Pst*I_AR, 0.5 μl of 100 μM *Mse*I_AF and 0.5 μl of 100 μM *Mse*I_AR adapters at 95°C for 5 min, followed by 15 min at room temperature. Successively, the restriction-ligation (RL) reaction was performed. The genomic DNA (500 ng) was digested with 5 U of the *Pst*I restriction enzyme (EURx, Poland) and 5 U of the *Mse*I restriction enzyme (New England Biolabs, United States). The RL solution was composed of 1 U of T4 DNA Ligase (EURx, Poland), 2 μl of double-stranded adapters, 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 μg/ml of BSA in a final volume of 20 μl.

The RL reaction was carried out for 1 h at 37°C. At the end of this reaction, each RL reaction mixture was diluted with an addition of 80 μl of sterile water and 1 μl of this solution was used as a template in the selective amplification reaction. The selective PCR amplification reaction was performed in a final volume of 5 μl which consisted of 2.5 μl of 2X Taq PCR Reaction Master Mix (EURx, Poland), 1 μl of diluted RL solution, 0.25 μl of 10 μM 6-FAM-*Pst*I+ACA primer (Genomed, Poland) and 0.25 μL of 10 μM *Mse*I+CA primer (Genomed, Poland). The reaction was performed in a Verti Fast thermal cycler (Applied Biosystems, United States) under the following conditions: 72°C for 120 s, followed by 7 cycles of 94°C for 15 s, 63°C with a touchdown of −1°C by cycle for 30 s, 72°C for 45 s, followed by 33 cycles of 94°C for 45 s, 56°C for 30 s, 72°C for 45 s and followed by a final step at 72°C for 60 s. At the end of this step, the exonuclease I – alkaline phosphatase purification step was performed. Hence, 2 μl of Exo-BAP Mix (EURx, Poland) was added to each reaction tube. The samples were incubated at 37°C for 15 min and then at 80°C for another 15 min. Next, 28 μl of sterile water was added into each PCR-product and 0.5 μl of this solution was combined with 0.25 μl of GS-600 LIZ Standard (Applied Biosystems, United States) and 9.25 μl of HiDi formamide (Applied Biosystems, United States). This mixture was incubated for 150 s at 95°C and cooled down in ice for 5 min. The amplicons were separated by capillary electrophoresis with an Applied Biosystems 3130 Genetic Analyser (Applied Biosystems, United States), in a 50 cm capillary array filled with NanoPOP-7 Polymer (McLAB, United States). The fragments were compared to the standard and visualized in the form of an electropherogram using a GeneMapper® version 4.0 software (Applied Biosystems, United States).

The cAFLP was performed on complementary DNA (cDNA). Therefore, total RNA was extracted from each of the five strains cultured on SD, BP, and WB. The RNA was extracted by a MagMAX™-96 Total RNA Isolation Kit (Thermo Fischer) with a modified homogenization step. 100 mg of mycelium was

TABLE 1 | Chemical properties of tested wastes.

Characteristics	Wheat bran	Oak sawdust	Beet pulp	Correlation coefficient
TS (%)	91.61 ± 0.11	94.11 ± 0.13	92.53 ± 0.09	0.22
VS (% TS)	96.26 ± 0.11	99.44 ± 0.05	95.99 ± 0.07	0.22
CA (% TS)	3.74 ± 0.12	0.56 ± 0.04	4.01 ± 0.08	0.48
N_{org} (% TS)	2.87	0.12	1.46	**0.79**
N_{NO3} (ppm)	47.11	15.50	27.80	**0.59**
N_{NH4} (ppm)	606.67	84.62	528.00	**0.73**
P (ppm)	6151.11	469.23	808	**0.91**
K (ppm)	10793.33	426.92	5808	**0.78**
CP (% TS)	17.94	0.75	13.51	**0.80**
CF (% TS)	3.77 ± 0.50	1.01 ± 0.08	0.31 ± 0.05	−0.17
NFC (% TS)	28.63 ± 1.77	12.21 ± 3.71	23.11 ± 0.05	**0.567**
NDF (% TS)	45.78 ± 0.92	85.47 ± 3.04	59.06 ± 0.04	−0.12
ADF (% TS)	12.06 ± 1.68	55.17 ± 1.23	23.43 ± 1.49	−0.35
ADL (% TS)	2.00 ± 0.15	14.35 ± 2.09	1.36 ± 0.04	−0.29
CEL (% TS)	11.03 ± 0.04	40.82 ± 2.09	22.07 ± 0.14	−0.35
HCEL (% TS)	32.89 ± 1.18	30.30 ± 2.23	35.63 ± 0.18	0.22
(CEL+HCEL)/ADL	21.96	4.95	42.43	

TS, total solids; VS, volatile solids; CA, crude ash; CF, crude fat; N_{org}, organic nitrogen; N_{NO3}, nitrate nitrogen; N_{NH4}, ammonium nitrogen; P, phosphorus; K, potassium; NFC, non-fiber carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; CEL, cellulose; HCEL, hemicellulose; NFC, non-fiber carbohydrates; n = 3. Pearson correlation coefficients ($p < 0.05$) were calculated between each characteristic and optical density comprising the ability of Petriella setifera to decomposition of waste measured in MT2 plate, within all the waste taken under consideration. The bolded values indicate significant correlation coefficients with $p < 0.05$.

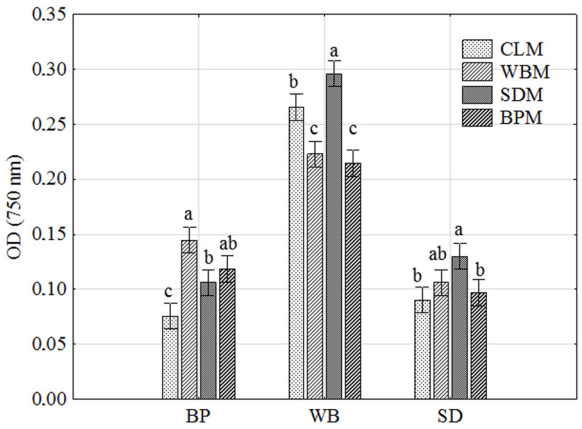

FIGURE 1 | *Petriella setifera* decomposition ability of waste-based substrate located in the MT2 plate® as influenced by preculturing. SD, oak sawdust; BP, beet pulp; WB, wheat bran; SDM, oak sawdust medium; BPM, beet pulp medium; WBM, wheat bran medium; CLM, control medium; OD, optical density. The vertical bars indicate the confidence intervals at 0.95. Different letters above the bars indicate errors in the significance of differences ($\alpha = 0.05$) between the mean values of OD [Tukey's test ($p < 0.05$)], $n = 4$.

suspended in 1 ml of nuclease-free water. Then the samples were mixed for 3 min and 165 µl of suspension was transferred on bead tubes (Thermo Fischer) containing 235 µl of Lyse F buffer (EURx). Then homogenization with a FastPrep-24 instrument (MP Bio) at 6.5 m/s with two cycles of 1 min each separated by 2 min of rest was performed. After that, samples were centrifuged at 16000 rcf for 210 s and 200 µl of lysate was transferred to a new tube. To clarify the lysate we centrifuged samples at 16000 rcf for 360 s.

After this step, the MagMAX^{TM}-96 Total RNA Isolation Kit protocol was performed. A reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer) with the addition of 10 µM of anchored Oligo(dT)$_{20}$ Primer. At the end of this step, the purification of exonuclease I – bacterial alkaline phosphatase was performed by the addition of 2 µl of Exo-BAP Mix (EURx, Poland) to 5 µl of each sample. The samples were incubated at 37°C for 15 min and then at 80°C for another 15 min.

The double-stranded *Pst*I and *Mse*I oligonucleotide adapters were formed as described for AFLP. The RL reaction was performed. The cDNA was digested with 5 U of the *Pst*I restriction enzyme (EURx, Poland) and 5 U of the *Mse*I restriction enzyme (New England Biolabs, United States). The RL solution was composed of 1 U of T4 DNA Ligase (EURx, Poland), 2 µl of double-stranded adapters, 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 25 µg/ml of BSA in a final volume of 20 µl.

The RL reaction was carried out for 1 h at 37°C. At the end of this reaction, each RL reaction was diluted by the addition of 80 µl of nuclease-free water and 1 µl of this solution was used as a template in the preamplification reaction. The preamplification reaction was performed in a final volume of 5 µl, which consisted of 2.5 µl of 2X Taq PCR Reaction Master Mix (EURx, Poland), 1 µl of diluted RL solution, 0.25 µl of 10 µM PstI+A primer (Genomed, Poland), and 0.25 µl of 10 µM MseI+C primer (Genomed, Poland). The reaction was performed in a Veriti Fast thermal cycler (Applied Biosystems, United States) under the following conditions: 72°C for 120 s followed by 35 cycles of 95°C for 15 s, 50°C for 30 s, 72°C for 45 s, and followed by a final step at 60°C for 30 s. After this, each reaction mixture was diluted by the addition of 45 µl of nuclease-free water and 1 µl of this solution was used as a template in the selective amplification reaction.

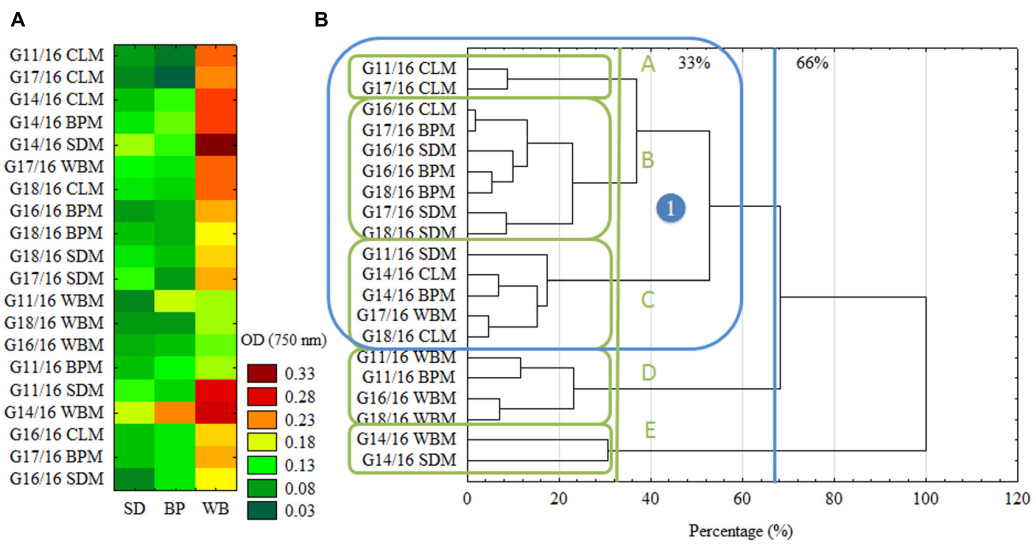

FIGURE 2 | Intraspecific metabolic variability of *P. setifera* isolates as influenced by preculturing. **(A)** The cluster analysis depending on the waste-based substrate decomposition in MT2 plate®, **(B)** clustering according to the stringent Sneath's criterion (33%) and less restrictive criterion (66%). For explanations: please see **Figure 1**.

The selective amplification reaction was performed in a final volume of 5 µl, which consisted of 2.5 µl of 2X Taq PCR Reaction Master Mix (EURx, Poland), 1 µl of diluted preamplification solution, 0.25 µl of 10 µM 6-FAM-PstI+ACA primer (Genomed, Poland), and 0.25 µL of 10 µM MseI+CA primer (Genomed, Poland). The reaction was performed in a Veriti Fast thermal cycler (Applied Biosystems, United States) under the following conditions: 72°C for 120 s followed by 7 cycles of 94°C for 15 s, 63°C with a touchdown of −1°C per cycle for 30 s, 72°C for 45 s followed by 33 cycles of 94°C for 45 s, 56°C for 30 s, 72°C for 45 s, and followed by a final step at 72°C for 60 s. At the end of this step, purification by Exo-BAP Mix (EURx, Poland) was performed. In the next step, 28 µl of sterile water was added into each selective amplification product and 0.5 µl of this solution was combined with 0.25 µl of GS-600 LIZ Standard (Applied Biosystems, United States) and 9.25 µl of HiDi formamide (Applied Biosystems, United States). This mixture was incubated for 150 s at 95°C and cooled down in an ice bath for 5 min. The transcriptome-derived fragments were separated by capillary electrophoresis as described for AFLP.

Statistical Analyses

Analysis of variance (ANOVA) was used to determine the differences in functional diversity indices and lignocellulose substrate utilization. *Post hoc* analyses were performed using a Tukey test (HSD). The data were presented as 95% confidence intervals. Statistical significance was established at $p < 0.05$. Pearson correlation coefficients ($p < 0.05$) were calculated between each characteristic and OD of *P. setifera* measured in the MT2 plate, within all the waste taken into consideration, values in bold indicate a strong correlation. Additionally, the cluster analysis for waste utilization (MT2 microplates), catabolic profiles (FF microplates) and genetic profiles (AFLP and cAFLP) on tested *P. setifera* isolates was used to detect groups in the data set through the calculation of Euclidian distance using the Ward method approach, which was calculated based on the average of all readings. The obtained results were depicted using dendrograms calculated using the Ward method cluster analysis with Sneath's dissimilarity criteria (single bindings agglomeration and Euclidean distance measure). All statistical analyses, described above were performed using Statistica software (version 10.0).

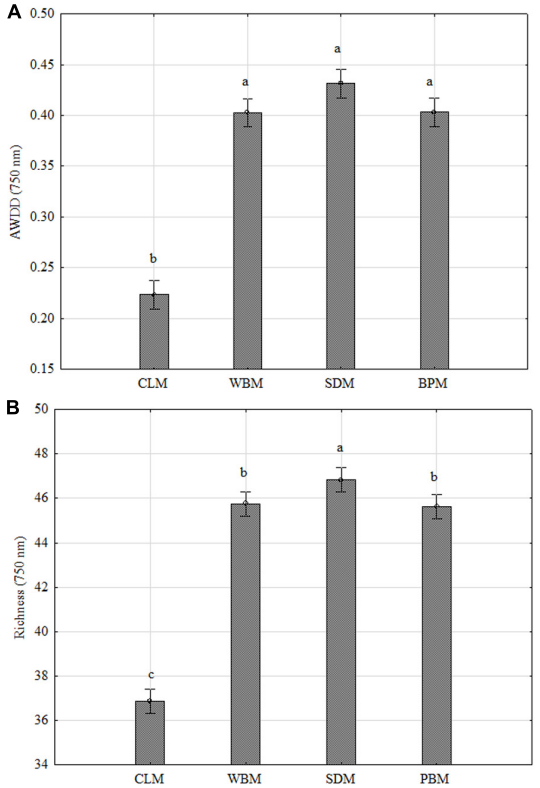

FIGURE 3 | FF plate® biodiversity indices based on carbon compounds utilization ability of *Petriella setifera* as influenced by preculturing. **(A)** Average Well-Density Development index (AWDD), **(B)** substrate Richness (R). Explanations: SDM - oak sawdust medium, BPM - beet pulp medium, WBM - wheat bran medium, CLM - control medium. The vertical bars indicate the confidence intervals at 0.95. Different letters above the bars indicate errors in the significance of differences (α = 0.05) between the mean values of OD [Tukey's test ($p < 0.05$)], $n = 3$.

RESULTS AND DISCUSSION

Intraspecific variation is a common feature of fungi which has been widely investigated on both a metabolic (Knapp and Kovács, 2016; Wang et al., 2016) and genetic level (Fedorova et al., 2009; Corradi and Brachmann, 2016). In this study the influence of three different materials (oak sawdust, dried BP, durum WB) on *P. setifera* catabolic and genetic properties were tested. The chemical characteristics of these materials are presented in **Table 1**. All wastes were represented by similar TS, VS, and CA content. WB encompassed the highest content of CF (3.77% TS), CP (17.94% TS), and NFC (28.63% TS), compared with oak sawdust and BP. WB also contained a relatively high amount of phosphorus (P) (6151 ppm), potassium (K) (10793 ppm), and nitrogen (N): 2.87 ppm, 47.11 ppm and 606 ppm of N_{org}, N_{NO3}, and N_{NH4}, respectively. Sawdust was in turn distinguished by a high content of all fractions of fiber: NDF (85.47% TS), ADF (55.17% TS), particularly ADL 14.35% TS) and a very low content of CA and macroelements (N, P, K), as well as CP. BP was characterized by a higher content of hemicellulose (HCEL), compared to other wastes and the lowest content of ADL. The results of chemical analyses were in accordance with other studies on similar materials (Mikiashvili et al., 2011; Stevenson et al., 2012).

Among the waste tested, the highest ratio of hemicellulose to lignin [(CEL+HCEL)/ADL] (42.43) for BP was evidenced, whereas in WB the ratio reached 21.96. The lowest ratio (4.95) was observed for sawdust. As mentioned, e.g., by Lyson and Sobolewska (2015) the high hemicellulose to lignin ratio means that this waste was regarded to be the most susceptible to biological decomposition, and therefore difficult, if the ratio is low. This is only partially consistent with our study, because for *Petriella,* a rather weak negative correlation was noted with

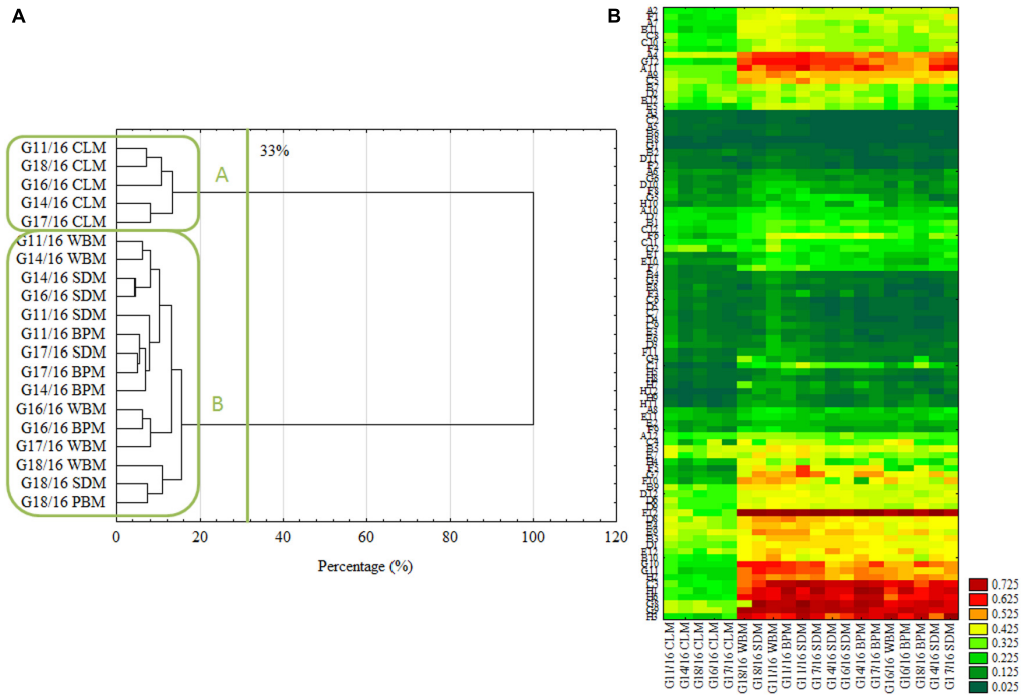

FIGURE 4 | Intraspecific metabolic diversity of *P. setifera* isolates as influenced by preculturing. **(A)** Clustering according to the stringent Sneath's criterion (33%) and less restrictive criterion (66%), **(B)** the cluster analysis depending on carbon-compound utilization located in the FF plate®. For explanations: please see **Figure 1**. Carbon compounds decoding according to (Frąc, 2012).

lignocellulose components, such as: NDF, ADF hemicellulose and lignin content (ADL), lignin (ADL), cellulose content (CEL) as well as CF (**Table 1**). At the same time, a strongly positive correlation with N, P, K, protein (CP) and simple sugars soluble in water, namely NFC was recorded. This means that *P. setifera* compost isolates are more capable of decomposing materials rich in protein, N, P, K and easily accessible sugars, than lignocellulose. Furthermore, improved decomposition of WB compared to BP was revealed, in spite of an almost twofold lower hemicellulose-to-lignin ratio. Therefore, more important for the decomposition ability of *P. setifera* is the accessibility of N, P, K and protein in the material, rather than the limiting hemicellulose to lignin ratio.

As presented in **Figure 1**, *P. setifera* was the most effective at decomposing such a waste substrate located on MT2 plates® as WB. BP and sawdust were generally significantly less rapidly decomposed. Notwithstanding it occurred that preculturing, nutritional condition with a high lignocellulose content significantly contributed to the improved ability of *P. setifera* to utilize substrates in MT2. The variability in that waste utilization was revealed. BP was preferably decomposed to a significant extent if the fungus is precultured with any waste-based medium [bran medium (WBM)], sawdust medium (SDM) or beet pulp medium (BPM), compared to the control (CLM). As far as WB is concerned, its utilization was significantly enhanced by preculturing on SDM, and significantly decreased if precultured on WBM and BPM. When it comes to sawdust, it was noticed that the preculturing of *P. setifera* on SDM significantly improved sawdust utilization, whereas just slightly if precultured on WBM. As tough nutritional condition (high lignocellulose content) as encountered while preculturing *P. setifera* on SDM, triggered genetic and metabolic changes at the cellular level clearly and were subsequently persistent regardless of the changed material. It is supposed that nutritional short-term history may result in the facilitation of the switching ability of *P. setifera* to follow a pathogen/saprophyte or endophyte mode of action.

What is more, the intraspecific differences among *P. setifera* isolates were also described for waste utilization in MT2 Plate® (**Figure 2A**). Most intensive catabolic properties for WB utilization were noted for strains G14/16 and G11/16 precultured on SDM. On the other hand, G18/16 and G16/18 strains precultured on WBM were the least active for this waste. Among strains tested against waste utilization in the MT2 plate®, there were five (A–E) groups revealed if the restrictive Ward's criterion (33%) was taken into consideration (**Figure 2B**). Groups A–C comprised group (1), standing out by comprising strains G16/16, G17/16 and G18/16. In group A, there were strains precultured on CLM clustered, in B and C mostly on SDM and BPM, and D on WBM. E and C groups were characteristic in so far as both strains of G14/16 were located. This suggests one notable intraspecific difference in metabolic properties of *P. setifera* as influenced by culture media composition.

In **Figure 3** biodiversity indices are presented: AWDD and Richness (R) calculated based on FF plates®. It shows the significant dependency of *P. setifera* abilities to catabolize C-compounds in FF plates®, on preculturing on media with all

the tested waste additives (WBM, SDM, BPM), twice compared to the control as far as AWDD. Among tested media SDM significantly influenced metabolism of *P. setifera* to increase the number of utilized substrates, which was evidenced by a 20% upswing in Richness. The result for C-compounds are consistent with the more complex substrates as tested in MT2. However, there was less diversity among strains found based on the FF approach, compared to MT2, which was shown in **Figure 4**.

In accordance with the previous report (Eisen et al., 1998) the cluster analysis revealed clear patterns of microbial properties. Similar research on FF plates® showing metabolic differences of closely related fungi were recently performed by, e.g., (Janusz et al., 2015; Jaber et al., 2017; Pinzari et al., 2017). Regardless of the restriction criterion in cluster analysis, *P. setifera* isolates revealed dichotomy clustering in A and B groups (**Figure 4A**). There were *P. setifera* strains that were precultured on CLM included in the A group. Whereas all the other strains of initially precultured variants comprised a separate group B. The catabolic activity of *P. setifera* precultured on waste was highly strengthened toward more effective utilization of some particular C-compounds located on the FF plate® (**Figure 4B**). These were particularly: *N*-Acetyl-D-Glucosamine (A4), L-Arabinose (A9), Arbutin (A11), belonging to Carbohydrates; D-Glucuronic Acid (C3) and Quinic Acid (F12), which contain the carboxylic and ketonic acid groups; polymer Glycogen (C5); L-Alanine (G8), L-Alanyl-Glycine (G9), L-Asparagine (G10), L-Aspartic Acid (G11), L-Glutaminic Acid (G12), Glycyl-L-Glutamic Acid (H1), L-Ornithine (H2), L-Phenylalanine (H3), L-Serine (H6) that belong to the amino acid class. At a slightly lower level the utilization of γ-Amino-Butyric Acid (F1), D-Mannose (D2), D-Xylose (E12), L-Rhamnose (D12) was also increased. These results were in compliance with the properties of soft-rot fungi (Schwarze, 2007; Mathieu et al., 2013). It was found that all of the isolates could degrade at a high level the substances that may be produced during the hemicellulose's and cellulose's degradation. Furthermore, it was revealed that all of the analyzed isolates degraded Quinic Acid, which is a monomer of lignin degraded by brown-rot fungi at a high rate (Albrecht et al., 2010). Both modes of action are characteristic for fungal pathogens and/or saprophytes. Verma et al. (2007) described that plant fungal pathogens typically secrete a number of plant cell-wall-degrading enzymes (cellulases, glucanases, polygalacturonases, xylanases), and may persist on dead plants as a saprophyte. However, fungal endophytes being in a mutual relationship with a plant must lose their ability to produce hydrolytic enzymes and already use simple compounds provided by the plant. Many of the C-compounds effectively utilized by *Petriella* are plant cell metabolites (Kot et al., 2015), that *Petriella* uses as being an endophyte. What is more, the growth of all tested *P. setifera* isolates was inhibited by amines and amides, especially by *N*-Acetyl-D-Mannosamine and Glucuronamide (**Figure 4B**). In conclusion, the FF Plates® results have also demonstrated slight intraspecific variability of the analyzed *P. setifera* strains to utilize particular C-compounds. *P. setifera* precultured on all waste-based media also revealed a wider diversity of C-compounds utilization, compared to the control. These results are consistent with MT2 findings and indicate that the nutritional history

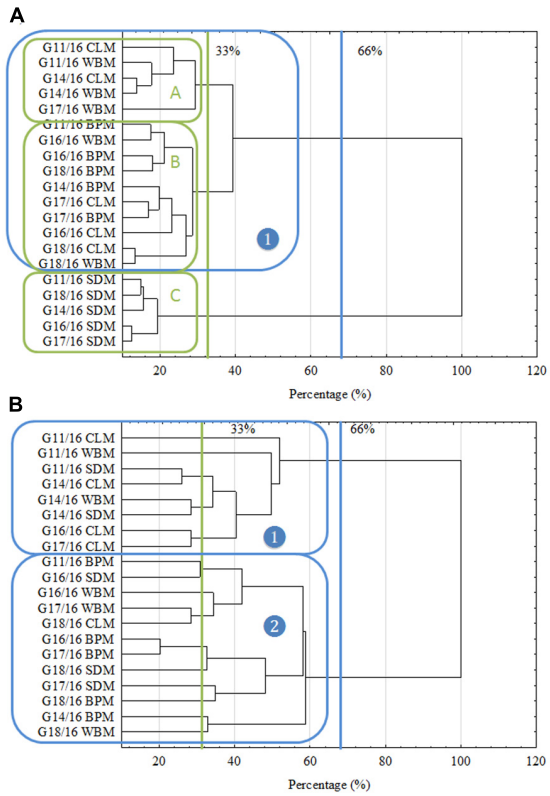

FIGURE 5 | Intraspecific genetic fingerprinting of *P. setifera* isolates as influenced by preculturing. **(A)** Genome-derived fragments clustering, **(B)** transcriptome-derived fragments clustering based on Amplified Fragment-Length Polymorphism analysis. For explanations: please see **Figure 1**.

imposes actual metabolic activity which may trigger *P. setifera* mode of behavior. This may pose a threat to the corps especially if they are fertilized with compost inhabited by *P. setifera*. Plant pathogens in compost constitute a serious problem (Bollen and Volker, 1996). Reliable analyses are needed for the evaluation of infestation of the finished compost product, as a soil conditioner.

Trying to link the metabolic differences described above with genetic shifts, Amplified Fragment-Length Polymorphism (AFLP) was performed. Also cAFLP genotyping was provided, showing shifts in transcript-derived fragments (**Figure 5B**). These approaches were previously presented for fungi, e.g., by Al-Hatmi et al. (2016) and Xiao et al. (2016). In our findings there were three different groups noted for AFLP fingerprinting based on the 33% criterion (**Figure 5A**). Groups A and B included isolates cultured on all the wastes, besides SDM, which formed an outlying cluster C. This guarantees a clarification of the findings of the metabolic properties of *P. setifera*. All of the changes which may be observed at a metabolic level are determined by many modifications at a genetic level. This might be accentuating the expression or including the expression of other genes that code enzymes responsible for decomposition, or/and launching new metabolic pathways. This could also be the result of post-translational modifications of enzyme proteins (Brown, 2006) or

epigenetic phenomena defined by reversible heritable changes in gene expression in the absence of changes in DNA sequence. These include, among others, DNA methylation, position effects, RNA silencing systems, and centromere location. Fungi share silencing systems, for instance RNA interference (iRNA) and DNA methylation (Smith et al., 2012). Similarly fingerprinting for isolates cultured on SDM resulted in specific DNA methylation since the *Pst*I restriction enzyme used is sensitive to cytosine methylation, predominately present at CpG or CpNpG sites. To be specific, *Pst*I is highly sensitive to the cytosine status in CpNpG sites because its recognition site involves two CpNpG trinucleotides (Cui et al., 2013). This may also be the effect of point mutations occurring on recognition sites: CTG CAG and TTAT for *Pst*I and *Mse*I, respectively (Montiel et al., 2006; Jiang et al., 2013). However, as was revealed by the cAFLP approach, other more complex scenarios were involved, since with transcriptome level modification no groupings were noted for particular isolates cultured on different wastes.

The influence of preculturing of *P. setifera* on selected waste type on metabolic and genetic properties was evidenced. Isolates were found to be better able to decompose waste materials such as WB and BP, rich in protein, N, P, K and easily accessible sugars compared to oak sawdust, rich in lignocellulose. Sawdust clearly triggered changes in the metabolic and genetic properties of *P. setifera*. However, intraspecific differences among *P. setifera* isolates were noted. Especially, the contribution to improve its ability to utilize waste substrates in the MT2 plate® and the two times increase in the ability to catabolize carbon compounds located in FF plates® was noted. Vivid metabolic properties following the preculturing of *Petriella* isolates on sawdust were in accordance with differing genotype profiles but not the transcriptome. Based on the study we may also conclude that amines and amides inhibited the growth of *P. setifera* isolates. Therefore, such compounds may be tested as potential agents in plant protection against this pathogen.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KO, JP, GP, AS, MO, and MF. Performed the experiments: KO, JP, GP, and AS. Analyzed the data: KO, MF, JP, and MO. Contributed reagents/materials/analysis tools: KO, JP, GP, AS, MO, and MF. Wrote the paper: KO and MF. Improvement and acceptance of the last version of manuscript: KO, JP, GP, AS, MO, and MF.

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Fungal Genetics and Functional Diversity of Microbial Communities in the Soil under Long-Term Monoculture of Maize Using Different Cultivation Techniques

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Fungal diversity in the soil may be limited under natural conditions by inappropriate environmental factors such as: nutrient resources, biotic and abiotic factors, tillage system and microbial interactions that prevent the occurrence or survival of the species in the environment. The aim of this paper was to determine fungal genetic diversity and community level physiological profiling of microbial communities in the soil under long-term maize monoculture. The experimental scheme involved four cultivation techniques: direct sowing (DS), reduced tillage (RT), full tillage (FT), and crop rotation (CR). Soil samples were taken in two stages: before sowing of maize (DS_{BS}-direct sowing, RT_{BS}-reduced tillage, FT_{BS}-full tillage, CR_{BS}-crop rotation) and the flowering stage of maize growth (DS_F-direct sowing, RT_F-reduced tillage, FT_F-full tillage, CR_F-crop rotation). The following plants were used in the crop rotation: spring barley, winter wheat and maize. The study included fungal genetic diversity assessment by ITS-1 next generation sequencing (NGS) analyses as well as the characterization of the catabolic potential of microbial communities (Biolog EcoPlates) in the soil under long-term monoculture of maize using different cultivation techniques. The results obtained from the ITS-1 NGS technique enabled to classify and correlate the fungi species or genus to the soil metabolome. The research methods used in this paper have contributed to a better understanding of genetic diversity and composition of the population of fungi in the soil under the influence of the changes that have occurred in the soil under long-term maize cultivation. In all cultivation techniques, the season had a great influence on the fungal genetic structure in the soil. Significant differences were found on the family level ($P = 0.032$, $F = 3.895$), genus level ($P = 0.026$, $F = 3.313$) and on the species level ($P = 0.033$, $F = 2.718$). This study has shown that: (1) fungal diversity was changed under the influence different cultivation techniques; (2) techniques of maize cultivation and season were an important factors that can influence the biochemical activity of soil. Maize cultivated in direct sowing did not cause negative changes in the fungal structure, even making it more stable during seasonal changes; (3) full tillage and crop rotation may change fungal community and soil function.

Keywords: fungal community, genetic diversity, metabolic profiles, biolog ecoplates, ITS-1 NGS

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INTRODUCTION

Research on the biological diversity of soil microorganisms is concentrated on three aspects of diversity: species, genetic and functional (Bundy et al., 2009; Bowles et al., 2014). In biodiversity analysis, it is important to evaluate the microbiome as a whole, not only its individual components (Daghino et al., 2012). Research on microbial activity in different environments is essential to increase knowledge about the ecology of their biocenosis and should be analyzed in connection with the existing environmental conditions, considering both biotic and abiotic factors (Bowles et al., 2014). Undoubtedly, an important aspect of such research is the selection and development of appropriate indicators and methods for assessing soil biodiversity and the activity of soil microorganisms, so that they can give the most reliable and reproducible results (Brussaard et al., 2007; Ghimire et al., 2014).

Investigation of soil biological activity as one of the indicators in the evaluation of tillage systems for the needs of sustainable agriculture was undertaken. It has been assumed that through the elaboration of the correct cropping technique, it will be possible to significantly increase the degree of fungal and bacterial diversity and reducing loss soil biodiversity (Danielsen et al., 2012).

Additionally, the introduction of such a cropping system should result in the reduction of energy consumption and labor-intensive tillage (Brussaard et al., 2007). The cultivation of plants in monoculture may be such a system. Tillage practices and cultivation techniques as well as residue management have an important effect on biological activities and the functional diversity of microorganisms (Lupwayi et al., 1998). Monoculture of plants can induce important changes in the soil environment and biological activity related to the reduction of fungal diversity (Liang et al., 2011). Many authors have suggested that long-term cropping of plants in monoculture induces degradation processes in the soil that can lead to a reduction in the a number of fungal species and a decline in organic matter (Brussaard et al., 2007; Liang et al., 2011; Han et al., 2017). Maize cultivation in monoculture is practiced in many countries. The cultivation of maize in direct sowing (zero-tillage) in Polish conditions is a good alternative but requires further systematic research on interactions between system factors: biotic and abiotic factors, soil environment, plants and fungi (Gałązka et al., 2017a,b). Maize (*Zea mays* L.) is one of the most important crops and is widely used in agriculture and industry, but its cultivation is very energy-intensive, hence agricultural practice is looking for a simpler solution (Liang et al., 2011; Gałązka et al., 2017c). The cultivation of some plants such as maize in a zero-tillage system is the most attractive and gives the biggest economic profits. Maize is grown increasingly in direct sowing and in this system leaves many crop residues are left on the surface of the field (Zhang et al., 2012).

The effects of long-term monoculture on soil quality, especially fungal genetic diversity, are not widely recognized for cultivation. Research on the effects of long-term cultivation of maize in monoculture on changes in soil quality, vegetation and yield of maize has been conducted at IUNG (Institute of Soil Science and Plant Cultivation, State Research Institute, Pulawy, Poland) for many years (Gałązka et al., 2017a,c). Long-term monoculture has a strong influence on soil parameters, especially on soil microorganisms and enzymes.

However, it is not known how the use of different techniques for cultivating maize growth may influence the composition of the fungal community. Biochemical and microbial properties of soil respond very rapidly to even small changes in environmental factors, such as temperature, moisture, tillage or cultivation techniques, and may have a strong impact on the core microbiome of fungi (Liang et al., 2011; Zhang et al., 2012). The term "core microbiome of fungi" can be understood as the group of fungi comprised of the members common to two or more fungal assemblages associated with a habitat (Shade and Handelsman, 2011). Evaluating the core fungal species is essential to unraveling the ecology of fungi consortia. Hence, bacterial and fungal communities are very often used as early indicators of soil alteration induced by agricultural management (Nannipieri, 2011).

Fungi constitute the biodiversity group agricultural soil and perform numerous important ecosystem functions such as influencing plant health (Wang et al., 2017). Fungal biodiversity in soil has been increasingly recognized as being beneficial for soil health (Fisher et al., 2012; Duniere et al., 2017). In current research on fungal biodiversity, a very important issue is not only the identification and distribution of this group, but also the definition of their important roles in ecosystems (Fisher et al., 2012). Comparison of the functional diversity of soil and the genetic diversity of fungi may enable a better understanding of their fundamental and ecological role and impact on plant health. Several environmental factors, including the physicochemical properties of soil, biological activities, soil moisture, biomass carbon and nitrogen, organic matter content, climate, season and also tillage systems, may significantly impact the diversity of the fungal genetic community in the soil environment (Liang et al., 2011).

The functional and structural diversity of soil fungi have been evaluated using several parameters, such as microbial biomass, respiration and enzymatic activities, as well as molecular methods, including next-generation sequencing (Lim et al., 2010; Xu, 2010; Zhang et al., 2012; Welc et al., 2014; Wang et al., 2017). Next-generation sequencing (NGS) of the hyper -alternating regions (16S rDNA for bacteria and ITS for fungi) enables definition of the genetic diversity of microorganisms without cell culture cultivation (Kozich et al., 2013; Orgiazzi et al., 2013). It relies on the isolation of total DNA from soil samples and preliminary amplification of hyper-alternating regions with the use of specific starters. In the next stage, correct adaptors and indexes are attached to amplicons (Ranjard et al., 2003; O'Brien et al., 2005; Schoch et al., 2012; Orgiazzi et al., 2013). The concentrations of samples are normalized and then all samples are combined into one cumulative sample that is designated for sequencing. The use of adequate indexes allows individual amplicons to be assigned to appropriate samples (Bartram et al., 2011; Kozich et al., 2013; Zoll et al., 2016).

In current paper, we propose that more insight should be gained into the determination of fungal structural diversity and community level and physiological profiling of microorganisms in the soil under long-term maize monoculture. The other objective of this work was to identify which groups of soil fungi are most sensitive to these techniques. Application of both genetic and functional methods will allow investigation of the composition of the fungal community and functionality of soil microorganisms directly in the soil. It will also allow explanation of the possible relationships between fungi and microbial community with using cultivation techniques.

MATERIALS AND METHODS

Field Experiment

The study was based on in the a long—term stationary field experiment. The soil in this experiment was classified as gray brown podsolic soil formed from light loam (USDA: SiL silt loam). This field experiment was established in 2004 at the Institute of Soil Science and Plant Cultivation's Agricultural Experimental Station (AES) in Grabow, Mazowieckie Voivodship (51°23' N; 21°38' E), Poland. The experimental scheme involved four cultivation techniques: direct sowing (DS), reduced tillage (RT), full tillage (FT), and crop rotation (CR). Soil samples were taken twice a year: before sowing of maize (the index bottom BS): DS_{BS}-direct sowing, RT_{BS}-reduced tillage, FT_{BS}-full tillage, CR_{BS}-crop rotation and in the flowering stage of maize growth (the index bottom F): DS_F-direct sowing, RT_F-reduced tillage, FT_F-full tillage, CR_F-crop rotation. The following plants were used in the crop rotation: spring barley, winter wheat and maize. The field experiment was carried out with the long strips with the mirror image of combinations. More information about the character and plan of this experiment can be found in Gałązka et al. (2017c).

In the full tillage technique, straw residues were left after the cob harvest, then shredded, and turned under (Gałązka et al., 2017c). By contrast, in the direct sowing the straw was shredded but left on the soil surface. Under the crop rotation management, all the crop species involved were grown each year and full doses of fertilization and herbicides were applied to maize. Maize cv. Delitop was seeded using a precision maize planter. Nitrogen was applied to the maize at a rate of 140 kg N ha^{-1} (70 + 70), phosphorus and potassium rates (kg·ha^{-1}) were P_2O_5 – 80 and K_2O – 125. Annual fertilizer rates applied to barley were: N – 60, P_2O_5 – 35 and K_2O – 50 kg ha^{-1}, and to wheat: N – 120, P_2O_5 – 40 and K_2O – 70 kg·ha^{-1}. The results of physicochemical properties and soil quality have already been published (Gałązka et al., 2017a,c).

Soil Samples

Soil samples were collected in 2016 according to (Polish Standard PN-ISO 10381-6, 1998) in two sampling times: before sowing and in the flowering phase of maize growth. The soil samples in three replicates were taken from a 0–30 cm layer (as bulk soil samples from a given field), sieved through a 2 mm sieve and stored in a refrigerator (4°C) until analysis.

Community Level Physiological Profiling (CLPP) Analysis Using Biolog Ecoplates

The metabolic potential of soil microbial communities was evaluated using Biolog EcoPlate (Biolog Inc., Hayward, CA, USA). The metabolic capacities of all soil microorganisms (bacteria and fungi together) were determined using the EcoPlates system with 31 different carbon sources. Soil suspension for the inoculation of wells in microplates was prepared as follows: 1 g of soil was weighed, transferred to conical flasks holding 99 cm^3 sterile 0.9% NaCl each, and vortexed for 30 min at 150 rpm and at 25°C, after which the samples were cooled for 30 min to 4°C (Pohland and Owen, 2009). After that, 120 mm^3 was transferred to each of the wells in an EcoPlate and incubated in the dark at 28°C for 168 h. The experiment included three replications. The results were read on the MicroStation ID system by the Biolog®.

The extent to which carbon sources were used was determined through the reduction of colorless tetrazolium chloride to red formasane ($\lambda = 490$ nm) (Insam and Goberna, 2004). The intensity of color development was recorded at $\lambda = 490$ nm for a period of 168 h at 24-h intervals. The most intensive metabolism of carbon substrates was observed after 120–168 h of incubation, but the results obtained at 168 h are presented in the paper. The activities of soil microorganisms are based on all carbon sources and on grouped sources defined as amines and amides, amino acids, carbohydrate, carboxylic acid and polymers (Pohland and Owen, 2009). The results were expressed as Average Well-Color Development (AWCD) and Shannon-Weaver (*H'*) indices.

DNA Extraction, ITS-1 Next- Generation Sequencing (NGS) and Bioinformatics Analyses

Total DNA was extracted from 0.5 g of soil using a FastDNA® SPIN Kit for Soil (MP Biomedicals, OH, USA), according to the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to determine the concentration and quality of the DNA. The fungal internal transcribed spacer-1 (ITS-1) region was amplified from each sample using primers ITS1FI2 (5′-GAACCWGCGGARGGATCA-3′) and 5.8S (5′-CGCTGCGTTCTTCATCG-3′), which provide a comprehensive coverage with the highest taxonomical accuracy for fungal sequences (Mello et al., 2011; Orgiazzi et al., 2013; Schmidt et al., 2013). The PCR was performed using Q5 Hot Start High-Fidelity 2x Master Mix, with reaction conditions according to the manufacturer's recommendations. The reaction was carried out according to the Illumina ITS-1 amplification protocol and sequencing was performed on an Illumina MiSeq (Genomed S.A., Warsaw, Poland). The reverse primer contained a 8-bp error-correcting barcode, unique to each sample. The libraries were prepared in analogously to the attached Illumina protocol. Sequencing was performed on a MiSeq by Illumina Inc. using paired—end (PE) technology, with 2 × 250 cycles with v2 chemistry, according to the manufacturer's recommendations. Automatic preliminary data

analyses were performed using an MiSeq Reporter (MSR) v2.6. These analyses consisted of following stages: adaptor sequences trimming— program cutadapt, quality control and trimming of low quality bases (quality < 20, min length 30)—program cutadapt, paired reads joining—fastq-join algorithm, OTU clustering with 97% sequence similarity—uclust algorithm, chimeras detection and removal—usearch61 algorithm and taxonomy assignment based on UNITE v7 database—blast algorithm.

Bioinformatics analyses, including classification of reads to species level, were performed using QIIME (Quantitative Insights Into Microbial Ecology) based on the reference databases UNITE v7 (Caporaso et al., 2010). Cluster generation was based on the chosen database of reference sequences, removal of chimer sequences and attribution of taxonomy. The results are presented in OTU (Operational Taxonomic Units) containing the classification and number of reads in every OTU in BIOM (Biological Observation Matrix) format.

Statistical Analysis

The main statistical analyses were performed using STATISTICA.PL (10) (Stat. Soft. Inc. USA). The data was subject to a three-way (fungal community, cultivation techniques, biodiversity indices from EcoPlate) analysis of variance (ANOVA) for the comparison of means. Significant differences were calculated according to Tukey's *post-hoc* HSD test at significance level $P < 0.05$. The Average Well-Color Development (AWCD) was evaluated according to (Garland and Mills, 1999) in with formula AWCD = Σ (C-R)/95; where C = the absorbancy in each well and R = the absorbancy in the control well. The Shannon—Weaver (H') index was evaluated in accordance with the formula $H' = -\Sigma \text{pi}(\ln \text{pi})$, where pi = the ratio of the absorbance of each well to the absorbance of all wells (Gomez et al., 2004). The results were also submitted to the PC (principal component) analysis in order to determine common relations between the fungal community and the soils collected using different cultivation techniques. For the principal coordinate analysis (PCoA) and Welch's test, the data was expanded using statistical analyses of fungal community profiles (STAMP 2.1.3) software (Parks et al., 2014). This analysis was employed to study statistically significant differential abundance of different-level taxa among soil fungal community. The results were calculated as each taxon-relative abundance, assuming all assigned reads per sample to be 100%. Welch's test (two-group analysis) was performed applying a *t*-test with 95% confidence intervals. Only taxonomic representatives that differed significantly ($P < 0.05$) among different soils were taken into account. Principal coordinate analysis (PCoA) was performed at the species level, using Euclidean distance measurements. Permutational multivariate analysis of variance (PERMANOVA) was used to compare the microbial community structure between soils taken from different sites and with different contamination times. This was performed with 999 permutations using the Adonis function of the PAST package (v 3.16) (Hammer et al., 2001).

RESULTS

Community Level Physiological Profiling (CLPP) of Soil

The effects of seasons and cultivation techniques on microbial community catabolic diversity as evaluated by main substrate utilization in the Biolog EcoPlate, were measured (**Figure 1**). The soil samples collected before the sowing of maize—DS_{BS}, RT_{BS}, FT_{BS}, and CR_{BS} were characterized by statistically lower indexes of biological activity of substrate utilization than the soil collected in the flowering stage of maize growth (DS_F, RT_F, FT_F, CR_F). This effect was present for all the evaluated substrate groups: amines and amides, carboxylic and acetic acids, carbohydrates, polymers, aminoacids and for the percent of total carbon source utilization in the soil. Also the effects of seasons and cultivation techniques on microbial community catabolic diversity was presented as the radar plot (Figure S1).

The soil samples collected before maize sowing from full tillage as the cultivation technique (FT_{BS}) were characterized by higher community level physiological profiling than the soils obtained in the maize flowering stage (FT_F). The highest diversity based on the Shannon-Weaver index was found in the soil from full tillage in both the before sowing and at flowering stages of maize growth (FT_{BS}, $H' = 3.31$ and FT_F, $H' = 3.34$) (**Figure 2**). Also, a higher diversity was found in the soil cultivated using reduced tillage from the flowering stage (RT_F, $H' = 3.29$) and crop rotation (CR_F, $H' = 3.31$) (**Figure 2**) than in other tillage systems. The lowest diversity was recorded for the soil collected before sowing from direct sowing fields (DS_{BS}, $H' = 3.14$).

The soils obtained in the flowering stage of maize cultivated using different sowing techniques were also characterized by a higher Average Well-Color Development (AWCD) index (**Figure 2**). The highest activity was found in the soil samples from reduced tillage (RT_F, AWCD = 4.47), followed by full tillage (FT_F, AWCD = 3.89) and crop rotation (CR_F, AWCD = 3.98). The lowest AWCD index was found in the soil from direct sowing (zero tillage) collected before the sowing of maize (DS_{BS}, AWCD = 3.14).

ITS-1 Next-Generation Sequencing

The taxonomic composition was determined based on the relative abundances of dominant class, orders, genera, and species. Fungi abundances were exported as a representative hit classification to avoid inflated hit counts, in accordance with the MG-RAST manual (ftp://ftp.metagenomics.anl.gov/data/manual/mg-rast-manual.pdf), section 4.5. Sequences and corresponding detailed analysis parameters are deposited in the MG-RAST server under sample identification numbers (**Table 1**). In addition, the raw data was also deposited in The European Nucleotide Archive (ENA) (ID project: PRJEB24318; http://www.ebi.ac.uk/ena/data/view/PRJEB24318 (**Table 1**). The classification rate summary (at least 97% sequence similarity) for all the analyzed soil samples is presented in Supplementary Materials (Table S1). The highest classification rate summary (at least 97% sequence similarity) was found for the soil sample RT_{BS} (the soil before sowing, reduced tillage) and equalled 237924 read classified, which constituted 72.38%.

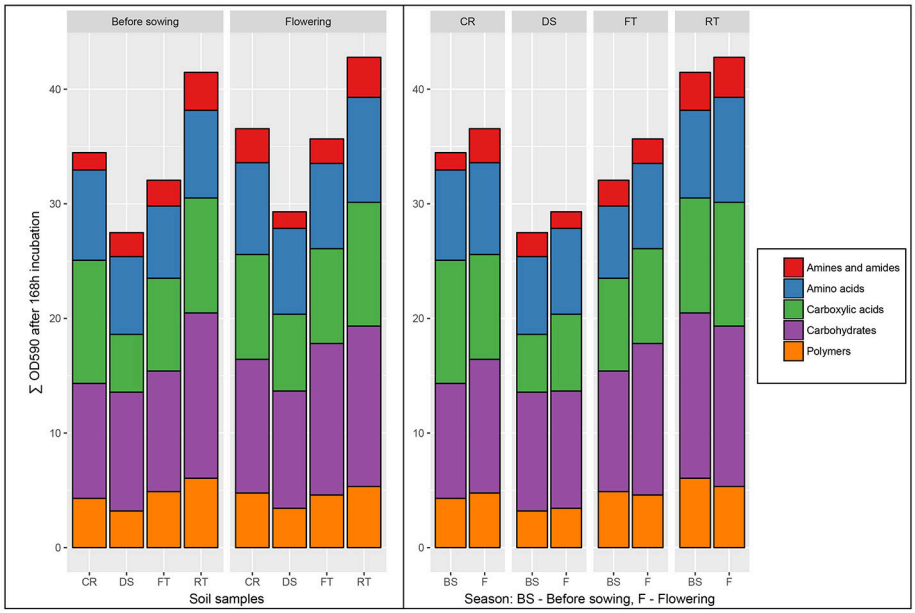

FIGURE 1 | The microbial community catabolic diversity in the soil collected from monoculture of maize according to Biolog EcoPlates incubated for 168 h. Treatment means separated by different letters are significantly different ($P < 0.05$). Soil collected before maize sowing: DS_{BS}, direct sowing; RT_{BS}, reduced tillage; FT_{BS}, full tillage; CR_{BS}, crop rotation. Soil collected in flowering stage of maize growth: DS_F, direct sowing; RT_F, reduced tillage; FT_F, full tillage; CR_F, crop rotation.

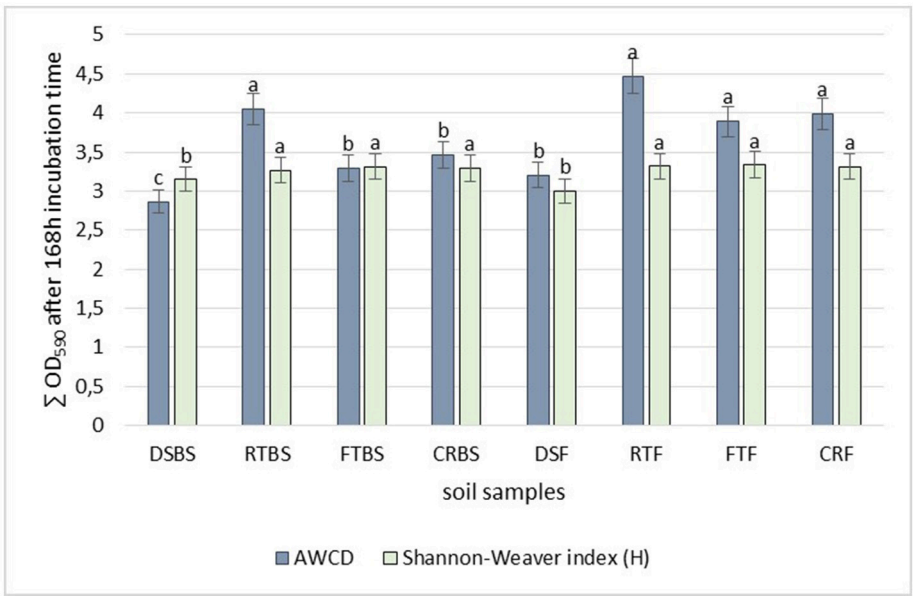

FIGURE 2 | Effect of different cultivation techniques on microbial community catabolic diversity as evaluated by: the Shannon's diversity index (*H*) and average well-color development ($AWCD_{590}$) in the Biolog EcoPlate incubated for 168 h ($P < 0.05$). Soil collected before maize sowing: DS_{BS}, direct sowing; RT_{BS}, reduced tillage; FT_{BS}, full tillage; CR_{BS}, crop rotation; Soil collected in flowering stage of maize growth: DS_F, direct sowing; RT_F, reduced tillage; FT_F, full tillage; CR_F, crop rotation. Treatment means separated by different letters are significantly different ($P < 0.05$).

The lowest classification rate summary (at least 97% sequence similarity) was found for the soil samples: CR_{BS} (soil before sowing, crop rotation) and DS_{BS} (crop rotation) and equalled 60746 read classified for the CR_{BS} sample or 44.01%, and 162114 reads classified for the r DS_{BS} sample. The table of main species along with the full name according to

TABLE 1 | Summary of deposited sequencing data.

Sample ID	Post-QC Sequences count	Post-QC Mean sequence length	Post-QC mean GC percent(%)	MG-RAST ID	ENA ID
DS_{BS}	61.095	255 ± 72 bp	51 ± 10	mgm4755961.3	ERS2075820
RT_{BS}	62.993	252 ± 70 bp	49 ± 9	mgm4755954.3	ERS2075821
FT_{BS}	54.558	258 ± 72 bp	50 ± 10	mgm4755955.3	ERS2075822
CR_{BS}	65.230	227 ± 72 bp	50 ± 10	mgm4755958.3	ERS2075823
DS_F	63.466	251 ± 68 bp	50 ± 10	mgm4755959.3	ERS2075824
FT_F	36.370	222 ± 74 bp	50 ± 10	mgm4755957.3	ERS2075825
FT_F	40.192	244 ± 65 bp	52 ± 9	mgm4755956.3	ERS2075826
CR_F	62.567	255 ± 71 bp	50 ± 10	mgm4755960.3	ERS2075827

Public data is available in the MG-RAST database (http://metagenomics.anl.gov/linkin.cgi?project=mgp81449); number of project: mgp81449. In addition, the raw data was also deposited in The European Nucleotide Archive (ENA) (ID project: PRJEB24318; http://www.ebi.ac.uk/ena/data/view/PRJEB24318. Soil collected before sowing: DS_{BS}, direct sowing; RT_{BS}, reduced tillage; FT_{BS}, full tillage; CR_{BS}, crop rotation. Soil collected in flowering stage of maize growth: DS_F, direct sowing; RT_F, reduced tillage; FT_F, full tillage; CR_F, crop rotation.

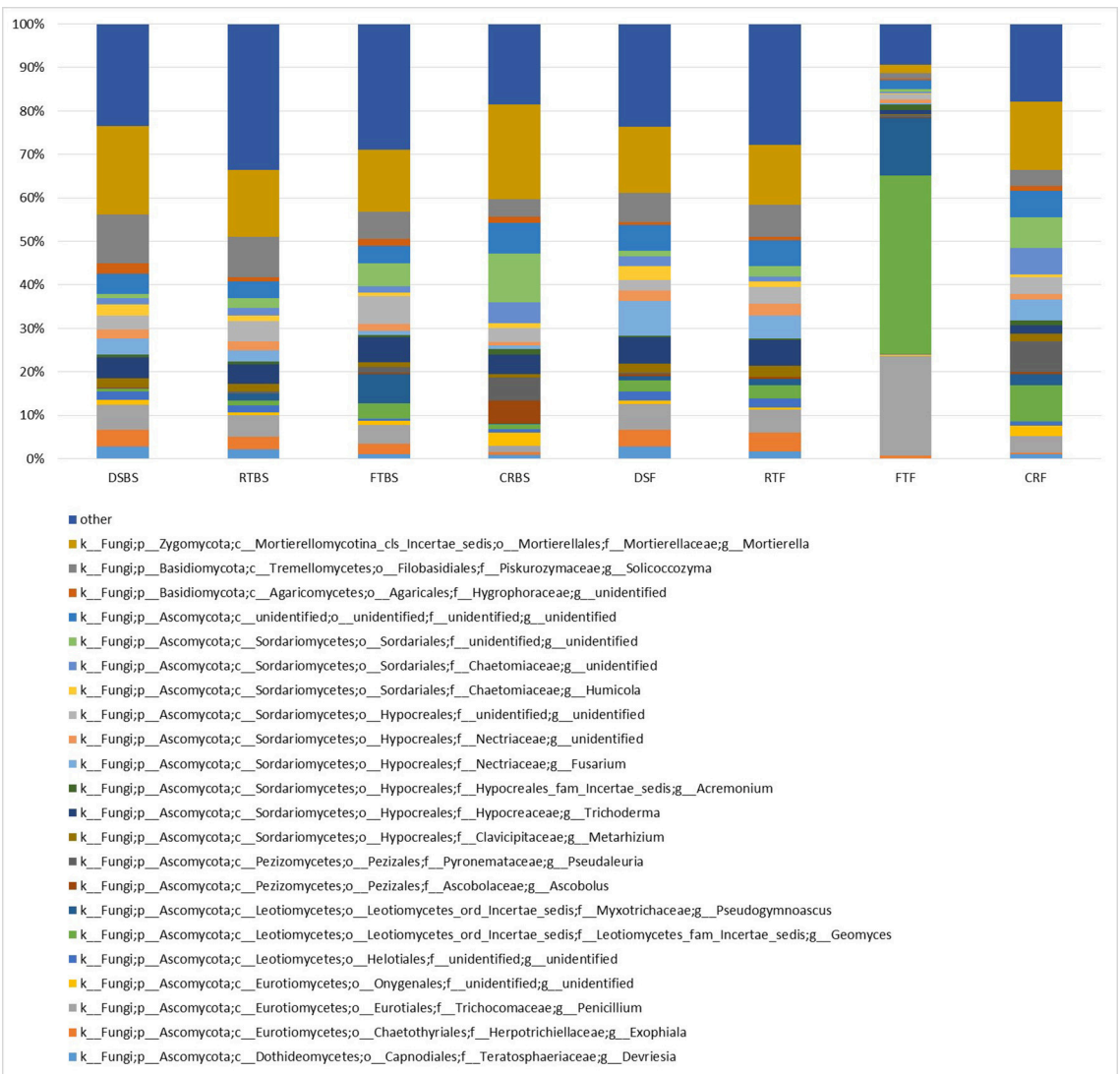

FIGURE 3 | The ITS-1 next generation sequencing for fungal genus. The classifications with less than 1% abundance are gathered into the category "other".

Index Fungorum was included in Supplementary Materials (Table S2).

Significant differences in the fungal community structure were found between soil taken from different sampling time (before the sowing and flowering stages of maize growth). These significant differences were found on the family level (PERMANOVA, $P = 0.032$, $F = 3.895$), genus level (PERMANOVA, $P = 0.026$, $F = 3.313$, and on the species level (PERMANOVA, $P = 0.033$, $F = 2.718$).

Furthermore, the allocation of the assembled contig sequences to fungal genome sequences based on ITS-1 next-generation sequencing for fungal genus sequences is presented in **Figure 3**. The main group among the fungi accounted for three phyla: *Zygomycota, Basidiomycota,* and *Ascomycota*. *Ascomycota* was the dominant phylum and was identified in all the analyzed soil samples (**Figure 3**). Six dominant fungal classes were also identified in the soils: *Dothideomycetes, Eurotiomycetes, Leotiomycetes, Pezizomycetes, Tremellomycetes,* and *Mortierellomycotina*. The significant differences in the genetic structure of the fungal community were observed in the soil collected from full tillage in summer in the flowering stage of maize (FT_F) (**Figure 3**). The dominant genera in this soil obtained in basic relative abundance were: *Penicillum* (28.3%), *Geomyces* (18.4%), *Mortierella* (12.3%), and *Pseudogymnoascus* (11.8%). Significant differences based on the allocation of the assembled contig sequences to fungal community were observed in the fungal genetic structure of soils collected before the sowing and flowering stages of maize growth. Also, the soil collected from full tillage fields was characterized by a different genus structure compared to direct sowing and other cultivation techniques. Soil collected from direct sowing of maize was characterized by the most stable fungal genetic structure, independing on the season compared with other cultivation techniques (**Figure 3**).

Correlation of the ITS fungal genera with the first (PC1) and second (PC2) components of principal components analyses is presented in **Table 2** (statistically significant at $P \leq 0.05$). The following genera were revealed in the analyzed soils: *Alternaria, Bionectria, Boeremia, Chaetomium*, *Cladosporium*, *Cochliobolus, Cylindrocarpon*, *Davidiella*, *Emericellopsis*, *Epichloe*, *Epicoccum*, *Fusarium*, *Gibberella*, *Glomerella*, *Hypocrea*, *Lecanicillium, Lewia*, *Paecilomyces*, *Peyronellaea*, *Phoma*, *Trichoderma*, *Verticillium*, *Conocybe*, *Cryptococcus*, *Guehomyces*, *Sporobolomyces*, *Olpidium*, *Mortierella*, *Mucor,* and *Zygorhynchus*.

To better understand the interdependence and correlation the fungal community structure, functional diversity of microbial community, different cultivation techniques and seasons, a biplot of principal component analysis (PC) was obtained. This analysis was performed for selected phylogenetic levels: class (**Figure 4**), order (**Figure 5**) and species (**Figure 7**). Based on biplot PC for fungal classes, the soils were grouped as follows: soils collected from full tillage from the flowering stage of maize growth (FT_F) with two dominant classes: *Leucoimycetes* and *Eurotiomycetes*, and the second group: soil collected before the sowing from full tillage (FT_{BS}) and reduced tillage (RT_{BS}). A third group was allocated to soils from other cultivation techniques with dominant classes: *Sacharomycetes*, *Sordariomycetes*, *Agariomycetes*, *Microbotrymycetes*, *Tremellomycetes*, *Pezizomycotina,* and *Glomeromycetes* (**Figure 4**). On the other hand, based on biplot PC for fungal orders, the soils were divided into three groups (**Figure 5**). The first group comprised soils collected from direct sowing (DS_{BS} and DS_F) and reduced tillage (RT_{BS} and RT_F) with the following dominant orders: *Pleosporales*, *Glomerales*, *Hypocreales*, *Chaetothyriales*. The second group consists of soils collected from full tillage before sowing (FT_{BS}) and soils collected from crop rotation (CR_{BS} and CR_F) with the following dominant orders: *Thelebolales*, *Pezizales*, *Saccharomycetales*, *Coniochaetales*. The third group was soil collected from full tillage from the flowering stage of maize growth with the dominant orders *Eurotiales*, *Leotiomycetes,* and *Melanosporales* (**Figure 5**). Based on biplot PC for fungal species the soils were divided into three groups but this clustering was strictly conditioned in terms of cultivation techniques (**Figure 6**). The first group were soils collected from direct

TABLE 2 | Correlation of ITS fungal genera with the first (PC1) and second (PC2) component (statistically significant ($P \leq 0.05$).

Genus	PC1 (33.23%)	PC2 (18.96%)
Alternaria	−0.556	
Bionectria		0.605
Boeremia	−0.754	−0.554
Chaetomium		0.747
Cladosporium		0.763
Cochliobolus	−0.680	
Cylindrocarpon	−0.695	−0.616
Davidiella		0.641
Emericellopsis		−0.792
Epichloe	−0.695	−0.616
Epicoccum		0.824
Fusarium	−0.921	
Gibberella	−0.826	0.517
Glomerella	−0.900	
Hypocrea	−0.693	
Lecanicillium	−0.868	
Lewia	−0.733	
Paecilomyces	−0.785	
Peyronellaea	−0.695	−0.616
Phoma		−0.597
Trichoderma	−0.689	−0.522
Verticillium		0.733
Conocybe	−0.520	0.670
Cryptococcus	−0.538	
Guehomyces	−0.520	0.670
Sporobolomyces		0.725
Olpidium	−0.695	−0.616
Mortierella	−0.731	
Mucor	−0.879	
Zygorhynchus		0.822

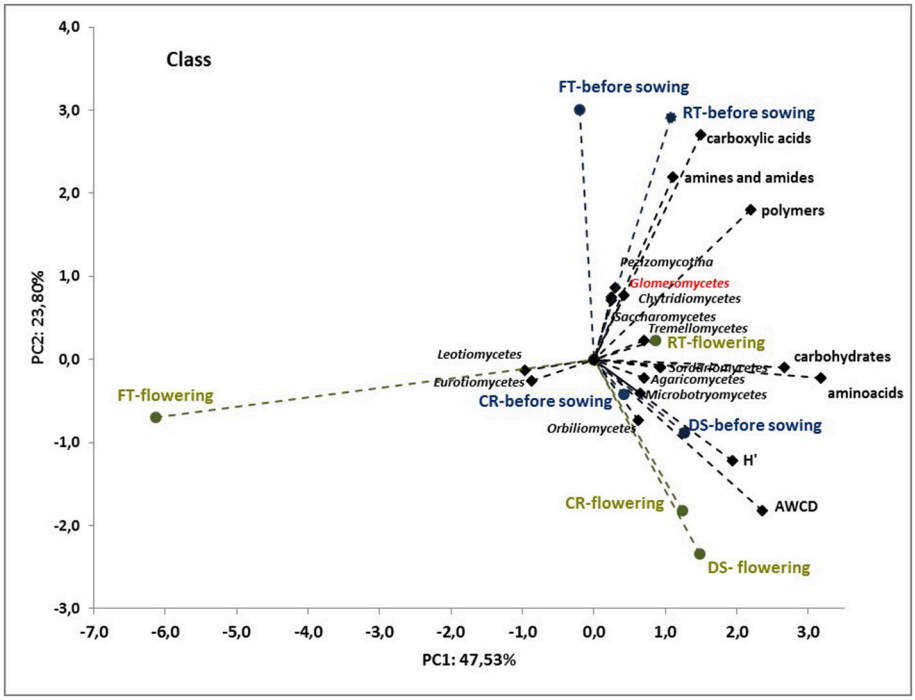

FIGURE 4 | Principal component analysis (PC) of ITS fungal class community and the Shannon's diversity index (*H*) and average well-color development ($AWCD_{590}$) in the Biolog EcoPlate incubated for 168 h ($P < 0.05$).

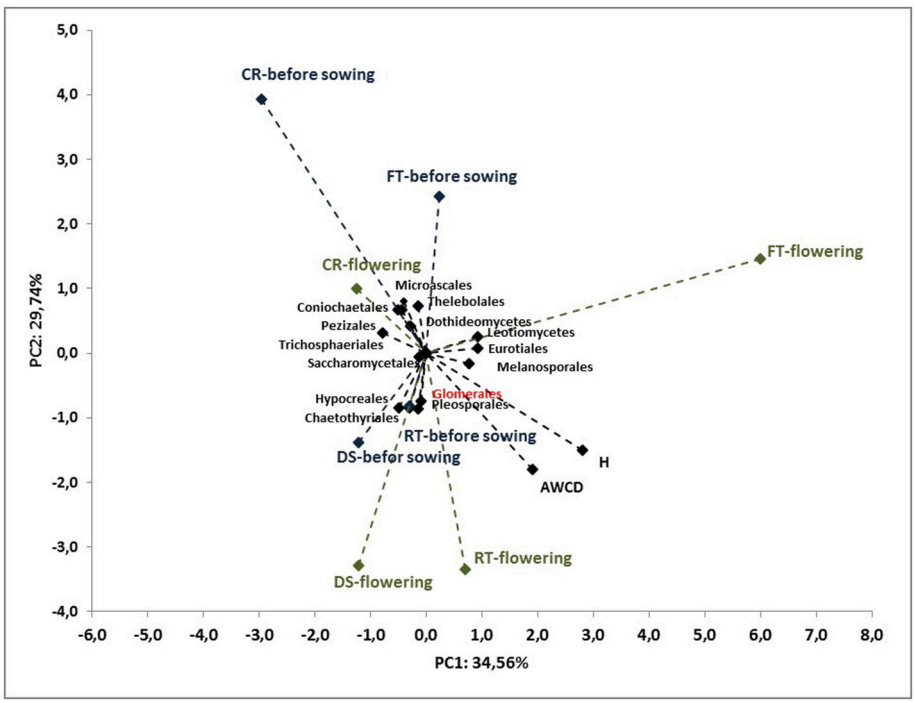

FIGURE 5 | Principal component analysis (PC) of ITS fungal orders community and the Shannon's diversity index (*H*) and average well-color development ($AWCD_{590}$) in the Biolog EcoPlate incubated for 168 h ($P < 0.05$).

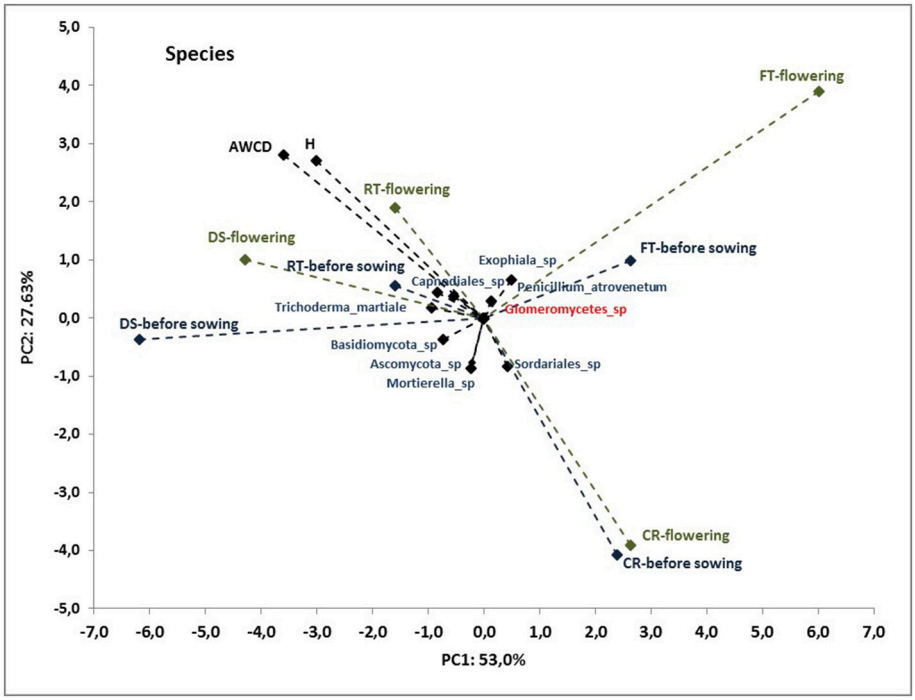

FIGURE 6 | Principal component analysis (PC) of ITS fungal species community and the Shannon's diversity index (*H*) and average well-color development ($AWCD_{590}$) in the Biolog EcoPlate incubated for 168 h ($P < 0.05$).

sowing (DS_{BS} and DS_F) and reduced tillage (RT_{BS} and RT_F) with the following dominant species: *Ascomycota* sp., *Basidiomycota* sp., *Trichoderma martiale*, *Glomeromycetes* sp., *Capnodiales* sp., and *Mortierella* sp. The second group comprised soils collected from full tillage (FT_{BS} and FT_F) with dominant species *Penicillium atrovenetum* and *Exophiala* sp. The last group was soils from crop rotation (CR_{BS} and CR_F) with the dominant species *Sordariales* sp. (**Figure 6**).

Both cultivation techniques and the sampling time had a great influence on the fungal community in the soil. A comparison of fungal species composition, depending on the seasons (before sowing, in the flowering stage) and cultivation techniques, is presented in **Figures 7–10**. Some fungal species dominated in the soils before sowing, others in the flowering phase of maize. The highest relative abundances of *Penicillium canescens*, *Verticillium dahlia*, *Paecilomyces carneus,* and *Hypocrea koningii* was observed in the soils from direct sowing collected before sowing of maize (**Figure 7**). But the highest relative abundances of other species such as *Epicoccum nigrum*, *Davidiella tassiana*, *Geomuyces pannorum*, *Trichoderma hamatum,* and *Penicillum coprophilum* were observed in the soils collected from direct sowing at the flowering stage of maize growth (**Figure 7**). The soils collected from reduced tillage were characterized by the dominant species at the flowering stage such as: *Penicillium ochrochloron*, *Mucor hiemalis*, *Boeremiaexigua*, *Penicillium aculeatum, Trichoderma hamatum* (**Figure 8**). But in soils sampled before sowing, other species were dominant: *Penicillium canescens, Geomyces pannorum, Phoma herbarum,* and *Emericellopsis terricola* (**Figure 8**). Similar differences were observed in the soils collected from full tillage (**Figure 9**) and crop rotation (**Figure 10**). The soils from full tillage were characterized by the highest relative abundance contents of fungal species with main dominant species in the soil taken before sowing such as: *Mortierella alpine*, *Tataromyces flavus*, *Emericellopsis terricola*, *Phoma herbarum,* and *Trichoderma viride* (**Figure 9**).

DISCUSSION

In our study, the soils taken before the sowing of maize were characterized by statistically lower indexes of biological activity of substrate utilization than the soil collected at the flowering stage of maize. The highest Shannon—Weaver index was found in the soil from full tillage in both sampling times: before the sowing and flowering stages of maize growth, but in contrast, the lowest diversity was recorded for the soil collected before sowing from direct sowing. Other authors suggest that the microbial community, especially enzymatic activities is comparatively active in the flowering stage, while before sowing, in spring there is a decrease in their activity (Nannipieri et al., 2003; Brussaard et al., 2007; Bowles et al., 2014; Gajda et al., 2016). This hypothesis was probably connected with the quantitative composition of different root exudates of plants (Fisher et al., 2012; Baetz and Martinoia, 2014). The plant root exudates are very rich in various substances such as aminoacids, hydrocarbons, vitamins, organic

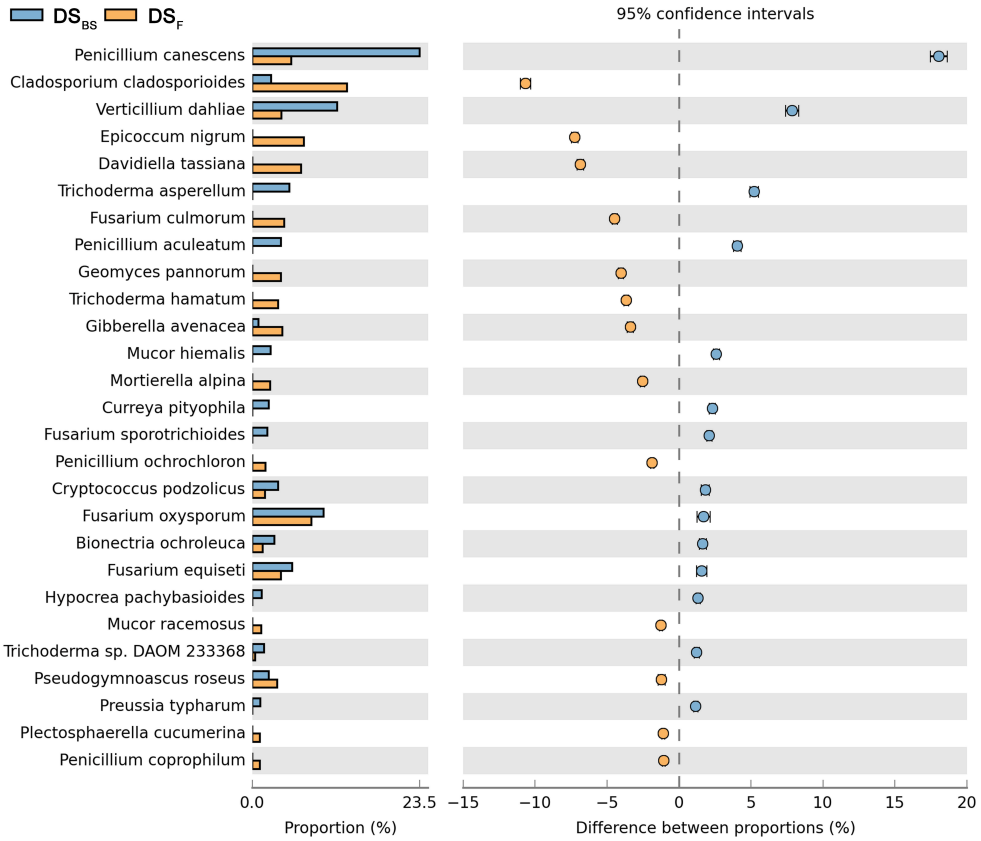

FIGURE 7 | Comparison of fungal species composition depending on the season: before sowing, flowering stage and direct sowing. The table of main species along with the full name according to **Index Fungorum** was included in Supplementary Materials (Table S1).

acids and enzymes (Liang et al., 2011; Razavi et al., 2016). These substances may inhibit or stimulate the growth and development of fungi. On the other hand, changes in the availability of different nutrient compositions coming from different sources such as dead plant cells, root exudates and plants metabolites may have a significant effect on the selection of main fungal species and change their function (Spedding et al., 2004). In our study, the highest Average Well-Color Development (AWCD) index was observed in the soils obtained in the flowering stage of maize growth cultivated using different sowing techniques. The soil community level physiological profiling is not only connected with the plant species but also depends on the amount of plant remains from the root system (Zhang et al., 2012; Gałązka et al., 2017a). Biolog EcoPlate is the very sensitive method used by many authors for indicating even small changes in microbial structure in the soil under the influence of different abiotic and biotic factors (Frac et al., 2012; Wang et al., 2017).

The current paper focuses on the evaluation of the genetic diversity of fungi against the background of the general functional diversity in soil. The authors have choosen the Biolog EcoPlates for research, instead of specialty plates, dedicated to fungi identification (FF or SF). The Biolog EcoPlates indicate even slight changes in microbial structure in the soil under the influence of different abiotic and biotic factors. Many authors confirm that the results of functional diversity obtained from comparison of functional activity in soil with the use of the Biolog EcoPlates, FF-Plates and SF-Plates may be significantly different (Preston-Mafham et al., 2002; Klimek and Niklinska, 2007). In opinion of other authors the Biolog SF-N plates help to avoid the toxicity of TTC dye (present in the EcoPlates) to fungi (Deacon et al., 2006). In current paper, the intention of the authors was to assess only the genetic diversity of fungi against the background of functional diversity of microbial communities in soils under long-term monoculture of maize using different cultivation techniques. That's way community-level physiological profiling (CLPP) method with Biolog multiwell plates was chosen to evaluation functional diversity of microbial communities.

The post-harvest residues in direct sowing positively affect soil properties (Zhang et al., 2012; Wang et al., 2017). Also, a plant cultivation technique is a very important factor that can influence the biochemical activity of soil, and fungal diversity. In the opinion of many authors, plant cultivation in a long-term monoculture and intensive cultivation have a negative impact on the soil quality and can cause changes in the structure of the bacterial and fungal communities (Rice and Gowda, 2011; Wang et al., 2017). Plant cultivation in permanent monoculture is accompanied by a one-sided exhaustion of nutritive components,

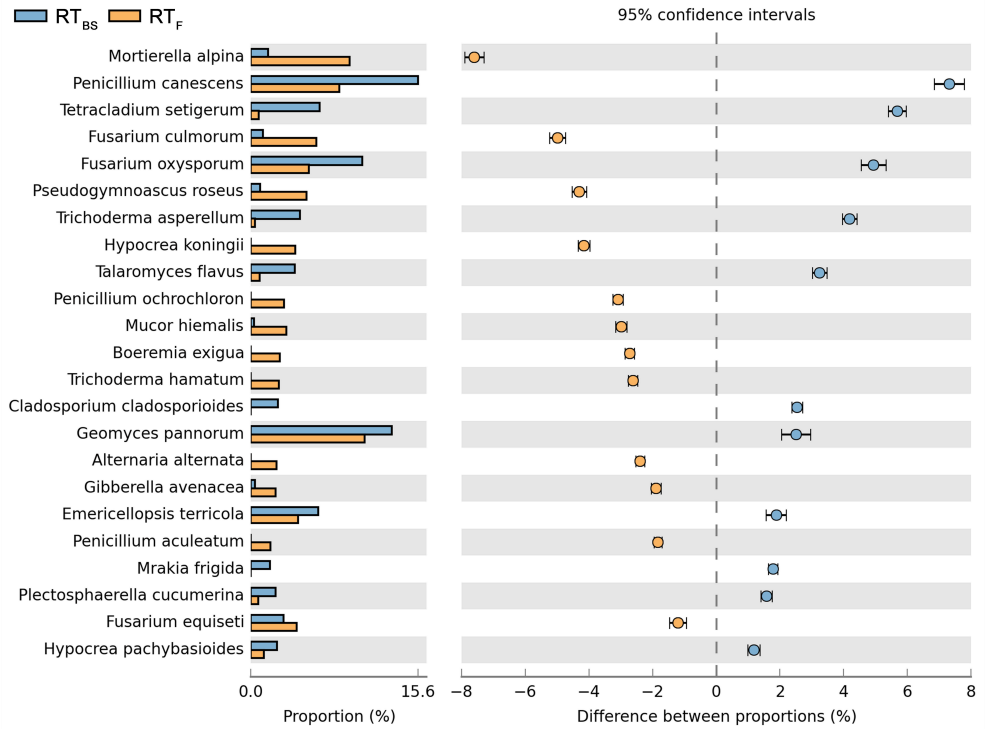

FIGURE 8 | Comparison of fungal species composition depending on the season: before sowing, flowering stage and reduced tillage. The table of main species along with the full name according to **Index Fungorum** was included in Supplementary materials (Table S1).

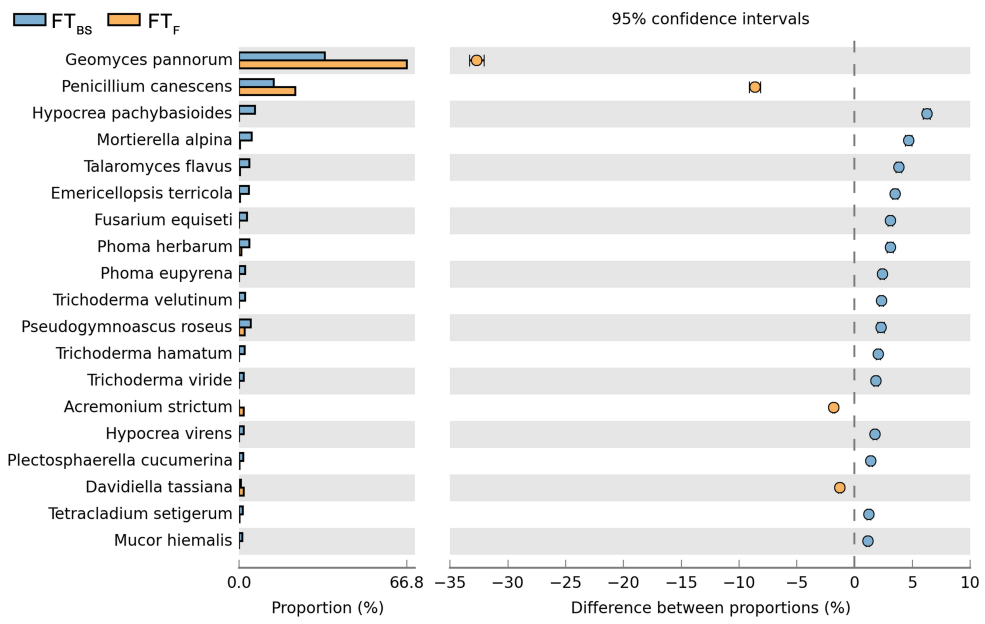

FIGURE 9 | Comparison of fungal species composition depending on the season: before sowing, flowering stage and full tillage. The table of main species along with the full name according to **Index Fungorum** was included in Supplementary Materials (Table S1).

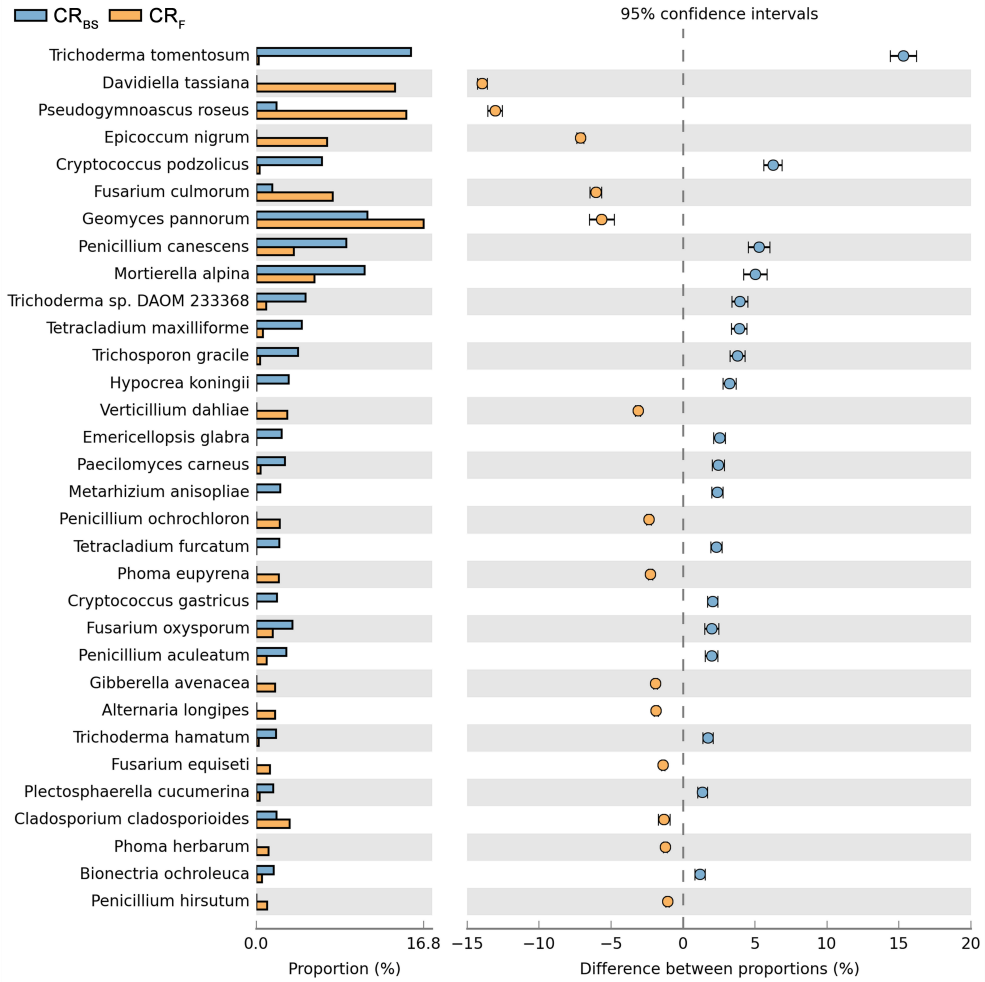

FIGURE 10 | Comparison of fungal species composition depending on the season: before sowing, flowering stage and crop rotation. The table of main species along with the full name according to **Index Fungorum** was included in Supplementary Materials (Table S1).

as well as by changes in the functional and genetic structure of soil microorganisms (Wang et al., 2009).

The results of our study indicate that the main group of fungi accounted for three phyla: *Zygomycota*, *Basidiomycota* and *Ascomycota*, while six dominant fungal classes identified as: *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes*, *Pezizomycetes*, *Tremellomycetes*, and *Mortierellomycotina*. *Ascomycota* was the dominant phylum identified in all the analyzed soil samples. Our results are consistent with the results of other authors (Ma et al., 2013). The soils collected from full tillage in summer at the flowering stage of maize were characterized by the highest diversity in the genetic structure of the fungal community.

Klaubauf et al. (2010) investigated fungal diversity in four arable soils and one grassland in Lower Austria. According to the results of their research all soils were dominated by the ascomycetous orders *Sordariales*, *Hypocreales*, and *Helotiales*, taxa that are known from conventional cultivation approaches occurring in agricultural soils (Klaubauf et al., 2010). Our results also confirm these relationships.

On the other hand, a higher fungal genetic diversity in the soil collected from full tillage indicates that the maize is a very good plant to be grown in monoculture due to the large amount of root exudates into the soil (Baetz and Martinoia, 2014). Han et al. (2017) have proven how biotic and abiotic factors affected fungal diversity in the soil. This study raises questions about the important roles and ecological implications of fungal diversity associated with plant and environmental factors. According to these results compared with edaphic properties controlling soil fungal community patterns, the plant growth stage was the dominant factor in determining their dynamics and development.

The differences in fungal genetic communities in soils under long-term monoculture using different cultivation techniques were significant and caused characteristic changes in the structure of the fungal population (Lupwayi et al., 1998; Brussaard et al., 2007; Bowles et al., 2014; Ghimire et al., 2014). In our study, there was a negative impact observed on community level physiological profiling and fungal genetic structure in the

soil under maize cultivated in long-term monoculture and full tillage. But, on the other hand, maize cultivated in direct sowing did not cause negative changes in the fungal structure making it more stable even during seasonal changes.

Direct sowing and reduced tillage are very good cultivation techniques for the development of the some group of fungi, especially mycorrhizal fungi. The large amount of crop residues left on the soil surface and slight interference with the soil structure may beneficially affect the stability of hyphae in the soil. In full tillage, the fungal hyphae are torn off, which significantly reduces their strangeness in the soil (Gianinazzi et al., 2010). In our study, the order *Glomerales* (belonging to mycorrhizal fungi) was identified in soils collected from direct sowing and reduced tillage. The order *Glomerales*, which includes the family *Glomeraceae* with genus the *Glomus*, is the very important group of arbuscular mycorrhizal fungi (AM) (Ngosong et al., 2010; Gałązka et al., 2017b). AM fungi participate in the bilateral exchange of carbon, phosphorus and other physiologically significant particles. These fungi also have the ability to produce and store in fungal filaments, the special fungal glycoproteins (glomalins) (Rillig, 2004). The carbon present in the glomalins has a large share of the organic carbon in the structure (Gianinazzi et al., 2010). In the natural environment, there is a large diversity of AM fungi (Ngosong et al., 2010). No other fungi, except for *Glomeromycota*, produce glomalins in a significant quantity.

Other genera that are very common in the soils collected from direct sowing and reduced tillage are *Fusarium* and *Penicillium*. Species of *Penicillium* are ubiquitous soil fungi preferring a moderate climate, commonly present wherever organic material is available (Duniere et al., 2017). *Fusarium* is commonly found in the soil and on underground and above - ground parts of plants, including seeds, plant residues and other organic substrates (Leslie and Summerell, 2013). *Fusarium* is common in temperate and tropical climate zones. This genus includes saprotrophic species and economically important species that are pathogenic for plants, causing significant economic losses in cereals (McMullen et al., 2012; Salgado et al., 2015). Of particular importance is the ability of *Fusarium* to produce a variety of toxins, very harmful to humans and animals, which contaminate plant foods with feed and food. *Fusarium* also produces phytotoxins that inhibit the growth of infected plants or cause their wilt, mainly seedlings (Salgado et al., 2015). They can also alter the metabolism of plants in an unfavorable way and act as virulence factors.

CONCLUSIONS

The main research objective presented in this paper involved examination of fungal structural diversity in the soil. The results of this study have contributed to a better understanding of genetic diversity and the composition of the population of fungi in the soil environment under the influence of the changes that take place in the soil under long-term maize cultivation. The cultivation techniques can modify the fungal community in the soil under long-term monoculture of maize. Maize cultivated in direct sowing did not cause negative changes in the fungal structure, making it more stable even during seasonal changes. The biggest changes in the fungal community were observed in full tillage of maize cultivation. In this study, soil under direct sowing and reduced tillage treatment had a higher biological activity, based on community level physiological profiling (especially in the soil samples collected from the flowering stage of maize growth), than that under full tillage and crop rotation treatment. The fungal genetic structure was significantly correlated with agricultural practices and seasons. Agricultural practices and seasons were two important factors affecting the fungal community. Further research is needed to determine the function of the main genera and species dominant in soil under monoculture of maize and to identify key organisms and their dynamics under maize growth using different agricultural management practices. Also, further studies need to quantify the fungal effects on soil biochemical cycling of nutrients and maize production. It is also necessary to conduct further studies on the bacterial identification and comparison of the bacterial and fungal relationship in the soil from long-term monoculture of maize.

AUTHOR CONTRIBUTIONS

AG: Contributions to the conception of the experiment; AG: Contributions to the design of the experiment; AG, JG: The acquisition and analysis of the data; AG, JG: The interpretation of data for the work; AG, JG: Drafting and writing the work; AG, JG: Revising the manuscript; AG, JG: Final approval of the manuscript and agreement to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00076/full#supplementary-material

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Ecology of Alpine Macrofungi - Combining Historical with Recent Data

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Historical datasets of living communities are important because they can be used to document creeping shifts in species compositions. Such a historical data set exists for alpine fungi. From 1941 to 1953, the Swiss geologist Jules Favre visited yearly the region of the Swiss National Park and recorded the occurring fruiting bodies of fungi >1 mm (so-called "macrofungi") in the alpine zone. Favre can be regarded as one of the pioneers of alpine fungal ecology not least because he noted location, elevation, geology, and associated plants during his numerous excursions. However, some relevant information is only available in his unpublished field-book. Overall, Favre listed 204 fungal species in 26 sampling sites, with 46 species being previously unknown. The analysis of his data revealed that the macrofungi recorded belong to two major ecological groups, either they are symbiotrophs and live in ectomycorrhizal associations with alpine plant hosts, or they are saprotrophs and decompose plant litter and soil organic matter. The most frequent fungi were members of *Inocybe* and *Cortinarius,* which form ectomycorrhizas with *Dryas octopetala* or the dwarf alpine *Salix* species. The scope of the present study was to combine Favre's historical dataset with more recent data, either with the "SwissFungi" database or with data from major studies of the French and German Alps, and with the data from novel high-throughput DNA sequencing techniques of soils from the Swiss Alps. Results of the latter application revealed, that problems associated with these new techniques are manifold and species determination remains often unclear. At this point, the fungal taxa collected by Favre and deposited as exsiccata at the "Conservatoire et Jardin Botaniques de la Ville de Genève" could be used as a reference sequence dataset for alpine fungal studies. In conclusion, it can be postulated that new improved databases are urgently necessary for the near future, particularly, with regard to investigating fungal communities from alpine regions using new techniques.

Keywords: *Dryas octopetala*, fungal communities, ectomycorrhiza, *Salix herbacea*, *Salix reticulata*, *Salix retusa*, *Salix serpyllifolia*, Swiss National Park

INTRODUCTION

The Swiss geologist Jules Favre was one of the first mycologists who explored the macrofungal communities in the alpine zones. Employed as a curator at the Natural History Museum of Geneva, he visited the region of the Swiss National Park (SNP) every summer between the years 1941 and 1957 making daily excursions to sample and record the fruiting bodies of fungi larger than 1 mm and easily visible to the naked eye (so-called "macrofungi"). He published the list of fungi occurring in the alpine zone (above timberline) in 1955 (Favre, 1955) and that one from the subalpine zone (below timberline) in 1960 (Favre, 1960). However, Favre (1955) not only presented a list of the occurring fungal taxa, moreover, he provided valuable ecological data, such as site location, elevation above sea level, geology, and associated plants. Hence, Jules Favre can be regarded not only as a pioneer of alpine mycology but also of alpine fungal ecology (Monthoux, 1973; Miskiewicz and Ronikier, 2002).

Other mycological studies from alpine zones were conducted decades later, mainly from the 1960s through the 1970s in the National Park "La Vanoise" in the French Alps (Bon and Géhu, 1973; Eynard, 1977; Kühner and Lamoure, 1986). In the arctic regions, the first mycofloristic investigations were made by Lange (1948, 1955, 1957) and Peterson (1977) in West Greenland, and by Ohenoja (1971) in Svalbard (Norway). In recent years, several studies of alpine fungi in association with the ectomycorrhizal host plants *Dryas octopetala*, *Salix* spp., *Kobresia myosuroides*, and *Bistorta vivipara* have been conducted in alpine regions of the Alps and Scandinavia. Investigations followed either a classical way by collecting fruiting bodies (Debaud et al., 1981; Senn-Irlet, 1987, 1988, 1993; Graf, 1994; Graf and Brunner, 1996) or a modern way by fingerprinting ectomycorrhizal root tips with genetic methods (Mühlmann and Peintner, 2008; Mühlmann et al., 2008; Ryberg et al., 2009, 2011; Bjorbækmo et al., 2010).

However, the vast mycological legacy of Favre (1955, 1960) still awaits a more in-depth analysis. Information from subalpine habitats (Favre, 1960) has been used in a few cases to analyse fungal communities of subalpine meadows and forests in the region of the SNP (Horak, 1985; Brunner and Horak, 1990). Because Favre (1955, 1960) also provided accurate information on sampling locations, his work can potentially serve as a basis for resurveys in the way historical records have been used for vascular plants (Pauli et al., 2012; Wipf et al., 2013). This task is urgent because biodiversity scenarios for the current century consistently forecast a loss of alpine habitats as well as of alpine plants (Thuiller et al., 2005; Engler et al., 2011), possibly resulting in a disappearance of their associated fungi. Few studies (Willis and MacDonald, 2011; Suz et al., 2015) highlight that the availability of long-term datasets is the strongest limiting factor in global change research, and that research linked to long-term monitoring plots will enhance the relevance of scientific results and conclusions to the practical application of mitigation measures.

In the present study, we aimed to analyse the comprehensive myco-ecological study of Favre (1955). The main goal was to locate the most relevant sampling sites of the fungi in the alpine zones of the SNP, to identify the fungal lifestyles, and to characterize the fungal communities associated to *Dryas octopetala* and the alpine dwarf willows *Salix* spp. In addition, we aimed to combine the database of Favre (1955) with the "SwissFungi" inventory (Senn-Irlet, 2010) as well as with more recent literature from the Swiss, French and German Alps in order to provide a more comprehensive basis on the diversity of alpine macrofungi. Furthermore, we intended to compare Favre's data with the newest results from high-throughput DNA sequencing (HTS) analyses from soils of the Swiss Alps (e.g., Frey et al., 2016). With the comparison of the fungal lists it was of interest, whether and which taxa listed by Favre (1955) could also be found with these new HTS techniques. Our ultimate goal was to establish a firm foundation with respect to future challenges related to alpine fungal ecology.

MATERIALS AND METHODS

The Historical Dataset of Favre (1955)

The main source of information was the book of Favre (1955). Because he listed the fungi taxonomically in his book including detailed ecological information assigned to each fungal species, the locations, the elevation above sea level, the geology, and the associated plants had to be extracted in a meticulous way. Additional information was found at the Botanical Garden of Geneva ("Conservatoire et Jardin Botaniques de la Ville de Genève") where the field-book of Jules Favre is kept. In that field-book, each one-day excursion from the years 1942 to 1957 is listed with the date, the routes, and locations in the alpine zone underlined. The Botanical Garden of Geneva also preserves Favre's dried fungal material ("exsiccata") including all the relevant information. The exsiccata are listed and displayed publicly (http://www.ville-ge.ch/musinfo/bd/cjb/chg/result.php?type_search=simple&lang=fr&criteria=favre&mode=tout).

The Swiss National Park

The SNP (http://www.nationalpark.ch) was founded in 1914 and, is the oldest National Park of the Alps. It is located in the southeast of Switzerland and has 170 km^2 of natural landscape including subalpine forests, alpine grassland, and nival peaks up to 3,174 m of height. The SNP is situated on a mosaic of various bedrocks, but dominated by dolomitic limestone and only occasionally interrupted by various other limestone formations. Some parts consist of acidic bedrock formed by granite gneiss of crystalline base or of sandstones, shales, and psephites of the Permian ("Münstertaler Verrucano"). What Favre (1955) called "triassic limestone" corresponds to a quaternary moraine, to dolomite, or to coral limestone. "Gneissic" and "granite soils" correspond to a muscovite granite gneiss and "verrucano" corresponds to a conglomerate of cemented gravel, sand, and rounded stones of different sizes. The predominant geological formations of the sampling area can be viewed with the Swiss geology data viewer (https://map.geo.admin.ch).

Naming the Fungal Taxa and Assigning Their Lifestyles

Naming of the fungal species followed the "Index Fungorum" (http://www.indexfungorum.org/names/names.asp). According to that "Index," many genus names have changed since Favre's descriptions, e.g., *Rhodophyllus* changed to *Entoloma, Geophila* to *Psilocybe*, and the genus *Hygrophorus* has been splitt into several genera, e.g., *Hygrophorus s.s.*, *Hygrocybe, Gliophorus,* or *Cuphophyllus* (Lodge et al., 2014). However, species of *Hygrophorus s.s.* were not found by Favre (1955) since they form ectomycorrhiza (ECM) with trees below the alpine zone.

The lifestyles of fungi were assigned according to the fungal guilds published in Nguyen et al. (2016), e.g., saprotrophs, ectomycorrhizal fungi, lichenized fungi, and many others. Due to new scientific evidence, members of the genus *Helvella* are classified as ectomycorrhizal fungi (Rinaldi et al., 2008; Tedersoo et al., 2010), and the genera *Hygrocybe* and *Entoloma* are considered saprotrophs (Högberg et al., 1999; Hobbie et al., 2001), although there are indications that members of the latter two genera can symbiotically associate with roots (e.g., Tello et al., 2014).

Recent Data from the Swiss, French, and German Alps

In order to compare the historical records of Favre (1955) with more recent fungal records collected in the Alps, the "SwissFungi" database (http://www.swissfungi.ch) and some major studies from the Swiss (Senn-Irlet, 1987; Graf, 1994), French (Bon and Géhu, 1973; Kühner and Lamoure, 1986), and German Alps (Schmid-Heckel, 1985) were considered. The "SwissFungi" database is part of the Swiss national data center for biodiversity initiated by the Federal Office for the Environment (FOEN) and located at the Swiss Federal Institute for Forest, Snow and Landscape Research WSL in Birmensdorf (Switzerland, Senn-Irlet, 2010). The main objective of "SwissFungi" is to present updated maps of the distribution of fruiting bodies for each species of fungi in Switzerland. These distribution maps serve as a base for the elaboration of the Red List of threatened species and inform authorities about the presence of species requiring protection in each region. Since 1992, more than half a million records of fungi have been stored, with a focus on macrofungi, in order to deepen the ecological knowledge about the species diversity at a national level.

High-Throughput DNA Sequencing Techniques of Swiss Alpine Soils

In order to compare the fungal records of Favre (1955) with most recent fungal records from the Alps, the lists of newly generated data using novel high-throughput DNA sequencing (HTS) techniques of alpine soils in Switzerland were used. Such novel DNA sequencing techniques allow the analysis of the entire fungal community in soils in different ecosystems, including alpine soils (Hartmann et al., 2014, 2015; Pellissier et al., 2014). HTS techniques allow an in-depth description of the microbial diversity and their potential relative abundance by massively parallel DNA sequencing to increase the number of sampled PCR amplicons in environmental surveys (Sogin et al., 2006). Here we compare the fungal records of Favre (1955) with the HTS results from the fungal communities of two studies carried out with soils of the Swiss Alps. Rime et al. (2015) investigated soils of a recently deglaciated area in front of the "Damma" glacier (2,000–2,100 m a.s.l.), and Frey et al. (2016) investigated soils near the summit of "Muot da Barba Peider" (2,960 m a.s.l.) with permanently frozen soils ("permafrost") in deeper layers. In both studies, the internal transcribed spacer region 2 (ITS2) of the eukaryotic ribosomal operon (fungi and some groups of protists and green algae) was amplified (Frey et al., 2016).

RESULTS

Excursion Destinations of Favre (1955)

Between 1941 and 1957, Favre usually spent several weeks during the summer months at one location and made daily excursions into the subalpine and alpine zones. Because of his advanced age, he stopped the excursions into the alpine zones in 1953. In total, he made 66 excursions, with one to ten excursions per year. He started his excursions either from Il Fuorn and Pass dal Fuorn (during six years) or from the village of S-charl (during 7 years) (**Figure 1A**). His earliest excursion started in late July

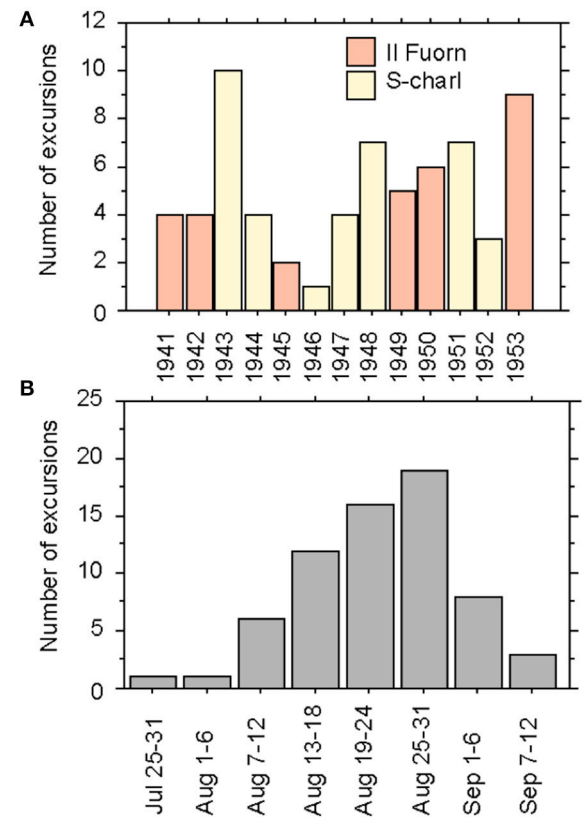

FIGURE 1 | Bar charts of the excursions made by Favre (1955) in the region of the Swiss National Park according to his field-book. **(A)** Number of the excursions made per year from 1941 to 1953 in the surroundings of Il Fuorn (orange) and S-charl (yellow), and **(B)** number of excursions made per time period in the months July to September.

and his latest in early September, but with a clear majority of the excursions taking place in the second half of August (**Figure 1B**).

From S-charl most of the excursions lead Favre into the nearby valleys Val S-charl (16x) and Val Sesvenna (13x) (**Table 1**). In the surroundings of Il Fuorn and Pass dal Fuorn he mainly visited the areas of Murtaröl (8x) and Munt la Schera (5x), as well as the nearby valleys Val dal Botsch (5x) and Val Nüglia (3x) (**Table 1**). An overview of the SNP and its surroundings, including the two main excursion areas Il Fuorn and S-charl, is given in **Figure 2**.

Sampling Sites of Favre (1955)

The analysis of Favre's (1955) book and his field-notes revealed about 26 sites where he sampled fungal fruiting bodies (**Table 2**, **Figure 2**). The majority of the sampling sites were on calcareous rocks (15 of 26 sites; dolomite, quaternary moraine, coral limestone) where the calciphilic ectomycorrhizal plants *Dryas octopetala*, *Salix retusa, S. reticulata*, and *S. serpyllifolia* grow. Ten sites on muscovite granite gneiss and one on verrucano were preferentially colonized by the also ectomycorrhizal, but acidophilic, *Salix herbacea*. A few sites on the quaternary moraine, dolomite, and muscovite granite gneiss hosted *D. octopetala* as well as *S. herbacea* (**Table 2**).

TABLE 1 | Excursion destinations, regions, and number of excursions (n) according to the field-book of Favre (1955).

Excursion destination	Region	*n*
IL FUORN AND SURROUNDING AREAS		
Murtaröl, Piz Daint, Taunter Pizza, Fuorn	Pass dal Fuorn	8
Val Nüglia	Pass dal Fuorn	3
Munt da la Bescha	Pass dal Fuorn	3
Val dal Botsch, Margunet, Val Stabelchod	Il Fuorn	5
Munt la Schera, Munt Chavagl	Il Fuorn	5
Murteras da Grimmels, Murteras d'Ivraina, Val Laschadura, Foppinas	Il Fuorn	3
Piz Murtèr	Il Fuorn	2
Val Mingèr	Val Mingèr	1
Total		30
S-CHARL AND SURROUNDING AREAS		
Ils Lajets	Val Sesvenna	4
Source of Sesvenna	Val Sesvenna	3
Blaisch dals Manaders	Val Sesvenna	3
Marangun	Val Sesvenna	2
Mot da l'Hom	Val Sesvenna	1
Mot dal Gajer	Val S-charl	4
Murters da Tamangur	Val S-charl	3
Costainas	Val S-charl	3
Valbella	Val S-charl	2
Munt Plazèr	Val S-charl	2
Mot Mezdi, Mot Madlain	Val S-charl	2
Blaisch Bella	Val Tavrü	2
Val Mingèr	Val Mingèr	5
Total		36

Some sites have a large elevation span, e.g., from about 2,000 to 2,600 m a.s.l. for Val Nüglia and Munt la Schera, and other sites have only a narrow range, e.g., from 2,150 to 2,200 m a.s.l. for Valbella and from 2,600 to 2,650 m a.s.l. for Munt Plazèr (**Table 2**).

The most relevant sampling sites with >30 fungal records are the sites Murtaröl and Val dal Botsch, and with 21–30 fungal records the sites Piz Mezdi, Val Mingèr, Valbella, Val Nüglia, and Munt la Schera/Munt Chavagl (**Table 2.** dark gray shadows). The largest number of fungal species in association with *D. octopetala* was observed by Favre (1955) in the calcareous sites Murtaröl (24 species), Val dal Botsch (20), and Val Nüglia (18), in association with the calciphilic *Salix* species in Valbella (13) and Murtaröl (11), and association with the acidophilic *S. herbacea* in Ils Lajets (17) (**Table 2**).

Fungal Taxa Recorded by Favre (1955)

In his book, Favre (1955) listed in total 202 fungal species in 1302 records mainly belonging to macrofungi. The majority of recorded species belonged to the Basidiomycota, whereas he only recorded 12 species belonging to the Ascomycota. A large majority of the fungi were already known from other regions of Europe. At least 63 of them were known from forests or meadows, seven from dung, and 11 from mires and swamps (Favre, 1955). However, Favre (1955) designated about 121 species as mainly alpine species. About one-third of them were unknown to him, and, thus, he described them as new species. In total, he described 46 new species and classified the majority of them as members of the genera *Inocybe* and *Cortinarius*. Additionally, for 20 species that were already known, he described several variations and forms. In some cases, other mycologists assigned such forms and variations to new species at a later stage, e.g., *Hebeloma alpinum* (=*Hebeloma crustuliniforme var. alpinum* according to Favre, 1955) by G. Bruchet, *Inocybe taxocystis* (=*I. decipientoides var. taxocystis* according to Favre, 1955) by E. Horak, or *Amanita oreina* (=*A. vaginata f. oreina* according to Favre, 1955) by R. Heim. On the other hand, two taxa described by Favre (1955), *Marasmius obscurus* and *Rhodophyllus umbella*, have not been accepted because no deposited exsiccata are available. All these modifications resulted in an overall species number of 204 instead of 202 as listed in Favre (1955) (see also below).

The genera of fungi with the highest number of recorded species were *Inocybe* with 40 species, *Cortinarius* with 26 species, and *Entoloma* with 14 species. Other abundant fungi were members of the genera *Hygrocybe, Omphalina, Marasmius,* and *Psilocybe*. The individual species with the largest number of observations by Favre (1955) were *Inocybe rimosa, I. dulcamara, Hebeloma marginatulum*, and *Hygrocybe conica,* each of which had >40 records (**Tables 3**, **4**). A rank-abundance diagram shows that most of the fungal taxa were found only once or only a few times: he recorded about 34% of the fungi only 1 time, and 76% of the fungi ≤5 times (**Figure 3**). The highest elevation from where Favre recorded fungal fruiting bodies was at 2,850 m a.s.l. (*Omphalina pyxidata, Arrhenia griseopallida*.)

The majority of the fungi recorded by Favre (1955) are symbiotrophs or saprotrophs. Among the symbiotrophs, ectomycorrhizal fungi (ECM) are dominant, and only

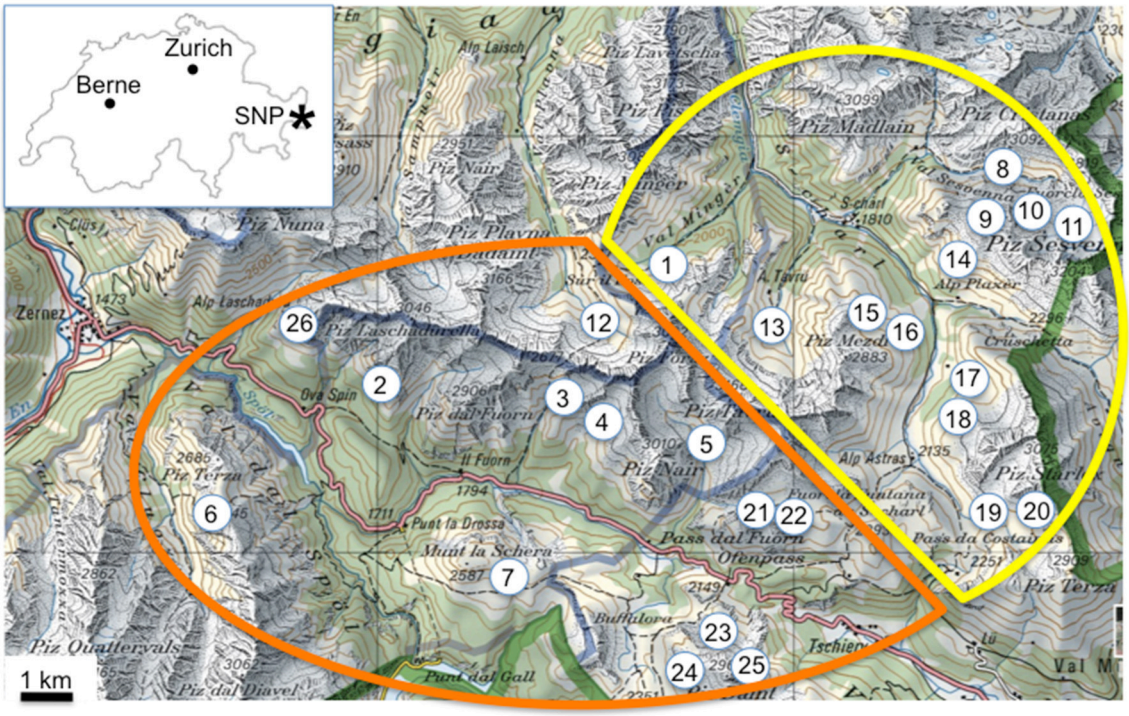

FIGURE 2 | Overview of the sampling sites of Favre (1955) in the region of the Swiss National Park (SNP). The sampling sites are in the surroundings of Il Fuorn (orange line) and of S-charl (yellow line). The inset graph shows the location of the SNP (*) within Switzerland. The green solid line shows the border between Switzerland and Italy, and the blue solid line shows the border of the SNP. Reproduced with permission from swisstopo (JA100118). Sites within SNP: (1) Val Mingèr, (2) Murteras da Grimmels, (3) Val dal Botsch, (4) Val Stabelchod, (5) Val Nüglia, (6) Alp Murtèr, Piz Murtèr, (7) Munt la Schera, Munt Chavagl. Sites outside of SNP: (8) Marangun, (9) Blaisch dals Manaders, (10) Ils Lajets (11) source of Sesvenna, (12) Val Plavna, (13) Blaisch Bella, Val Tavrü, (14) Munt Plazèr, (15) Mot dal Gajer, (16) Piz Mezdi, (17) Valbella, (18) Murters da Tamangur, (19) Costainas, (20) Piz Starlex, (21) Chaschlot, (22) Munt da la Bescha, (23) Murtaröl, (24) Taunter Pizza, Jufplaun, (25) Piz Daint, (26) Val Laschadura, Foppinas.

a few species were lichenized, such as the basidiolichen *Lichenomphalina* spp. Other lichenized fungi were not recorded, although known to be abundant in the alpine zone. Overall, 95 ectomycorrhizal, 105 saprotrophic, and 4 lichenised taxa were recorded, resulting in a total of 204 taxa (**Tables 3**, **5**, Tables S1, S2).

Ectomycorrhizal Fungi Recorded by Favre (1955)

Overall, Favre (1955) recorded 27 ECM fungal taxa with >5 records (**Table 3**). Among them are *Inocybe rimosa s.l., I. dulcamara s.l., Hebeloma marginatulum, H. alpinum* and *Laccaria laccata.* However, only *Hebeloma marginatulum* and *H. alpinum* seem to be confined to alpine habitats (**Table 3**), whereas the *Inocybe* species and *L. laccata* are known from lower altitudes as well. Most of the ECM taxa, however, were only occasionally found (≤5 records), and some were recorded only once.

From the whole range of the ECM species found by Favre (1955) 22% were found to be associated with *Dryas* as well as with all of the four dwarf willows (**Table 3**, **Figure 4A**). Thirty percent of the ECM taxa grew specifically with *Dryas*, 22% specifically with *Salix herbacea*, and 7% specifically with the three *Salix* species on calcareous soils (Table S1). About one third of these ECM fungal taxa were newly described by Favre (1955) (indicated with an * in the lists).

In most cases Favre (1955) indicated the putatively associated alpine plants *Dryas octopetala* and the four dwarf willows. Only in a few cases, Favre cited other potential hosts, such as *Juniperus communis* (presumed mycorrhizal partners: *Cantharellus cibarius, Russula sanguinea, Tricholoma inamoenum*; **Table 5**), *Loiseleuria procumbens* (*Cortinarius cinnamomeus, Hebeloma marginatulum, Russula emetica, R. pascua*), and *Bistorta vivipara* (*Inocybe dulcamara*). However, only *B. vivipara* is known to host ECM fungi, whereas *J. communis* hosts preferentially arbuscular mycorrhizal (AM) fungi and *L. procumbens* ericoid fungi (Read and Haselwandter, 1981). Besides *D. octopetala*, *Salix* spp. and *B. vivipara,* it is known that *Kobresia myosuroides, Arctostaphylos uva-ursi, A. alpina,* and *Helianthemum* spp. also form ECM, and even some *Carex* species may live in an ectomycorrhizal symbiosis (see also Read and Haselwandter, 1981).

Saprotrophic Fungi Recorded by Favre (1955)

About 20 taxa of the more than 100 saprotrophic fungi were abundantly found with >5 records. The most common species were *Hygrocybe conica* and *Psilocybe montana,* which were

TABLE 2 | Sampling sites and number of fungal species of Favre (1955).

Sampling sites	Coordinates (N/W)	Elevation (m a.s.l)	Geology	Fungal records in association			No.
				D	*Srrs*	*Sh*	
Murtaröl	46°38′/10°17	2,250–2,500	Quaternary moraine	24	11	-	23
Piz Mezdi	46°41′/10°20′	2,200–2,500	Quaternary moraine	16	5	-	16
Val Mingèr	46°43′/10°16′	1,900–2,550	Quaternary moraine	14	8	-	1
Valbella	46°41′/10°22′	2,150–2,200	Quaternary moraine	9	13	-	17
Alp Murtèr/Piz Murtèr	46°41′/10°09′	2,300–2,700	Coral limestone	6	9	-	6
Val Stabelchod	46°41′/10°16′	2,250–2,600	Quaternary moraine	6	8	-	4
Chaschlot	46°39′/10°17′	2,250–2,500	Dolomite	8	1	-	21
Murteras da Grimmels	46°41′/10°11′	2,400–2,650	Dolomite	6	1	-	2
Piz Daint	46°38′/10°17′	2,300–2,450	Dolomite	3	2	-	25
Val Plavna/Spadla Sura	46°42′/10°16′	2,100–2,400	Quaternary moraine	2	2	-	12
Blaisch Bella/Val Tavrü	46°41′/10°18′	2,300–2,450	Muscovite granite gneiss	1	7	-	13
Val dal Botsch	46°41′/10°15′	2,000–2,800	Quaternary moraine	20	8	3	3
Val Nüglia	46°41′/10°17′	2,000–2,600	Quaternary moraine	18	5	3	5
Munt la Schera/M. Chavagl	46°39′/10°13′	2,000–2,550	Dolomite	15	7	2	7
Munt da la Bescha	46°39′/10°18′	2,200–2,550	Dolomite	6	3	4	22
Blaisch dals Manaders	46°43′/10°22′	2,400–2,700	Muscovite granite gneiss	3	1	5	9
Costainas	46°39′/10°23′	2,400–2,650	Muscovite granite gneiss	2	5	2	19
Marangun	46°44′/10°23′	2,200–2,400	Muscovite granite gneiss	2	1	3	8
Murters da Tamangur	46°40′/10°22′	2,350–2,500	Muscovite granite gneiss	1	1	3	18
Mot dal Gajer	46°42′/10°20′	2,350–2,750	Muscovite granite gneiss	1	1	1	15
Val Laschadura/Foppinas	46°42′/10°09	2,100–2,700	Muscovite granite gneiss	1	1	1	26
Ils Lajets	46°43′/10°23′	2,400–2,700	Muscovite granite gneiss	-	1	17	10
Source of Sesvenna/moraine	46°43′/10°24′	2,250–2,650	Muscovite granite gneiss	-	3	5	11
Munt Plazèr	46°42′/10°23′	2,600–2,650	Muscovite granite gneiss	-	3	5	14
Taunter Pizza/Jufplaun	46°37′/10°17′	2,300–2,650	Verrucano	-	5	5	24
Piz Starlex	46°39′/10°24′	2,250	Dolomite	2	-	-	20

The macrofungi were growing in association with the calciphilic host plants Dryas octopetala (D) and Salix reticulata, S. retusa and S. serpyllifolia (Srrs), and with the acidophilic host plant Salix herbacea (Sh). The sampling sites are listed with their coordinates, elevation and the predominant geological bedrock.
Number of fungal records, 1–10, 11–20, 21–30, 31–40. No., Site number according to ***Figure 2****.*

recorded in grasslands as well as in mats of *Dryas* and all four dwarf willows (**Table 4**). This clearly dominant group of fungi represented by 36 species grew exclusively in grasslands: 17 taxa on acidic soils, 10 taxa on calcareous soils, and 9 taxa on both soil types (**Table 4**, **Figure 4B**, Table S2). Another large group with 16 taxa was found exclusively in association with *Dryas*, e.g., *Clitocybe dryadicola, Rhizomarasmius epidryas, Infundibulicybe lateritia,* and *Gymnopus dryophilus*, probably decaying leaves or woody parts of *Dryas*. In contrast, *Arrhenia obatra* grew mainly in association with *Salix spp*. on calcareous as well as on acidic soil. Some abundant taxa found in the alpine meadows are also known from meadows of lower altitudes, e.g., *Bovista nigrescens* and *Lycoperdon umbrinum* (**Table 4**).

Fungi recorded in bogs and swamps were mainly confined to these types of habitat. In total, 14 taxa were recorded, e.g., *Galerina annulata*, *Bovistella paludosa* and *Psilocybe elongata* (**Table 4**, Table S2). Five taxa were found to grow exclusively in "cowpads" on alpine pastures outside the SNP, e.g., *Panaeolus semiovatus* (**Table 5**).

Exsiccata from Favre (1955) Deposited at the Conservatory and Botanical Garden in Geneva

A large proportion of the fungal specimens recorded including those newly named in Favre (1955), are stored at the "Conservatoire et Jardin Botaniques de la Ville de Genève." The list of fungi is provided at http://www.ville-ge.ch/musinfo/bd/cjb/chg. Overall, 392 exsiccata of fungal specimens collected by J. Favre are deposited in Geneva, of which 249 specimens were from the region of the SNP (Favre, 1955, 1960). Of these, 188 were sampled in the alpine zone above 2,000 m a.s.l., with six specimens classified as "typus," 20 as "holotypus," four as "isotypus," 28 as "lectotypus," 124 as "syntypus," three as "isosyntypus," two as "isolectotypus," and one was not assigned to a particular type. In our study, and according to the "Index Fungorum," we recorded 59 type specimens indicated with (*) (**Tables 3**, **4**, Tables S1, S2). The majority are members of the genera *Inocybe* (20 newly described taxa) and *Cortinarius* (20), followed by *Entoloma* (3), and *Hebeloma*, *Arrhenia*, and *Clitocybe*

TABLE 3 | Abundant ectomycorrhizal fungal species (>5 records) of Favre (1955).

Species name	Species name (in Favre, 1955)	Fungal records in association with		
		Dryas o.	*Salix rrs.*	*Salix h.*
ASSOCIATED WITH ALL *DRYAS* AND *SALIX* SPECIES				
Inocybe rimosa	(=*I. fastigiata, f. alpestris+alpina*)		59	
Inocybe dulcamara	(=*I. dulcamara, all forms)*		47	
Hebeloma marginatulum *	(=*H. versipelle var. marginatulum*)		46	
Laccaria laccata	(=*L. laccata var. montana*)		43	
*Hebeloma alpinum**	(=*H. crustuliniforme var. alpinum*)		35	
*Inocybe geraniodora**			29	
*Cortinarius minutulus**			27	
*Cortinarius tenebricus**			27	
Hebeloma mesophaeum			20	
Cortinarius hinnuleus	(=*C. hinnuleus, all forms and varieties)*		16	
Cortinarius anomalus			15	
Inocybe nitidiuscula	(=*I. Friesii, all forms;*		15	
	=*I. ovalispora-subbrunnea f. brunneola)*			
Laccaria pumila	(=*L. laccata var. pumila*)		12	
*Cortinarius pauperculus**			8	
Inocybe decipiens	(=*I. decipiens, all varieties)*		8	
Helvella corium	(=*H. arctica var. macrosperma; =H. corium f. alpestris)*		6	
ASSOCIATED WITH CALCIPHILIC *DRYAS* AND *SALIX* SPECIES				
*Inocybe canescens**			25	-
Inocybe fuscomarginata			14	-
*Inocybe oreina**			6	-
ASSOCIATED WITH ALL *SALIX* SPECIES				
Cortinarius alpinus		-	26	
Cortinarius cinnamomeus		-	22	
*Cortinarius phaeopygmaeus**		-	14	
Inocybe praetervisa		-	14	
*Amanita oreina**	(=*A. vaginata f. oreina*)	-	11	
ASSOCIATED WITH *SALIX HERBACEA*				
Russula pascua	(=*R. xerampelina var. pascua)*	-	-	21
*Lactarius nanus**		-	-	18
Inocybe lacera	(=*I. lacera, all forms and varieties; =I. rhacodes)*	1	-	14

The macrofungi were growing in association with the calciphilic Dryas octopetala (Dryas o.) and Salix reticulata, S. retusa and S. serpyllifolia (Salix rrs.) on calcareous rocks, and with the acidophilic Salix herbacea (Salix h.) on gneiss or verrucano.
Number of fungal records, 6–10, 11–20, 21–30, 31–40, 41–60.
**Named by Favre.*

(each 2). With so many type specimens assigned, all sampling sites of Favre (1955) (see **Table 2**) within and around the SNP can be considered as "type locations."

In recent years, several microscopical re-examinations of Favre's dried specimens from the botanical garden in Geneva have led to taxonomic revisions of whole genera, subgroups, or single specimens, e.g., Horak (1987a) and Senn-Irlet (1992) of *Cortinarius*, Horak (1987b) of *Inocybe*, Horak (1993) of *Entoloma,* and (Beker et al., 2016) of *Hebeloma*. However, DNA analyses of Favre's type specimens are rare and have only been used in a few studies. Altogether, the DNA sequences of eight type specimens (e.g., *Cortinarius*, *Entoloma, Hebeloma,* and *Inocybe* species) have been included recently in mycological surveys and phylogenetic analyses by Cripps et al. (2010), Kokkonen and Vauras (2012), Kokkonen (2015), Eberhardt et al. (2015), Liimatainen et al. (2015), and Garnica et al. (2016) (**Table 6**).

The "Swissfungi" Database and Other Records from the Swiss, French, and German Alps

A check through the "SwissFungi" database in connection with other studies from the alpine zone from the Swiss, French, and German Alps revealed that the diversity of ECM fungi is high (210 species). About half of these fungi are generalists (117 species, 56%) and associated with *Dryas octopetala* as well as with the four alpine *Salix* species (Table S3). A smaller portion of fungi (38, 18%) is exclusively associated with calciphilic *D. octopetala* or with acidophilic *Salix herbacea* (23, 11%). A smaller portion is

TABLE 4 | Abundant saprotrophic fungal species (>5 records) of Favre (1955).

Species name	Species name (in Favre, 1955)	Fungal records in association with					
		D	*Srrs*	*Sh*	Gc	Ga	B
GROWING UBIQUITOUS							
Hygrocybe conica	(=*Hygrophorus conicus et var. nigresc.)*			42			-
Psilocybe montana	(=*Geophila atrorufa)*			30			-
GROWING WITH *DRYAS OCTOPETALA*							
*Clitocybe dryadicola**	(=*C. rivulosa var. dryadicola*)	39	-	-	-	-	-
Rhizomarasmius epidryas	(=*Marasmius epidryas*)	30	-	-	-	-	-
*Infundibulicybe lateritia**	(=*Clitocye lateritia*)	28	-	-	-	-	-
Gymnopus dryophilus	(=*Marasmius dryophilus*)	11	-	-	-	-	-
PREFERENTIALLY GROWING WITH ALL *SALIX* SPECIES							
*Arrhenia obatra**	*Omphalina obatra*	-		19	-	-	-
Entoloma conferendum	(=*Rhodoph. staurosporus, var. Rickeni)*	-		5	-	2	-
PREFERENTIALLY GROWING WITH *SALIX HERBACEA*							
*Entoloma alpicola**	(=*Rhodophyllus clypeatus f. alpicolus;* =*Rhodophyllus sericeus var. nanus)*	-	-	11	-	11	-
PREFERENTIALLY GROWING IN GRASSLANDS							
Bovista nigrescens		1	-	-		27	-
Lycoperdon umbrinum		1	1	-		23	-
Bovista tomentosa		3	1	-		14	-
Cuphophyllus pratensis	(=*Hygrophorus pratensis)*	-	-	-		12	-
Lycoperdon nigrescens		1	1	-		11	-
Bovista plumbea		1	-	-		10	-
Melanoleuca strictipes	(=*M. evenosa)*	-	-	-		8	-
Entoloma sericeum	(=*Rhodophyllus sericeus f flexipes* + *f. rubellotactus* + *f. luridofuscus)*	1	-	1		6	2
Hygrocybe marchii	(=*Hygrophorus Marchii)*	3	-	-		5	-
Melanoleuca stridula		3	-	-		3	-
GROWING IN BOGS							
*Galerina annulata**	(=*Galera rubiginosa var. annulata)*	-	-	-	-	-	6

The macrofungi were growing in association with calciphilic Dryas octopetala (D) and/or Salix reticulata, S. retusa and S. serpyllifolia (Srrs) and/or acidophilic Salix herbacea (Sh), and/or in alpine grassland on calcareous (Gc) and/or acidic soils (Ga) and/or in bogs (B).
Number of fungal records, 6-10, 11-20, 21-30, 31-40, 41-60.
**Named by Favre.*

only associated with the four *Salix* species (32, 15%). Dominant genera are *Inocybe* with 99 species (49%) and *Cortinarius* with 54 species (26%). Besides the "SwissFungi" database, which included 130 species, the compilation of the French Alps from Kühner and Lamoure (1986) contributed considerably with 116 species to this list of ECM fungi.

High-Throughput DNA Sequencing of Alpine Soils from Switzerland

The forefield of the "Damma" glacier (Rime et al., 2015) has several ECM forming plant species including *Salix herbacea* and *S. helvetica,* but also *Alnus viridis* and *Pinus mugo* at the lower margin of the site. Investigation of the soils resulted in 2,390 fungal operational taxonomic units (OTUs; a proxy to fungal species) in total. More than two thirds of the OTUs were assigned to the Ascomycetes, about 25% to the Basidiomycetes, and 5% remained unclassified. In the Basidiomycetes, a large group were Agaricales, with 120 OTUs, whereas Russulales, Boletales, and Cantharellales were less abundant. The majority of the classified Agaricales found were saprotrophic and not typical alpine species (e.g., *Marasmius rotula, Galerina marginata*). The genera *Cortinarius, Inocybe*, and *Entoloma,* with 5 to 6 OTUs each, were not particularly abundant, and some of their identified members were not typically known as alpine species (*Cortinarius decipiens, Inocybe lacera, I. jacobi, I. ochroalba, I. soluta, Entoloma griseocyaneum, E. pleopodium,* and *E. vernum*).

Frey et al. (2016), investigating the area near the summit of "Muot da Barba Peider," observed only a few plant species of the genera *Poa, Cerastium* and *Senecio*. Thus, no ECM plants were present. In total, the authors recorded in the soils 840 fungal OTUs. However, about 20% could not be classified. About 400 of the classified OTUs were assigned to Ascomycetes, and 222

OTUs were assigned to Basidiomycetes. Fifty-three OTUs were assigned to Agaricales, with *Cortinarius, Inocybe*, and *Entoloma* having together 19 OTUs. The species *Cortinarius malicorius, C. uraceus, C. traganus, C. cotoneus, C. dilutus, Inocybe cookie*, and *I. soluta,* however, have ECM partners that are not in the alpine zone. Similarly, the recorded members of the Boletales have trees as ECM partners, which are not found in alpine areas.

DISCUSSION

Favre (1955) indicated for a majority of the recorded macrofungi that they grow in carpets of *Dryas octopetala* or *Salix* spp. Either these fungi are saprotrophs and decompose plant litter, or they form ECM associations with these plants. In particular due to this symbiotrophic relationship with the plant roots, the fungal flora in the alpine environment is remarkably rich. Favre (1955) lists about 90 ECM fungi in association with these hosts. Based on the fungal list of the Swiss, French, and German Alps including Favre (1955) and the "SwissFungi" database, we come to a total of 210 ECM taxa. With the 99 species of *Inocybe* and 54 species of *Cortinarius*, it cannot be excluded that some species are identical, in particular in *Cortinarius*, where microscopic characteristics are rare. In addition, the harsh alpine climatic conditions with strong rains, drought, snow, freezing and thawing, and high solar irradiation potentially strongly affect the morphological appearance of fruiting bodies. Therefore, colors, veil remnants, odor, and many other features might not be stable, which can result in vague descriptions and make identification of fungal species difficult, as already indicated by others (e.g., Horak, 1987b).

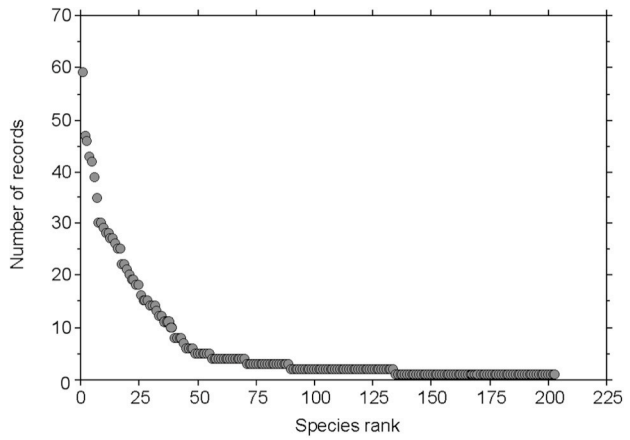

FIGURE 3 | Rank-abundance diagram of fungal taxa recorded by Favre (1955). The rank-abundance diagram displays the contribution of the fungal taxa (number of records) to a community against their ranks, with the most common species ranked first and the most rare species ranked last.

We are aware that there is more literature from the Alps, in particular from Italy. The book of Jamoni (2008) compiles a large collection of the fungi (193 taxa) recorded in the Italian Alps. Similarly as in Favre (1955), Jamoni (2008) listed the species taxonomically, and he added, besides outstanding pictures, taxonomical descriptions and ecological data such as associated plants. However, precise information about the sampling sites (coordinates, geology, elevation a.s.l.) is not given. Another noteworthy publication is from a glacier forefield of the Austrian Alps. Here, Horak (1960) listed 34 species and included taxonomically as well as ecological data.

Due to this high diversity of alpine fungi, it seems that there is truly a need for genetic characterisation of the type specimens. Up to now, however, <10 type specimens of Favre (1955) have been genetical characterized. In particular, with the upcoming

TABLE 5 | Number of fungal records of Favre (1955) not growing in association with *Dryas* or *Salix*.

Species name	Species name (in Favre, 1955)	Host or substrate	No. of records
ECTOMYCORRHIZAL SPECIES			
Cantharellus cibarius		*Juniperus communis*	1
Inocybe leptophylla	*(=I. Casimiri)*	Peat bog	1
Russula sanguinea		*Juniperus communis*	2
Thelephora terrestris	*(=Phylacteria terrestris)*	Black humic soil	1
Tricholoma inamoenum		*Juniperus communis*	1
LICHENIZED SPECIES			
Lichenomphalia alpina	*(=Omphalina flava)*	On acidic soils	1
Lichenomphalia hudsoniana	*(=Omphalina luteolilacina)*	On acidic soils	2
Lichenomphalia umbellifera	*(=Omphalina umbellifera)*	On acidic soils	19
Lichenomphalia velutina	*(=Omphalina grisella)*	On acidic soils	2
COPROPHILOUS SPECIES			
Stropharia umbonatescens	*(=Geophila umbonatescens)*	Cow dung ("cowpat")	1
Conocybe coprophila		Cow dung ("cowpat")	1
Panaeolus papilionaceus	*(=P. campanulatus)*	Cow dung ("cowpat")	several
Panaeolus semiovatus	*(=P. separatus)*	Cow dung ("cowpat")	3
Coprinus niveus		Cow dung ("cowpat")	2

The macrofungi were either ectomycorrhizal, lichenized, or coprophilous.

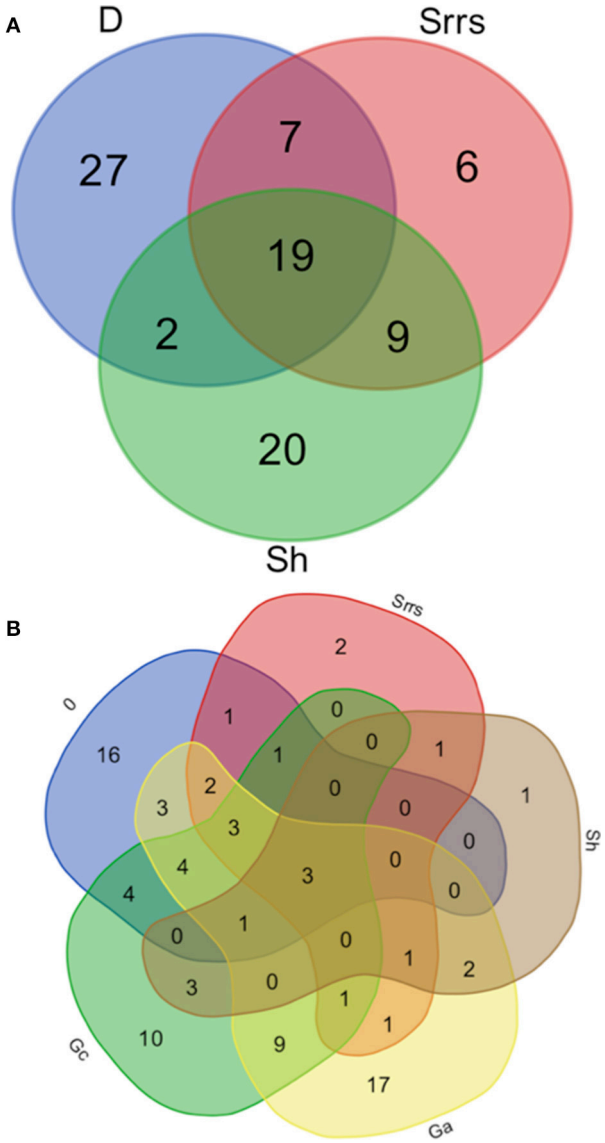

FIGURE 4 | Venn diagrams of the fungal species recorded by Favre (1955). **(A)** Venn diagram displaying the relationships between ectomycorrhizal fungal species growing in association with *Dryas* (D) on calcareous bedrock, with alpine *Salix* (Srrs) on calcareous bedrock, and with alpine *Salix* (Sh) on acidic bedrock. **(B)** Venn diagram displaying the relationships between the saprotrophic fungal species growing in association with *Dryas* (D) on calcareous bedrock, with alpine *Salix* (Srrs) on calcareous bedrock, with alpine *Salix* (Sh) on acidic bedrock, with alpine grassland on acidic bedrock (Ga), and with alpine grassland on calcareous bedrock (Gc). D, *Dryas octopetala;* Srrs, *Salix reticulata, S. retusa, S. serpyllifolia;* Sh, *Salix herbacea;* Ga, grassland on acidic bedrock; Gc, grassland on calcareous bedrock.

new sequencing techniques well-founded databases are needed. It is one of the aims of the present study to highlight that type specimens of Favre (1955) are available at the botanical garden in Geneva. As an example of combining DNA analysis from deposited fungal fruiting bodies and modern HTS technologies, Geml et al. (2012) were able to identify more OTUs at the species level from an arctic environment. From the 109 phylogroups in total, the authors were able to identify and assign 62 to known species or species complexes, allowing them to detect more lineages than with either fruiting bodies or soil HTS alone.

TABLE 6 | ITS sequences of type specimens of Favre (1955) deposited at the NCBI GenBank.

Species name	Species name (in Favre, 1955)	NCBI accession no.
Entoloma anthracinum	*(=Rhodophyllus anthracinus)*	LN850598
Hebeloma alpinum	*(=H. crustuliniforme var. alpinum)*	KM390768
Inocybe dulcamara	*(=I. dulcamara f. pygmaea)*	GU980629
Inocybe giacomi		JN580864
Inocybe taxocystis	*(=I. decipientoides var. taxocystis)*	JN580884

About half of the 210 fungal ECM species in our list of the Swiss, French, and German Alps are generalist fungi (56%) associated with *Dryas octopetala* as well as with the four *Salix* species, and only a minority is associated with only one host plant, with *D. octopetala* (38 taxa, 18%) or with *Salix herbacea* (23 taxa, 11%). Based on the DNA identification of ECM root tips from the three ECM plants *D. octopetala*, *Salix polaris* and *Bistorta vivipara* in Svalbard (Norway), Botnen et al. (2014) came to a similar conclusion. Their analysis of the shared and unique OTUs showed that the majority of them (138 OTUs) were shared among the three EMC plants, whereas only between 24 and 38 OTUs were uniquely associated with each plant species, respectively.

Notwithstanding the extraordinary pioneering achievement of Favre (1955), his work has its weak points. Because he focussed on fruiting bodies easily visible to the naked eye, he clearly favored Basidiomycetes over Ascomycetes. In addition, his main sampling period was late summer, thus, the fruiting bodies of the spring and early summer period are lacking from his observation. Here, isolating fungal cultures or fungal DNA from soils or ECM root tips could potentially overcome this weakness (Brunner et al., 2011; Geml et al., 2012). For example, Geml et al. (2012) commonly encountered in arctic soils members of the order Sebacinales, which are missing in Favre's book, because their fruiting bodies were probably overlooked because they are very small or inconspicuous.

The potential of HTS techniques to record alpine fungi is given. Rime et al. (2015) in the glacier forefield recorded fungal taxa that are known from alpine and arctic regions, such as *Cortinarius aureomarginatus (=C chrysomallus, =C. saniosus), C. croceus,* and *C. diasemospermus* (Senn-Irlet, 1987; Graf, 1994; Lindström et al., 2008; Niskanen et al., 2008). Some other fungi also known from alpine regions were detected as well, e.g., *Psilocybe montana, Arrhenia lobata, A. griseopallida, Hygrocybe conica, H. miniata,* and *Galerina vittiformis*. However, in another HTS study with soils from the Swiss Alps (Frey et al., 2016), identification on a species level was rare.

New HTS techniques potentially can detect entire fungal communities of soils, and several bioinformatics tools and workflows provide quality filtering, clustering and identification, as well as sequence count matrices of OTUs (e.g., Schloss et al., 2009; Abarenkov et al., 2010). Nevertheless, problems associated with these new techniques are manifold, including sequencing errors, chimeric sequence formation during the polymerase chain reaction (PCR), and preferential amplification of taxa due to primer bias (see also Balint et al., 2016). An OTU is an operational definition used to classify groups of closely related individuals by similarity. At the genus level these identifications and comparisons are reliable, but at the species level the situation is often less clear. Ecological studies focused on individual species are usually based on an OTU similarity threshold ≥97% (see also Bjorbækmo et al., 2010). However, for species of the genera *Cortinarius* and *Inocybe*, it has been shown that similarity threshold ≥99% would yield more accurate results (Barge et al., 2016; Garnica et al., 2016).

Overall, we are convinced that there is an urgent need to improve databases with sequences from historic type material. As stated by Nilsson et al. (2016), precise and robust taxonomic assignment of many ITS sequences is not possible at present due to the lack of similar reference sequences in public databases, e.g., NCBI (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov), INSDC (International Nucleotide Sequence Databases Collaboration; www.insdc.org), or UNITE (User-friendly Nordic ITS Ectomycorrhiza Database; unite.ut.ee). Thus, we propose an enhanced effort to sequence type material from fungal collections according to accepted standards, which include reliable sequence data combined with a correct taxonomic name as well as collection and voucher information (Schoch et al., 2014).

CONCLUSION

The book of Favre (1955) represents a unique historical dataset of alpine macrofungal communities, and the combination with more recent fruiting body data provides a comprehensive compilation of ECM macrofungi of the Swiss, French and German Alps. In addition, we also identify the potential plant partners, with the conclusion, that many of the ECM taxa form symbiotrophic associations with more than one host plant. This fungal list can also potentially serve as a baseline for future alpine fungal ecological studies. Once the sequences of Favre's type specimens are incorporated in public DNA databases, they will enable a reliable identification of fungi in alpine soils analyzed with new HTS techniques. Thus, it is important to bridge historical data with new molecular methods for determining biodiversity of fungi in a region. Moreover, it will make it possible to assess possible shifts of alpine fungal communities under a changing climate when soil samples are taken at different time points in a standardized way and analyzed with standard HTS technologies. Such shifts have already been reported for alpine plant communities (e.g., Pauli et al., 2012; Wipf et al., 2013). It is likely that changing alpine plant communities will force the accompanying fungal communities to go along.

AUTHOR CONTRIBUTIONS

IB, FG, and BS analyzed the data of Favre (1955). FG and BS contributed relevant fruiting body data of the Alps. BF and MH contributed relevant HTS data. SZ contributed relevant geology data of the SNP. LS, TN, and MB contributed signicifant information on OTUs recorded in alpine studies. All authors contributed to the writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02066/full#supplementary-material

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Functional Diversity of Fungal Communities in Soil Contaminated with Diesel Oil

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The widespread use and consumption of crude oil draws the public's attention to the fate of petroleum hydrocarbons in the environment, as they can permeate the soil environment in an uncontrollable manner. Contamination of soils with petroleum products, including diesel oil (DO), can cause changes in the microbiological soil properties. The effect of diesel oil on the functional diversity of fungi was tested in a model experiment during 270 days. Fungi were isolated from soil and identified. The functional diversity of fungal communities was also determined. Fungi were identified with the MALDI-TOF method, while the functional diversity was determined using FF-plates made by Biolog®, with 95 carbon sources. Moreover, the diesel oil degradation dynamics was assessed. The research showed that soil contaminated with diesel oil is characterized by a higher activity of oxireductases and a higher number of fungi than soil not exposed to the pressure of this product. The DO pollution has an adverse effect on the diversity of fungal community. This is proved by significantly lower values of the Average Well-Color Development, substrates Richness (R) and Shannon–Weaver (H) indices at day 270 after contamination. The consequences of DO affecting soil not submitted to remediation are persistent. After 270 days, only 64% of four-ringed, 28% of five-ringed, 21% of 2–3-ringed and 16% of six-ringed PAHs underwent degradation. The lasting effect of DO on communities of fungi led to a decrease in their functional diversity. The assessment of the response of fungi to DO pollution made on the basis of the development of colonies on Petri dishes [Colony Development (CD) and Eco-physiological Diversity (EP) indices] is consistent with the analysis based on the FF MicroPlate system by Biolog®. Thus, a combination of the FF MicroPlate system by Biolog® with the simultaneous calculation of CD and EP indices alongside the concurrent determination of the content of PAHs and activity of oxireductases provides an opportunity to achieve relatively complete characterization of the consequences of a long-term impact of diesel oil on soil fungi.

Keywords: fungal communities, functional diversity, diesel oil, pollution, degradation of PAHs, FF PLATE®

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INTRODUCTION

The widespread use of crude oil and petroleum products in the world leads to an increasing contamination of the natural environment (Baran et al., 2004; Albert and Tanee, 2011; Lebrero et al., 2012; Mohsenzadeh et al., 2012; Souza et al., 2014; Nwaichi et al., 2015). One of the underlying reasons is the technical, industrial and economic progress. Each year, around

0.10 to 0.25% of petroleum products pervade the natural environment (Garcia-Lor et al., 2012). According to the International Energy Agency [IEA] (2016), the global demand for crude oil in the first quarter of 2016 was 95.0 mln barrels d^{-1}. The consumption of biodiesel oil in the world increases dramatically. Hence, the contamination of the natural environment with products originating from crude oil processing raises concern in both industrial and developing countries (Lebrero et al., 2012). Because petroleum products are a mixture of hydrocarbons with low bioavailability, which are often carcinogenic and mutagenic compounds (Souza et al., 2014), they are considered to be among the most toxic and dangerous pollutants in particular compartments of nature, especially in soil, which is the major pool of their accumulation (Sutton et al., 2013; Covino et al., 2016; Marchand et al., 2017). They can cause changes in of fungal biodiversity of soil. This can have an unfavorable impact on the soil health and grown plants (Alrumman et al., 2015).

The presence of petroleum products in the natural environment is a grave problem, because it causes gradual soil degradation in many parts of the world (Albert and Tanee, 2011), and sometimes leads to the permanent destruction of soil, loss of its fertility and disappearance of the plant cover (Wyszkowska et al., 2015). Petroleum substances have a high potential to accumulate in the soil environment, where they can interfere with the soil's microbiome (Kucharski et al., 2010; Wyszkowska and Wyszkowski, 2010; Lipińska et al., 2013, 2014a,b; Kaczyńska et al., 2015; Nwaichi et al., 2015; Wyszkowska et al., 2015).

Much importance is attached to fungi in bioremediation of soils contaminated with petroleum products, one reason being their adaptability to extreme conditions in habitat (Schadt et al., 2003; Rousk et al., 2010). In general, fungi demonstrate quite a large range of pH in which they can obtain an optimal growth (Rousk et al., 2010). The biomass of fungi is positively correlated with their diversity (Setälä and McLean, 2004). They respond positively to the input of organic matter to soil (Gomez et al., 2006; Islam et al., 2011; Mohsenzadeh et al., 2012). They can be effective in the removal of petroleum products from soil, e.g., some species of the genera *Aspergillus* (Díaz-Ramírez et al., 2013; El-Hanafy et al., 2017) and *Candida* (Fan et al., 2014; Silva et al., 2015). High capacity to degrade diesel oil is also ascribed to such fungi as *Alternaria alternaria, Aspergillus terreus, Cladosporium sphaerospermum, Eupenicillium hirayamae, Paecilomyces variotii, Trichoderma tomentosum,* and *Fusarium oxysporum* (Ameen et al., 2016; Jiang et al., 2016; Marchand et al., 2017). On the one hand, the fungi present in soil polluted with petroleum products are beneficial for this environment as they contribute to the removal of PAHs from soil. On the other hand, when such fungi appear in fuel tanks, they can add to the deterioration of petroleum products (Bentoa and Gaylardeb, 2001). The ability of fungi to survive in environments contaminated with petroleum products and petroleum fuels alone suggests a reciprocal relationship between these fungi and the products mentioned (Alrumman et al., 2015). Next to bacteria, fungi affect transformations of these products, whereas petroleum products produce some influence on the growth of fungi (Kucharski and Jastrzębska, 2005; Alrumman et al., 2015).

Precise identification of the impact of petroleum products on the natural environment is still difficult. One reason is that our knowledge of the diversity of microorganisms, especially fungi, present in environments contaminated with these products is incomplete and fragmentary (Jiang et al., 2016; Marchand et al., 2017). In such soils, changes may occur in proportions between counts of fast and slow growing microorganisms (Kucharski and Jastrzębska, 2005; Wyszkowska and Kucharski, 2005), and the succession of microorganisms is a necessary condition for effective soil purification from petroleum products. In addition, PAHs contained in petroleum products can be an excellent source of energy for some microorganisms (Sun et al., 2010).

In view of the above, the following study has been undertaken in order to determine the abundance and functional diversity of fungal communities, based on an analysis of the metabolic profile in soil polluted with diesel oil. This problem was scrutinized in the context of the duration impact (from 7 to 270 days) of diesel oil on soil fungal assemblages. Also, the percentage degradation of PAHs by soil microorganisms was determined. The assessment was supported by determinations of the activity of selected enzymes participating in processes of carbon transformation.

MATERIALS AND METHODS

Soil Material

Model tests were performed on soil samples with the textural composition of loamy sand, collected from the arable humic horizon (depth of 0–20 cm) of proper eutrophic brown earths, which were classified according to the World Reference Base of Soil Resources (IUSS Working Group WRB, 2014) as Eutric Cambisols. The soil used for further studies originated from fields used for research purposes and located at the Research Station in Tomaszkowo (NE Poland, 53.7161° N, 20.4167° E), a village in the Olsztyn Lake District. Selected properties of the soil are presented in **Supplementary Table S1**. The physicochemical characteristics of soil were determined with the following methods: the grain-size distribution by the areometric method (PN-R-04032, 1998), pH potentiometrically in 1 Mol KCl dm^{-3} (ISO 10390, 2005), hydrolytic acidity and the sum of exchangeable base cations by the Kappen method (Carter, 1993), content of organic carbon by the Tiurin method, total content of nitrogen by the Kjeldahl method (Nelson and Sommers, 1996), and the content of exchange cations K^+, Na^+, Ca^{2+}, Mg^{2+} by flame photometry (Harris, 2006).

Characteristics of Diesel Oil Ekodiesel ULTRA

Ekodiesel Ultra class B, purchased at a PKN ORLEN petrol station, was tested in the experiment. This product is used to power self-ignition engines in road transport. It is popular mostly in large agglomerations and protected nature areas. The density of diesel oil is 820–845 g dm^{-3}, while the maximum content of polycyclic aromatic hydrocarbons (PAHs) is 7% (m/m), solid impurities – 24 mg kg^{-1}, sulfur – 10 mg kg^{-1}.

More detailed information about Ekodiesel ULTRA can be found online[1].

The Experiment

Having selected soil for the tests, the second stage of the study was conducted under strictly controlled conditions in order to eliminate the impact of variable factors. Dark glass containers, 1.0 dm^3 in capacity, were each filled with 1 kg of properly prepared, air dry soil, previously passed through a 2 mm mesh sieve. Each batch of soil (1 kg) was applied a single dose of Ekodiesel Ultra B equal 50 cm^3 per 1 kg d.m. of soil. The soil material was carefully mixed with diesel oil, after which demineralised water was added to achieve 40% of capillary water capacity. The whole set of containers was covered with perforated foil and incubated in a thermostat at 22°C for 270 days. The soil moisture was monitored throughout the whole experiment and any water loss was replenished. The control sample consisted of soil unpolluted with diesel oil. The experiment included three replicates.

Isolation and Identification of Fungi in Soil

At all dates of analyses (days 7, 30, 60, 90, and 270 of incubation), counts of fungi were determined using the method of dilutions of soil smears for both unpolluted soil and soil contaminated with Ekodiesel Ultra B. Fungi were grown on Martin's medium (1950) with addition of rose bengal and aureomycin antibiotic addition. Microorganisms were cultured on Petri dishes, with five replicates, at a constant temperature of 28°C. The number of colony forming units (cfu) was determined using a colony counter. In addition, the influence of diesel oil on the structure of fungal communities and their biodiversity was identified. To this aim, appropriate dilutions (10^{-4} and 10^{-5}) of a soil solution suspension were transferred in parallel onto Petri dishes, in five replications, and then incubated in an incubator at 28°C. For 10 days, the grown colonies of fungi were counted every day, after which values of the colony development index (CD) and the eco-physiological diversity index (EP) were calculated (**Supplementary Table S2**).

On day 270 of the experiment, fungi were isolated from unpolluted and diesel oil polluted soil. Isolated fungi were inoculated 10 times in order to obtain pure cultures. The isolated fungi were identified with mass spectrometry MALDI-TOF MS, based on matrix-assisted laser desorption/ionization coupled with the use an ion flow tube (**Supplementary Figure S1**). The mass spectrometry method relies on an analysis of the unique protein profile, specific for each species of microorganisms, which is referred to as a molecular "fingerprint" (Wieser et al., 2012). Analysis of the protein profile of a microorganism and its comparison with the reference set of proteins of microorganisms allow us to identify the species of the examined microorganism (Chalupová et al., 2014).

[1]www.orlen.pl

The Catabolic Profile of Soil Fungal Community Achieved with FF MicroPlates®

On the same dates when counts of fungi and activity of dehydrogenases and catalase were determined, i.e., days 7, 30, 60, 90, and 270 of the experiment, the metabolic profile of soils contaminated with diesel oil was determined in three consecutive replications. The metabolic capacities of fungi were determined using the system FF MicroPlate® with 95 carbon sources. Soil suspension for the inoculation of wells in microplates was prepared as follows. 1 g of soil was weighed, transferred to conical flasks holding 99 cm^3 sterile peptone water (Buffered Peptone Water, Biocorp®) each, and vortexed in an Infors HT Multitron Provortex mixer for 20 min at 130 rpm and at 22°C, after which the samples were cooled for 20 min to 4°C (Pohland and Owen, 2009). Next, 100 mm^3 was transferred to each of the wells in a FF MicroPlate® and incubated in dark at 28°C for 216 h. The experiment included three replications. The results were read on MicroStation ID systems by the Biolog® company. The extent to which individual carbon sources were used was determined through the reduction of colorless tetrazolium chloride to red formasane ($\lambda = 490$ nm) (Islam et al., 2011). Intensity of color development (ICD) was recorded at $\lambda = 490$ nm for a period of 216 h at 24-h intervals. The most intensive metabolism of carbon substrates was observed after 48 h of incubation, and therefore the results obtained at that time are presented in the paper. The activity of fungi described in this paper is based on all carbon sources and on grouped sources defined as amines/amides (AN); amino acids (AC); carbohydrate (CB); carboxylic acids (CA); polymers (PY); others (OT). The results were expressed as Average Well-Color Development (AWCD). In addition, the Shannon–Weaver (H) and Substrate Richness (R) indices were calculated from the formulas presented in **Supplementary Table S2**. Finally, four classes of substrate consumption were distinguished, based on OD_{490}. They are: OD > 0.75 – high; OD = 0.51–0.75 – good; OD = 0.25–0.50 – medium; OD < 0.25 – low.

Activity of Dehydrogenases and Catalase

At days 7, 30, 60, 90 and 270, in three replications, samples of unpolluted and polluted soils were submitted to determination of the activity of dehydrogenases with the method described by Öhlinger (1996) and catalase according to the method of Alef and Nannipieri (1998). For each date, a separate series of objects was prepared. At each of the above dates, a series of the experiment was terminated and the whole batch of soil from that series was carefully mixed. This soil served to prepare samples, with three replications, where 6 g were weighed to 25 cm^3 glass test tubes each for determination of the activity of dehydrogenases, and 2 g of soil were transferred to 100 cm^3 Erlenmeyer flasks each for determination of the activity of catalase. To determine the activity of dehydrogenases, 60 mg $CaCO_3$, 2.5 cm^3 H_2O, and 1 cm^3 of 3% 2,3,5-triphenyltetrazolium chloride (substrate) were added to each test tube with a 6-gm soil sample. Afterward, the samples were incubated an incubator for 24 h in at a temperature of 37°C. Next, triphenylformazan produced during the incubation was

extracted from the soil with 96% ethyl alcohol, and the extinction was measured on a Perkin-Elmer Lambda 25 spectrophotometer (Waltham, MA, United States) at a wavelength (λ) equal 485 nm. To determine the activity of catalase, 40 cm^3 of demineralised H_2O and 5 cm^3 of 3% H_2O_2 were added to each of the flasks holding 2-gm soil samples. The contents were shaken for 20 min next, an amount of 5 cm^3 of 0.85 M H_2SO_4 was added to each flask and the filtrate was submitted to the determination of the activity of catalase by titration with 0.02 M of potassium permanganate. The activity of dehydrogenases was expressed in μM TFF kg^{-1} d.m. of soil h^{-1}, and the activity of catalase in M O_2 kg^{-1} d.m. of soil h^{-1}. By taking into account the activity of dehydrogenases and catalase in unpolluted and polluted soil, the impact factor of diesel oil (IF_{DO}) on the activity of soil enzymes was calculated (**Supplementary Table S2**).

Determination of PAHs

The content of 9 PAHs: naphthalene (NAP), phenanthrene (PHE), anthracene (ANT), fluoranthene (FTH), benzo(a)antracene (BaA), chrysene (CHR), benzo(a)fluoranthene (BaF), benzo(a)pyrene (BaP) and benzo(ghi)perylene (BghiP), was determined in samples of polluted and Ekodiesel Ultra polluted soil samples after 270 days of the experiment (**Supplementary Figure S2**). The accuracy of determination for all PAHs was 0.005 mg kg^{-1}, and the recovery yields were form benzo(k)fluoranthene – 87.3% to benzo(a)antracene 120,8%. The content of PAHs was determined on a gas chromatograph Agilent 7890A coupled with a mass spectrometer Agilent 5975C equipped with a source of ions EI. PAHs were determined in WESSLING Company in Cracow, Poland. Hydrocarbons were extracted according to the ISO 18287 standard (ISO 18287, 2006).

Statistical Analyses

The results were processed statistically in the software program Statistica 12.0 (Statsoft Inc., 2015). Homogeneous groups were identified by using the Tukey's range test at the significance level $P = 0.01$ and applying analysis of variance ANOVA. The results were also submitted to the PCA (principal component analysis) in order to determine the most intensively metabolized substrates.

RESULTS

Activity of Oxireductases and Degradation of PAHs in Soils

Diesel oil significantly raised the activity of oxireductases in soil (**Figure 1**). Under the influence of diesel oil, the activity of these enzymes was 4.2-fold higher at day 7 after its application, 6.2-fold at day 30, 4.8-fold at day 60 and 3-fold higher at day 90. This trend did not last infinitely long as the activity of dehydrogenases decreased by 0.25-fold at day 270. On the other hand, the activity of catalase in response to diesel oil did not rise so high, ranging from a 1.4-fold increase at day 7 to 0.3-fold at day 270.

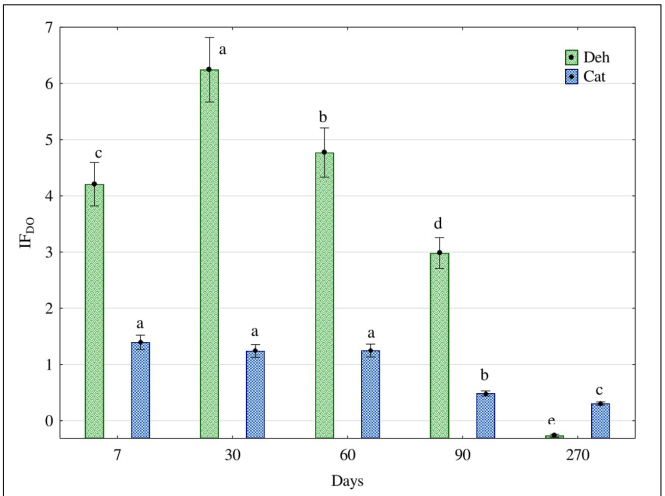

FIGURE 1 | Effect of diesel oil (IF_{DO}) on activity of dehydrogenases (Deh) and catalase (Cat). The same letters show homogeneous groups separately for dehydrogenases and separately for catalase. Different letters show significant differences separately for dehydrogenases and separately for catalase (one way ANOVA, Tukey test, $P = 0.01$). Error bars represent standard error of the mean for $n = 3$.

With time, both the activity of the tested enzymes and the soil content of PAHs decreased (**Figure 2**). Four-ringed hydrocarbons underwent the highest degradation, while six-ringed ones were the least degraded. At day 30, the concentration of four-ringed PAHs decreased by 44%, while the concentrations of the other PAHs were lower by 3–9%. After 270 days, 64% of four-ringed PAHs, 28% of five-ringed, 21% of 2–3-ringed and 16% of six-ringed PAHs had been degraded.

Degradation of hydrocarbons in soil was associated with a higher enzyme activity, which was a consequence of the stimulating effect of diesel oil on the counts (**Figure 3**) and structure (**Figure 4**) of fungi. In soil polluted with diesel oil, the number of fungi was significantly higher than in control soil (**Figure 3**). From days 30 to 270, the number of fungi was stablised in a range from $80{\cdot}10^8$ cfu·kg^{-1} of soil dry matter (270 d) to $93{\cdot}10^8$ cfu (60 d).

Diversity of Fungi in Soil Polluted with Diesel Oil

In diesel oil polluted soil (**Figure 4**), the colony development index (CD) for fungi increased significantly, while their diversity index (EP) decreased. The average value of the CD index for fungi isolated from polluted soil, irrespective of the day of analysis, was 44.3, compared to 37.6 for fungi isolated from control soil. Reversely, the value of EP for fungi isolated from control soil was 0.55, and its average value for fungi from polluted soil, regardless of the day of analysis, was 0.38. Moreover, polluted soil was dominated by fungi from the genera (**Figure 5**): *Fusarium* (37.9%), *Candida* (13.8%), *Microsporum* (13.8%) and *Penicillium* (13.8%), whereas the dominant fungi in control soil were: *Penicillium* (26.5%), *Microsporum* (21.1%), *Fusarium* (21.1%), and *Candida* (15.8%).

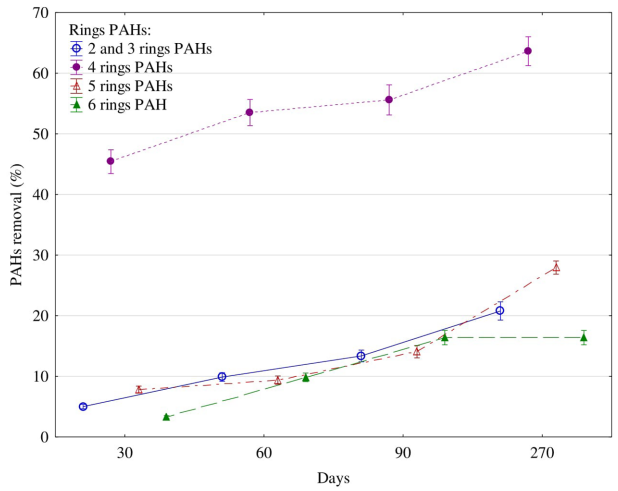

FIGURE 2 | Polycyclic aromatic hydrocarbons (PAHs) removal (%) from soil contaminated with diesel oil after 30, 60, 90, and 270 days. Error bars represent standard error of the mean for $n = 3$.

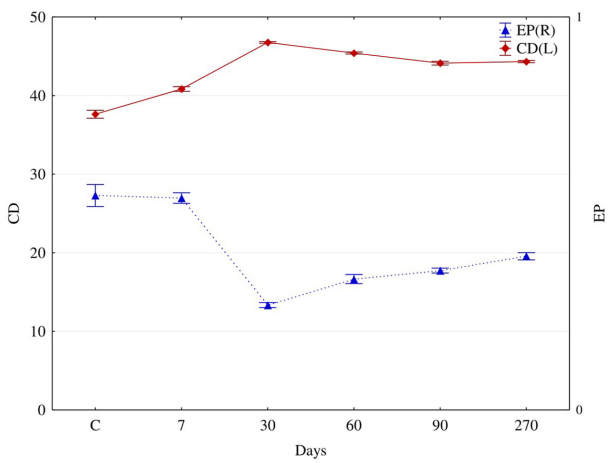

FIGURE 4 | The colony development (CD) and eco-physiological diversity (EP) indices for fungi isolated from soil contaminated with diesel oil (DO) after 7, 30, 60, 90, and 270 days (C – control without diesel oil). Error bars represent standard error of the mean for $n = 3$.

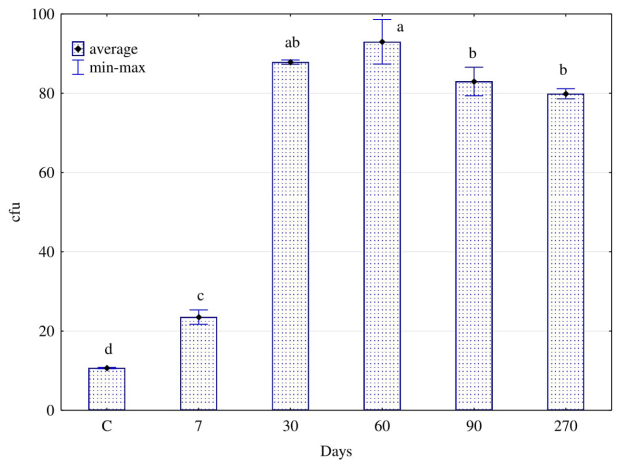

FIGURE 3 | Numbers of fungi (cfu 10^8 kg^{-1} of d.m. soil) in the soil contaminated with diesel oil after 7, 30, 60, 90, and 270 days (C – control without diesel oil). The same letters show homogeneous groups. Different letters show significant differences between objects for soil fungi (one way ANOVA, Tukey test, $P = 0.01$). Error bars represent standard error of the mean for $n = 3$.

Catabolic Profile of Soil Fungal Communities, Using FF MicroPlates®

The retention time of diesel oil in soil had a significant influence on the activity of fungi, although the effects were not unambiguous. The AWCD decreased from day 7 (0.60) to day 30 (0.48), to reach the highest value at day 60 (0.71), while being the lowest at day 270 (**Figure 6A**). Also, the Richness index (R) was the lowest at day 270, when it equalled 43, whereas at the other dates of analyses it was relatively even, ranging from 84 at day 7 to 80 at day 90 (**Figure 6B**). This is reflected by the Shannon–Weaver (H) index of fungi, whose value at day 270 was merely 3.6, fluctuating from 4.3 to 4.4 at the other days of analyses (**Figure 6C**).

The values of these indices depended on the ability of isolated fungi to use various sources of carbon. **Supplementary Table S3** shows that this was a varied capability. 11 sources of carbon were used to a high degree, 53 – to a good degree, 21 – to an average degree and 10 – to a low degree. The best sources of carbon were: Gentobiose (B9), α-Methyl-D-Glucoside (D7), D-Mannitol (D1), D-Melibiose (D4), i-Erythritol (B4), D-Mannose (D2), α-Methyl-D-Galactoside (D5), D-Galactose (B7), D-Glucosamine (B11), L-Fructose (B6) and D-Fructose (B5), whereas the worst ones were: D-Arabinose (A8), Amygdalin (A7), Adonitol (A6), *N*-Acetyl-D-Glucosamine (A4), *N*-Acetyl-D-Galactosamine (A3), L-Arabinose (A9), D-Arabitol (A10), *N*-Acetyl-D-Mannosamine (A5), Tween 80 (A2), and Arbutin (A11).

The ability of fungi to utilize particular groups of organic compounds was diverse (**Figure 7**). For 90 days, carboxyl acids and amino acids were utilized the best. The AWCD index calculated on the basis of decomposition for the former group of compounds ranged from 0.57 to 0.79, and for the latter – from 0.56 do 0.75. At day 270, the AWCD value decreased to 0.16 and to 0.21, respectively. The PCA results illustrate the above relationships very clearly (**Figure 8**). It is evident that fungi isolated from soil samples contaminated with diesel oil used carbohydrate and polymers as the best sources of carbon, while amines and amides were the poorest sources of carbon. At the other days, carboxyl acids and amino acids were ustilised the best. Utilization of the other groups of organic compounds was much poorer.

DISCUSSION

One of the most severe threats to the natural environment in central and south-eastern Europe is soil degradation (Günal

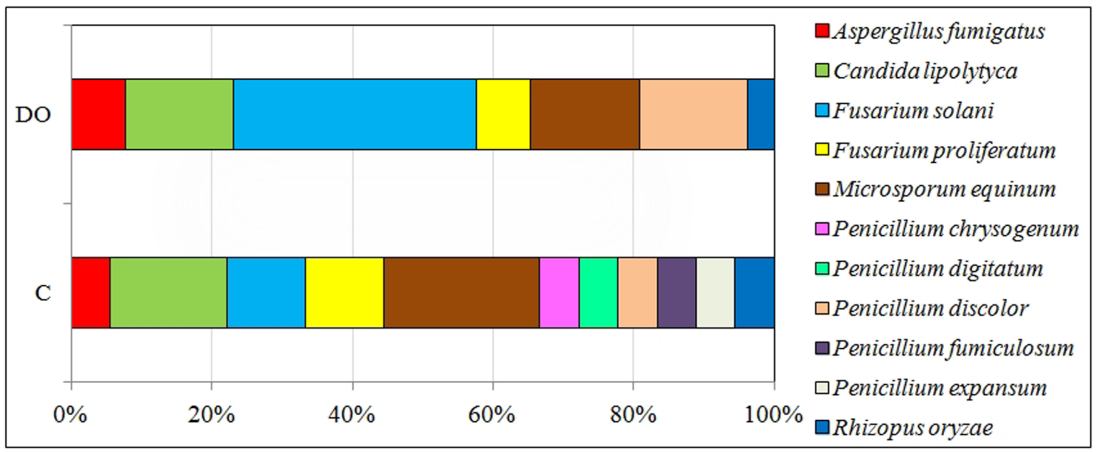

FIGURE 5 | Percentages of fungi identified with the MALDI-TOF method from soil unpolluted (C) and diesel oil polluted (DO).

et al., 2015), in which a large role is played by persistent organic pollutants, including PAHs (Wang et al., 2010; Agnello et al., 2016). Petroleum products which permeate into the soil profile cause changes in the physicochemical properties of soil (Kucharski and Jastrzębska, 2005; Wyszkowska and Kucharski, 2005; Griffiths and Philippot, 2013). In consequence, soil clumping may occur. Thus, petroleum products contribute to the deterioration of physicochemical characteristics of soil (Semrany et al., 2012), which has a negative influence on the growth and development of plants (Sivitskaya and Wyszkowski, 2013; Wyszkowska et al., 2015), but – on the other hand – they are an excellent energy source for certain microorganisms (Kucharski and Jastrzębska, 2005; Wyszkowska and Kucharski, 2005). For these reasons, when describing the quality of soil it does not suffice to determine physical and chemical parameters, even though they are determinants of the growth and development of plants as well as the dynamics of soil metabolism (Aparicio and Costa, 2007; Plaza et al., 2013), but it should be borne in mind that the above paradigm does not account for real-time changes in the soil quality (Lagomarsino et al., 2012; Nannipieri et al., 2012). In our study, the fact that diesel oil could be used by fungi as a source of carbon and energy (Wu et al., 2014; Alrumman et al., 2015; Marchand et al., 2017) probably explained why the number of fungi in DO polluted soil increased and so did the activity of soil oxireductases (**Figure 1**), which are a sensitive indicator of changes in soil quality in real time (Hawrot-Paw et al., 2010; Mohsenzadeh et al., 2012; Kaczyńska et al., 2015). A change in the activity of dehydrogenases and catalase in soil was reflected by the indicators showing the effect of diesel oil on these enzymes. The determination of these indices is fundamental to an objective assessment of the stability of a soil ecosystem exposed to the pressure of a petroleum product. Our experiment lasted for 270 days, which was long enough to observe a tendency for a lowering pressure of the petroleum product tested on dehydrogenases and catalase. However, it should be added that deposition of diesel oil into the soil was performed only once, at the onset of the experiment.

Some petroleum products stimulate the proliferation of fungi (Fan et al., 2014; Silva et al., 2015; El-Hanafy et al., 2017), and bacteria in soil (Sutton et al., 2013; Jiang et al., 2016). Hence, large changes in the activity of the enzymes were determined in soil polluted with diesel oil. For instance, the activity of dehydrogenases increased by as much as 6.2-fold at day 30 of the experiment, while that of catalase was 1.4-fold higher ar day 7 (**Figure 1**), The main reason being the fact that microorganisms are the major source of soil enzymes (Ameen et al., 2016; Stręk and Telesiński, 2016; Zaborowska et al., 2016).

On the other hand, not all hydrocarbons contained in diesel oil were an optimal source of carbon for the community of microorganisms. This most probably stems from the fact that two- and three-ringed PAHs have the highest share in the total polycyclic hydrocarbons (Nganje et al., 2007; Wyszkowska et al., 2015). Also, in this study, throughout the whole experiment, that is 270 days, the prevalent share of all PAHs consisted of 2- and 3-ringed compounds, while six-ringed hydrocarbons made up the smallest proportion (**Supplementary Table S4**). According to Baran et al. (2004), 2- to 4-ringed PAHs are responsible for about 70% of the global PAH contamination. The principal determinants of the diverse composition of PAHs in soil are: the type of pollution caused by petroleum products (Wyszkowski and Ziółkowska, 2013), physicochemical properties of soil (Gałązka et al., 2012) and species of grown plants (Wyszkowski and Ziółkowska, 2013; Nwaichi et al., 2015). In this research, tetravalent hydrocarbons were oxygenated to a higher degree than hexa-, penta-, or di-trivalent ones (**Figure 2**). After 270 days of degradation, the percentage of degraded compounds was 64% of four-ringed, 28% of five-ringned, 21% of 2–3-ringed and 16% of six-ringed PAHs. Alrumman et al. (2015) point to a significant correlation between the degree of degradation of PAHs in soil and the number of fungi This was one of the reasons why the structure and diversity of fungi changed. In polluted soils, the K strategy fungi decreased in number while the r strategy ones became more numerous. Changes in the structure of fungi observed during this study were a response to the biotic stress induced by the

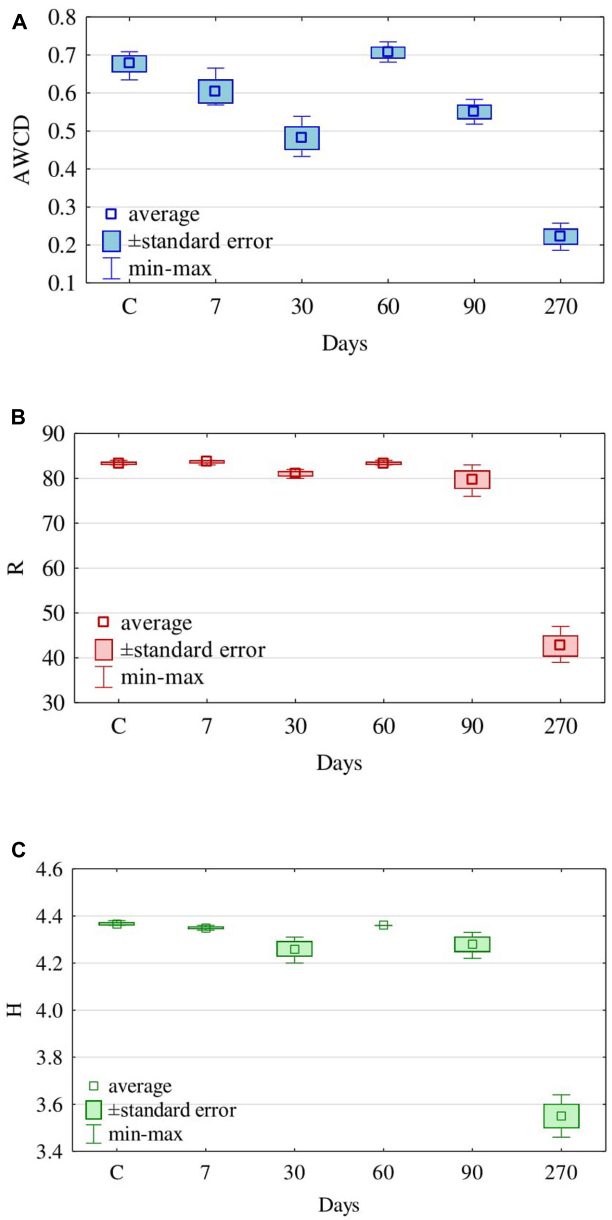

FIGURE 6 | Average **(A)** Well-Color Development (AWCD), **(B)** Richness (R) of carbon substrates and **(C)** Shannon–Weaver index (H) for fungal communities of soil contaminated with diesel oil (DO) using carbon substrates on FF MicroPlates, depending on the duration of soil exposure to diesel oil (C – control without DO).

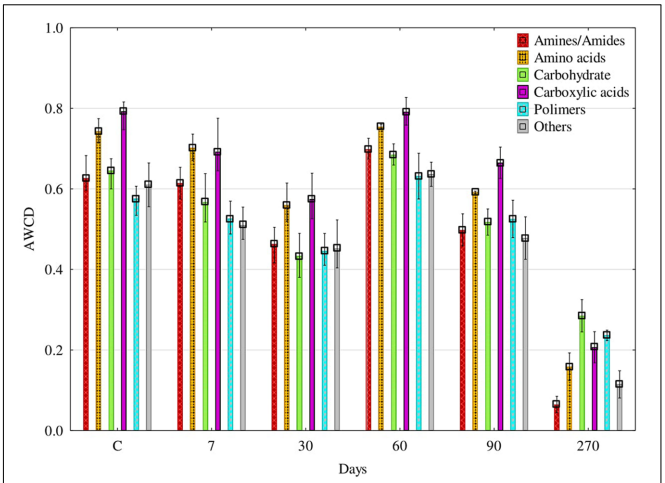

FIGURE 7 | Utilization of different carbon sources groups on the FF MicroPlates® by soil fungal communities depending on the duration of soil exposure to (DO – diesel oil; C – control without DO); AN – amines/amides; AC – amino acids; CB – carbohydrate; CA – carboxylic acids; PY – polymers; OT – others; AWCD – Average Well-Color Development index. Error bars represent standard error of the mean for $n = 3$.

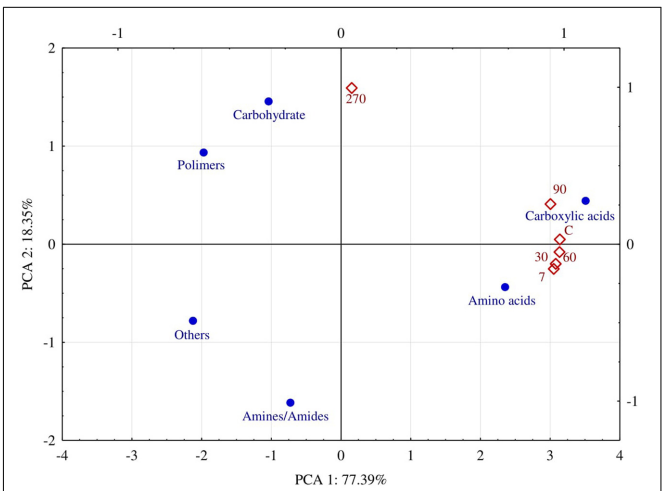

FIGURE 8 | Average Well-Color Development (AWCD) of fungi on FF MicroPlates® and isolated from soil polluted with diesel oil (DO) depending on the duration of soil exposure to diesel oil and carbon sources (C – control without DO, days 7, 30, 60, 90, 270).

pollution with diesel oil. This conclusion is supported by values of the indices CD and EP. The CD and EP indices provide information on changes in ratios between slowly (K-strategists) and rapidly growing microorganisms (r-strategists). The CD index value ranges from 0 to 100. If it approximates 100, a rapid growth of a microbial population in a short time span is implicated. The EP index ranges from 0 to 1, and reflects the evenness of the growth of microorganisms in a given time period. If the value of this index is 1, the growth of microorganisms in a given environment is very uniform (De Leij et al., 1994). The EP index equal 0 suggests a very low functional diversity as all microorganisms grow at the same time. In our study, communities of copiotrophic fungi, quick at responding to the supply of fresh organic matter, including diesel oil, reproduced more intensively. Copiotrophic fungi are essential for efficient utilization of pollution, including contamination with diesel oil. However, they are less representative to individual types and kinds of soils than K-strategy fungi, which can be called oligotrophic fungi. This explains the difference in the dominant species of fungi between the control and DO-polluted

soil. The theory of r-strategists and K-strategists presumes that there are genetic differences between microorganisms, which are responsible for the adaptation of fungi to different environments (De Leij et al., 1994). In DO-contaminated soil, some species of fungi can undergo lysis, and are then replaced by new populations of fungi, which are able to degrade PAHs owing to their intensive metabolism (Semrany et al., 2012; Wyszkowska et al., 2015). In unpolluted soil, the dominant species were *Microsporum equinum* and *Candida lipolytica* (**Figure 5**), whereas the dominant species in DO-contaminated soil was *Fusarium solani*, followed by much less dominating species, such as *Candida lipolytica* and *Microsporum equinum*. The differentiated response of autochtonous soil microorganisms to the applied diesel oil could have been caused by the succession of microorganisms (Vazquez et al., 2013; Wu et al., 2014). In the authors' own studies, no presence of *Penicillium chrysogenum, Penicillium digitatum,* or *Penicillium discolor* was detected in soil exposed to the pressure of diesel oil. In turn, diesel oil was conducive to the development of *Fusarium solani* (**Figure 5**).

Tests on plates from the FF MicroPlate® system by Biolog guarantee obtaining a faithful description of both pure cultures of microorganisms (Mishra and Nautiyal, 2009; Bourdel et al., 2016; Frąc et al., 2016) and their communities in the soil environment (Frąc et al., 2014; Pająk et al., 2016). The functional diversity of fungi isolated from the soil not polluted with DO, determined on FF MicroPlates®, was higher than that of fungi isolated from the soil polluted with DO, especially at day 270. This finding is supported by the values (**Figure 6**) of the indices AWCD, substrate Richness (R) oraz Shannon–Weaver (H). There are two reasons behind, one being the depressed ability to use carbon substrates by fungi isolated from polluted soil, and the other consisting of a change in the preference for a carbon source. Soil microorganisms mostly used carboxyl acids and amino acids for the first 90 days and carbohydrates as well as polymers in 270 days (**Figure 8**). The latter can be connected with the need to adapt to the environment. The observation is in accordance with the report of Frąc et al. (2014), who concluded that carbon sources located in the FF MicroPlate® provide a wide range of compounds, which can be used to estimate the functional diversity of soil.

Noteworthy is the coincidence between our evaluation of the functional diversity of fungi identified with the Biolog® system and the assessment of the physiological diversity (EP), based on the culture method carried out on Petri dishes for 10 days. The EP index (**Figure 3**) thus obtained, similarly to the Shannon–Weaver (H) index (**Figure 6C**), was the lowest for fungi isolated from the soil polluted with diesel oil for 270 days.

In conclusion, it is worth underlining that any disturbances in the metabolism of soil and fungal biodiversity should be viewed in the context of soil and plants health and possible recovery of the balance in an ecosystem. This conclusion is in agreement with the European Union Biodiversity Strategy to 2020, which imposes an obligation on all EU member states to make assessments of the condition of soil ecosystems (European Commission, 2014). The initiative is further supported by the Sustainable Development Goals.

CONCLUSION

(1) Soil contaminated with diesel oil is characterized by a higher activity of oxireductases and a higher count of fungi than soil not exposed to the pressure of this product.
(2) Soil pollution with diesel oil has an adverse effect on the diversity of fungal populations. This is proved by significantly lower values of the AWCD, Richness Substrates (R) and Shannon–Weaver (H) indices at day 270 after the pollution event.
(3) Effects of diesel oil in soil not submitted to remediation are persistent. After 270, degradation affected only 64% of four-ringed PAHs, 28% – of five-ringed, 21% – 2–3-ringed and 16% of six-ringed PAHs. The long-term effect of DO on fungal communities led to a decline in their functional diversity. This can reduce soil health and grown plants.
(4) The experiment shows that an assessment of the response of fungi to soil pollution with diesel oil made on the basis of colony development on Petri plates (the CD and EP indices) coincides with the results of an analyses performed on microplates (FF MicroPlate by Biolog®).

AUTHOR CONTRIBUTIONS

AB: conceived and designed the experiments; performed the experiments; isolation and identification of hyphal fungi, determination of: functional diversity of fungi (FF MicroPlates®), dehydrogenases and catalase activity, analyzed the data; wrote the manuscript. JW: methodical consultation, participation in the creation of concept the manuscript, verification of the manuscript editorial. KO: participation in the determination of functional diversity of fungi (FF MicroPlates®).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01862/full#supplementary-material

FIGURE S1 | Spectra for the fungi of soil analysis.

FIGURE S2 | Spectra for the PAHs of soil analysis: **(A)** control - soil without diesel oil; **(B)** soil contaminated with diesel oil after 7 days; **(C)** soil contaminated with diesel oil after 30 days; **(D)** soil contaminated with diesel oil after 60 days; **(E)** soil

contaminated with diesel oil after 90 days; **(F)** soil contaminated with diesel oil after 270 days.

TABLE S1 | General characteristics of experimental soil.

TABLE S2 | Used patterns.

TABLE S3 | Utilisation of different carbon sources by fungal community incubated on microplates FF MicroPlate® (ICD 490).

TABLE S4 | The effect of diesel oil (DO) on the content of polycyclic aromatic hydrocarbons in the soil on day 60 of the experiment, mg PAHs kg^{-1}d.m. of soil.

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Regulatory Mechanisms of a Highly Pectinolytic Mutant of *Penicillium occitanis* and Functional Analysis of a Candidate Gene in the Plant Pathogen *Fusarium oxysporum*

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Penicillium occitanis is a model system for enzymatic regulation. A mutant strain exhibiting constitutive overproduction of different pectinolytic enzymes both under inducing (pectin) or repressing conditions (glucose) was previously isolated after chemical mutagenesis. In order to identify the molecular basis of this regulatory mechanism, the genomes of the wild type and the derived mutant strain were sequenced and compared, providing the first reference genome for this species. We used a phylogenomic approach to compare *P. occitanis* with other pectinolytic fungi and to trace expansions of gene families involved in carbohydrate degradation. Genome comparison between wild type and mutant identified seven mutations associated with predicted proteins. The most likely candidate was a mutation in a highly conserved serine residue of a conserved fungal protein containing a GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domain and a fungus-specific transcription factor regulatory middle homology region. To functionally characterize the role of this candidate gene, the mutation was recapitulated in the predicted orthologue *Fusarium oxysporum*, a vascular wilt pathogen which secretes a wide array of plant cell wall degrading enzymes, including polygalacturonases, pectate lyases, xylanases and proteases, all of which contribute to infection. However, neither the null mutant nor a mutant carrying the analogous point mutation exhibited a deregulation of pectinolytic enzymes. The availability, annotation and phylogenomic analysis of the *P. occitanis* genome sequence represents an important resource for understanding the evolution and biology of this species, and sets the basis for the discovery of new genes of biotechnological interest for the degradation of complex polysaccharides.

Keywords: pectinases, cellulases, complex polysaccharide degradation, Clb-like transcription factor, gene regulation

INTRODUCTION

Pectins are complex heteropolysaccharides that are present mainly in the middle lamellae and primary cell walls of higher plants. Four substructures of pectin have been identified. The most abundant one is polygalacturonic acid (PGA) which consists of α backbone of D-galacturonic acid (GA) residues linked by α-1,4-glycosydic bonds (Hoondal et al., 2002; Mohnen, 2008). Pectinases encompass a family of enzymes able to degrade the pectin polymer. Microbial pectinolytic activity derives from the secretion of different classes of pectinases with distinct modes of action on pectin and PGA. For example, endo-polygalacturonases (endoPGs) specifically cleave the α-1,4 -glycosydic bond between two non-methylated GA residues.

The production of microbial pectinases is often subject to substrate induction and carbon catabolite repression (De Vries et al., 2002). Coordinated induction of genes encoding extracellular enzymes and sugar uptake systems in fungi is often mediated by Zn2Cys6 TFs that bind to conserved promoter elements in the co-regulated genes (Tani et al., 2014). With few exceptions (Alazi et al., 2016), the mechanisms underpinning transcriptional regulation of pectinase genes remain unknown in most fungal species.

Fusarium oxysporum is a devastating fungal pathogen that secretes a wide array of plant cell wall degrading enzymes (CWDEs), including polygalacturonases (Bravo-Ruiz et al., 2016), lipases (Bravo-Ruiz et al., 2013), pectate lyases (Huertas-González et al., 1999), xylanases (Gómez-Gómez et al., 2001, 2002), and proteases (Di Pietro et al., 2001), all of which contribute to plant infection. Many of these functions are encoded by redundant genes, which difficults the characterization of their roles by targeted gene deletion. One way to overcome the limitation of functional redundancy is to inactivate the transcription factors that regulate the coordinate induction or repression of multiple genes.

The pectinolytic system of *Penicillium occitanis* represents a model system for enzymatic regulation that has been intensely studied (Hadj-Taieb et al., 2002; Trigui-Lahiani and Gargouri, 2007). A mutant exhibiting constitutive and specific overproduction of pectinases was previously isolated by classical nitrous acid mutagenesis. The mutant secretes large amounts of different pectinolytic enzymes both when grown under inducing (pectin) or repressing conditions (glucose) (Trigui-Lahiani et al., 2008). The nature of the mutation(s) causing the pectinolytic overproduction phenotype is unknown.

In the present study we set out to identify the regulatory mechanism responsible for the pectinolytic overproduction phenotype of the CT1 mutant. To this aim we report, for the first time, the genomic organization of *P. occitanis* and compare the complete genome sequence of the parental and mutant strain. We further identified a putative Cys_6Zn_2 transcription factor which carries a mutation in a conserved residue in the CT1 mutant as a possible candidate for pectinolytic enzyme deregulation, and functionally tested this hypothesis by recapitulating the mutation in the orthologous gene of *F. oxysporum*.

MATERIALS AND METHODS

Fungal Isolates and Culture Conditions

We note that given the closer relatedness of *P. occitanis* with *Talaromyces* species than to other *Penicillium* species, the denomination is phylogenetically inconsistent and will probably be subject to revision (Houbraken et al., 2014). However, given the current absence of an accepted alternative name, and the widespread use of *P. occitanis* in the literature, we will keep the *P. occitanis* denomination throughout the manuscript. The CT1 mutant was selected after a single round of nitrous acid (HNO_2) mutagenesis from the wild type strain CL100 of *P. occitanis* (Hadj-Taieb et al., 2002) that was originally provided by G. Thiraby, Toulouse, France. Strains CL100 and CT1 are deposited at the culture collection of the Centre de Biotechnologie de Sfax, Tunisia, under reference numbers CTM10246 and CTM10496, respectively. The CT1 strain was routinely propagated on potato dextrose agar and maintained as spores in 20% glycerol at −80°C. *F. oxysporum* f.sp. *lycopersici* strain 4287 (race 2) was reported before (5). Microconidial suspensions were stored at −80°C with 30% glycerol. For extraction of DNA and microconidia production, cultures were grown in potato dextrose broth (PDB) (Difco, BD, USA) at 28°C as described (5). For induction conditions 5×10^8 *Fusarium* microconidia were grown in PDB media for 14 h. The germlings were collected by filtration and transferred to flasks containing synthetic medium (SM) (Benoit et al., 2012) supplemented with one of the following carbon sources: 0.5% (w/v) pectin from citrus fruit (Sigma-Aldrich, Germany), 0.5% (w/v) glucose, 0.5% (w/v) pectin plus 0.5% (w/v) glucose, or 2.5% of tomato vascular tissue (TVT), as indicated, and cultures were maintained in a rotary shaker at 150 rpm and 28°C. Vegetative growth of mutants was determined on solid SM containing one of the following carbon sources: 1% (w/v) sucrose, 1% (w/v) cellobiose, 1% (w/v) insoluble cellulose, 1% (w/v) carboxymethylcellulose (CMC), 1% CMC+1% glucose, aliquots of 5 μL containing serial dilutions of freshly obtained microconidia (10^2, 10^3, or 10^4) were spotted onto the agar plates and incubated at 28°C for 4 days before being scanned. Dry weight accumulation of mutants and wild type strain were determined in media containing complex (CMC, cellulose, or cellobiose) and simple carbon source (sucrose) after 120 h incubation at 28°C and 170 rpm.

Genome Sequencing and Assembly

The two strains of *P. occitanis* (CL100 and CT1) were sequenced at the sequencing core facility of the Centre for Genomic Regulation using Illumina HiSeq 2000 technology and 50 bp-long single-end sequencing approach. Roughly 70 million reads were obtained for each strain providing a coverage between 91X and 102X.

Reads were filtered using a quality threshold of 10 and a minimum read length of 31. Genomes were then assembled using SPAdes (Bankevich et al., 2012) (see **Supplementary Table 1** for statistics on the genome assembly). Gene prediction was performed using Augustus v2.0 (Keller et al., 2011) with parameters inferred for *A. nidulans* as used before in the annotation of *Penicillium* genomes (Ballester et al.,

2015; Banani et al., 2016). The final annotation comprised 11,269 protein-coding genes. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NPFJ00000000 (*P. occitanis* CT1), and NPFK00000000 (*P. occitanis* CL100). The version described in this paper is version NPFJ01000000.

Phylome Reconstruction

A phylome, meaning the complete collection of phylogenetic trees for each gene encoded in a genome, was reconstructed for *P. occitanis* CL100. The pipeline used was previously described in Huerta-Cepas et al. (2004). Briefly the pipeline mimics the steps taken to reconstruct a phylogenetic tree while trying to optimize for accuracy and time. For each gene encoded in a genome (seed genome) a blast search is performed against a proteome database. In this case the proteome database was built using 29 species (**Supplementary Table 4**). Blast results were then filtered ($e < 1e^{-05}$, overlap > 0.5) and the closest 150 homologs were aligned. The multiple sequence alignment was reconstructed using three different alignment programs MUSCLE v 3.8 (Edgar, 2004), MAFFT v6.712b (Katoh et al., 2005) and kalign (Lassmann and Sonnhammer, 2005), and each of the algorithms was used to align the proteins in forward and reverse orientation (Landan and Graur, 2007). A consensus alignment was obtained using M-coffee (Wallace et al., 2006) and then trimmed using trimAl v1.4 (Capella-Gutiérrez et al., 2009) (consistency-score cut-off 0.1667, gap-score cut-off 0.1). This final alignment was then used to reconstruct phylogenetic trees. Each tree was first reconstructed using a NJ approach as implemented in BIONJ (Gascuel, 1997), the likelihood of the topology was assessed using seven different models (JTT, LG, WAG, Blosum62, MtREV, VT, and Dayhoff). The model best fitting the data was then used to reconstruct a ML tree using phyML v3.0 (Guindon et al., 2010). Four rate categories were used and invariant positions were inferred from the data. Branch support was computed using an aLRT (approximate likelihood ratio test) based on a chi-square distribution. The best tree according to the AIC criteria was then chosen. The phylome, recorded with phylomeID 369 finally was formed by 11,004 trees. All the information is stored in phylomeDB v4 (http://phylomedb.org) (Huerta-Cepas et al., 2014).

Phylome Analysis

The phylome was scanned for the presence of species-specific expansions in *P. occitanis*. For each tree reconstructed in the phylome, ETE v3.0 (Huerta-Cepas et al., 2016) was used to search for monophyletic nodes that contained more than two sequences belonging to *P. occitanis*. The phylome was also scanned in order to identify orphan genes, understood as those genes that had no homologs in any of the species included in the phylome. 98 orphan genes were found that were present in the two *P. occitanis* strains. Orthologs and paralogs among the species considered were predicted using the species-overlap algorithm (Huerta-Cepas et al., 2007).

Species Tree Reconstruction

A species tree was reconstructed using data obtained in the phylome of *P. occitanis*. 505 one-to-one orthologs that had only one copy in each of the 29 species used in the phylome were selected. The trimmed alignments of these proteins were then concatenated to form a single multiple sequence alignment, which was then used to reconstruct the species tree. The concatenated alignment had 3,77,573 amino acid positions. Fasttree (Price et al., 2010) was used to reconstruct the species tree using default parameters (**Figure 1**).

Gene Tree Reconstruction

A broader tree of the transcription factor was reconstructed. We used the transcription factor found in *P. occitanis* and performed a blast search against uniprot, we limited our search to fungi. We selected the best 250 hits and filtered out strains and proteins that did not belong to a completely sequenced genome so as to limit the size of the resulting tree. The sequence of the identified *P. occitanis* Cys_6Zn_2 transcription factor was then added to the list and the tree was reconstructed by following the same pipeline used in the phylome reconstruction. ETE v3.0 was used to detect duplication and speciation nodes using a species overlap algorithm that assumes that a node is a duplication node if there are shared species at either side of the node, if this is not the case, the node will be a speciation node (Huerta-Cepas et al., 2007). The tree was rooted at the *Leotiomycetes* group.

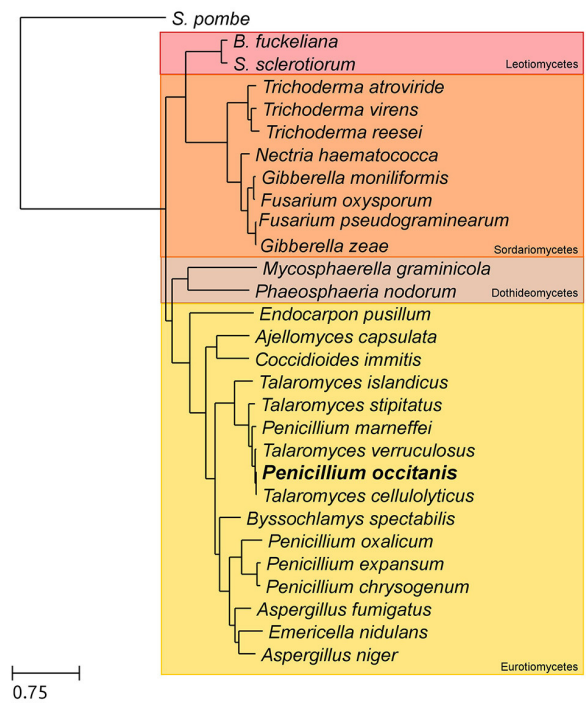

FIGURE 1 | Species tree depicting the evolutionary relationships between the species included in the phylome. The tree is based on a concatenated alignment of 505 genes found in single copy in all 29 species included in the tree. In bold is the newly sequenced species *P. occitanis*.

Comparison of *P. occitanis* Strains

Reads of the two strains of *P. occitanis* were mapped to the assembly of the opposite strain using bwa v0.7.3a (Li and Durbin, 2009) aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. SAMtools (Li et al., 2009) was then used to transform the sam files into bam files. GATK (McKenna et al., 2010) was then used to predict and filter SNPs. SNPs were then characterized depending on whether they were found in non-coding regions, promotor regions or coding regions. SNPs predictions were also compared between the two strains and a list of reciprocal SNPs was compiled (**Table 1** and **Supplementary Tables 5**, **6**).

Nucleic Acid Manipulation

Total RNA and genomic DNA were extracted from mycelium according to previously reported protocols (Raeder and Broda, 1985; Chomczynski and Sacchi, 1987). Southern analyses and probe labeling were carried out as described previously (Di Pietro and Roncero, 1998) using the non-isotopic digoxigenin labeling kit (Roche Life Sciences, Barcelona, Spain). Sequencing of DNA clones used in fungal transformation experiments was performed at STAB-VIDA (Setubal, Portugal). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI) (Bethesda, MD) or at Broad Institute *Fusarium* comparative website http://www.broadinstitute.org. PCR reactions were routinely performed with the High Fidelity Template PCR system (Roche Life Sciences, Barcelona, Spain) or the Biotaq DNA polymerase (Bioline, Taunton, MA, USA).

Transformation-Mediated Gene Replacement and Analysis of Transformants

Targeted gene replacement of the FOXG_08883 *gene* was performed using the split marker technique (Catlett et al., 2003) as reported (López-Berges et al., 2010). The two overlapping DNA fragments were used to transform protoplasts of *F. oxysporum* wild type strain 4287, as reported previously (Di Pietro and Roncero, 1998). The resulting hygromycin resistant transformants were initially identified by PCR, and the homologous recombination event was confirmed by Southern analysis of selected transformants using an internal gene fragment as probe. Site-directed mutant allele FOXG_08883$^{160Ser>Cys}$ was obtained by replacing the cytosine at position 599 of the sequence by guanine and the adenine 600 by thymine, using PCR amplification with a primer pair carrying these mutations (**Table 2**). This construct was used for co-transformation of protoplasts of the ΔFOXG_08883 mutant together with the phleomycin resistance cassette (Phl^R) as selective marker (Punt and van den Hondel, 1992). The resulting phleomycin resistant transformants were initially identified by PCR and Southern analyses, and the presence of the mutated allele was confirmed by DNA sequencing.

TABLE 1 | List of proteins that contained a reciprocal mutation found in the two *P. occitanis* strains.

Protein PENO1	Protein PENOC	Function
PENO1_019200	PENOC_048850	Ribonuclease CAF1
PENO1_011980	PENOC_010810	Hypothetical protein
PENO1_031020	PENOC_073480	Peptidase C48
PENO1_108040	PENOC_095050	Pre-rRNA-processing protein TSR2
PENO1_091170	PENOC_088330 (298V→298E)	Nucleic acid-binding, OB-fold
PENO1_027960	PENOC_037760 (198C→198S)	Transcription factor
PENO1_004030	PENOC_004030	alpha-ketoglutarate-dependent taurine dioxygenase

First column indicates the code of the protein involved in the reference strain, the second column indicates the protein affected in the mutant strain and the third column indicates putative function.

Biochemical Enzyme Characterization Assays

PG activity was screened on SM plates as described previously (Scott-Craig et al., 1990) with minor modifications. Plates containing 1% (w/v) PGA, 1.5 M $(NH_4)_2SO_4$, and 14% (w/v) MES (Sigma-Aldrich, Germany) were adjusted to pH 5.2 with NaOH. For total PG or PL activity, the wild type and mutant strains were induced on 0.5% pectin for different time periods. Cultures were filtered through nylon monodur (pore size 10 nm) to separate and discard the mycelia. The filtrate was centrifuged and the supernatant transferred to dialysis tubing (12 kDa cut-off), dialyzed overnight against distilled water and concentrated 50-fold by placing the tubing on solid polyethylene glycol (35 k Mr; Fluka Chemika-Biochemika, Switzerland). Aliquots from induced supernatants were used as enzyme source for PG or PL activity assays. Protein quantities were determined by using the BioRad protein assay (BioRad, Hercules, CA) with bovine serum albumin as standard. Total PG activity was determined by measuring the release of reducing groups from polygalacturonic acid (PGA) using the method of Nelson-Somogyi as described previously (Di Pietro and Roncero, 1996). Appropriate controls without either enzyme or substrate were run simultaneously. The quantity of reducing sugar released was calculated from standards of D-galacturonic acid. Enzyme activity is expressed in μg of D-galacturonic acid released per min and μg of protein used. Each assay was carried out three times in 96-well microtiter plates. Total PL activity was determined by measuring the release of reducing sugar from PGA using the method described previously (Muslim et al., 2015) with some modifications. Significance of activity data was calculated by student's *t*-distribution statistic test (> 0.05).

Expression Analyses of Polygalacturonase, Xylanase, and Pectate Lyase Genes

Expression patterns of genes encoding the two major PGs (FOXG_14695, *pg1,* and FOXG_15415, *pgx6*) (Bravo-Ruiz et al., 2016), a pectate lyase gene (FOXG_12264, *pl1*) (Huertas-González et al., 1999) and two xylanases (FOXG_13415, *xyl3,* and FOXG_15742, *xyl4*) (Gómez-Gómez et al., 2002), were

TABLE 2 | Oligonucleotides used in this study.

Name	Sequence 5′–3′	Position to ATG	Experimental use
FOXG_08883			
FOXG_08883-2	CTTTCAAGCGGGCGTGTCATA	−1474 (s)	Site-directed mutagenesis
FOXG_08883-4	GATGCTCGGGGTGTTATCTCT	+2696 (s)	Split marker
FOXG_08883-5	GCTGGGGGTCCTTCTCAACA	+3902 (as)	Site-directed mutagenesis/split marker
FOXG_08883-6	CCAGCCCTATACGACGGTCA	+3925 (as)	Site-directed mutagenesis/split marker
FOXG_08883-7	CCTGTCGCCAGGCAAACATG	+700 (s)	Site-directed mutagenesis
FOXG_08883-8	CATGTTTGCCTGGCGACAGG	+700 (as)	Site-directed mutagenesis
FOXG_08883-9	ggtacacttgtttagaggtaatcaactgAACTTACAGGTACAGAAC	+2 (as)	Split marker
FOXG_08883-11	ACTGTCGTGGCGGCAGGGA	−779 (s)	Split marker
FOXG_08883-12	GGCGTCGTTGATCGGGAGG	−691 (s)	Split marker
FOXG_08883-13	GGAGAGTTGAGAAGGATTTTGC	−430 (s)	Site-directed mutagenesis
FOXG_08883-14	ATGTTCTGTACCTGTAAGTTCAG	+1 (s)	Southern / mutants sequencing
FOXG_08883-16	CGATGCGACTGCTGCCAAAG	+1037 (as)	Southern / mutants sequencing
FOXG_08883-17	CCTTGTAACCAATGCGAGTCA	+183 (s)	RT-qPCR
FOXG_08883-18	ACCGATAATGAAGCCAGGTCT	+410 (as)	RT-qPCR
PG			
pg1-1	GTCACTTCGGGTACAAACATC	+790 (s)	RT-qPCR
pg1-2	CCTTGATGAACTTGATGCCGC	+1169 (as)	RT-qPCR
pgx4-1	GTACAGCATTGCCTCGCCAC	+275 (s)	RT-qPCR
pgx4-2	CGGGTTTCTCATTCGCAGGTT	+662 (as)	RT-qPCR
pg5-1	GCCTGGTCGCCTCCGTACT	+28 (s)	RT-qPCR
pg5-2	TCTTCTTGCCGCCGTTGCTGCCCTTGCCGT	+418 (as)	RT-qPCR
pgx6-1	GAAGTCATCGCAAGGTCTATAC	+136 (s)	RT-qPCR
pgx6-2	AGAACAGAATAGGTCGGAGGTA	+635 (as)	RT-qPCR
HYGROMYCIN CASSETTE			
hyg-G	CGTTGCAAGACCTGCCTGAA	+289 (s)	Split marker
hyg-Y	GGATGCCTCCGCTCGAAGTA	+735 (as)	Split marker
TtrpC-8B	AAACAAGTGTACCTGTGCATTC	+1727 (as)	Split marker
FOXG_08883-10	agagataactccccgagcatcGGAGAGACGGACGGACGCA	−675 (s)	Split marker
PHLEOMYCIN CASSETTE			
TtrpC-8B	AAACAAGTGTACCTGTGCATTC	+1133 (as)	Site-directed mutagenesis
PgpdA-15B	CGAGACCTAATACAGCCCCT	−1193 (s)	Site-directed mutagenesis
ACTIN			
act-q7	ATGTCACCACCTTCAACTCCA	+1278 (s)	RT-qPCR
act-q8	CTCTCGTCGTACTCCTGCTT	+1578 (as)	RT-qPCR

Lowercase is the overlapping region with 3′ end of the Hyg^R cassette to generate DNA fragment fusions by PCR amplification. Positions are referred to the start codon: (+) downstream, (−) upstream of the ATG. s, Sense; as, antisense.

performed under pectin or TVT induction (see above), or under glucose repression conditions. RNA from germlings grown 4 h on 0.5% pectin, on 0.5% pectin plus 0.5% glucose; or on 2.5% TVT for 4 and 24 h, was analyzed by quantitative RT-qPCR. Quality of extracted nucleic acids was verified by running aliquots in ethidium bromide stained agarose gels (0.7% w/v in TAE buffer) and further visualized under UV light. Additionally, they were quantified in a NanoDrop ND-1000 Spectrophotometer. The isolated RNA was treated with Desoxyribonuclease I (DNase I, Fermentas, USA) and then used to synthesize cDNA using the Transcriptor Universal cDNA Master (Roche Life Sciences), according to the manufacturer instructions. Quality of extracted nucleic acids was verified by running aliquots in ethidium bromide stained agarose gels (0.7% w/v in TAE buffer) and further visualized under UV light. Additionally, they were quantified in a NanoDrop ND-1000 Spectrophotometer. Total cDNA obtained was subject to RT-qPCR using the appropriate gene-specific primer pairs (see **Table 2** for primers). Three simultaneous technical replicate amplifications were carried out for each cDNA sample. Amplification reactions were performed in 96-well microtiter plates (BioRad, Hercules, USA) and each reaction was made up using aliquots from the same master mix. PCRs were performed in an iCycler iQ5 real-time PCR System (BioRad, Hercules, USA) using the following cycling protocol: an initial step of denaturation (5 min, 94°C) followed by 40 cycles of 30 s at 94°C, 30 s at 62°C, 45 s at 72°C, and 20 s at 80°C for measurement of the fluorescence emission. After this, a melting curve program was run for which measurements were made at

0.5°C temperature increases every 5 s within a range of 55–95°C. Relative levels of the RT-qPCR products were determined using the ΔΔCt method (Livak and Schmittgen, 2001). Expression values are presented as relative to those of wild type strain and normalized to the Ct value of the actin gene (**Table 2**). Samples without cDNA were used as negative control reactions. The assay was repeated with three independent biological replicates.

Virulence Assays

Root inoculation assays were performed as described previously (Di Pietro and Roncero, 1998). Briefly, 14-day-old tomato seedlings (cultivar Monika, seeds kindly provided by Syngenta Seeds, Almeria, Spain) were inoculated with *F. oxysporum* f. sp. *lycopersici* strains by immersing the roots in a suspension of 5×10^6 microconidia mL^{-1} for 30 min, planted in vermiculite and maintained in a growth chamber. Ten plants were used for each treatment (Di Pietro and Roncero, 1998). Severity of disease symptoms and plant survival was recorded daily for 35 days as previously described (López-Berges et al., 2010). Virulence experiments were performed at least three times with similar results. Plant survival was calculated by the Kaplan-Meier method and compared among groups using the log-rank test. Data were analyzed with the software GraphPad Prism 4.

RESULTS AND DISCUSSION

Comparative Genome Analysis of *P. occitanis*

The genomes of the wild type strain and the hyperpectinolytic mutant of *P. occitanis* were sequenced, assembled and annotated (see Materials and Methods). The resulting assemblies comprised roughly 1,600 contigs, 80 of which were larger than 100 kb (**Supplementary Table 1**), the total estimated genome size for both strains was of 36.3 Mb. Compared to its closely related, fully sequenced, species, *P. occitanis* is roughly the same size as its two closest relatives *Talaromyces cellulolyticus* (36.2 Mb) and *T. verruculosus* (36.7 Mb) and much larger than the human pathogen *Penicillium marneffei* (28.7 Mb). The gene prediction showed that *P. occitanis* encoded between 11,233 and 11,269 proteins, which is a bit higher than its closest sequenced relative *T. cellulolyticus* (10,910) but within normal parameters for *Talaromyces* species (from 10,001 in *P. marneffei* to 12,996 in *T. stipitatus*). The phylome, the complete collection of phylogenetic trees of each gene encoded in the *P. occitanis* genome was reconstructed (see Materials and Methods). Each individual gene tree was automatically analyzed with phylogeny-based algorithms that predict orthology and paralogy relationships (Gabaldón, 2008), and detect and date duplication events (Huerta-Cepas and Gabaldón, 2011). The reconstructed trees, alignments and orthology and paralogy predictions are accessible to browse or download at the PhylomeDB database (Huerta-Cepas et al., 2014). 505 one-to-one orthologs present across the 29 considered species (**Supplementary Table 2**) were used to reconstruct a species tree using an alignment concatenation approach (see Materials and Methods).

As seen in **Figure 1**, *P. occitanis* groups with the *Talaromyces* species and not with the other *Penicillium* species. Therefore, *P. occitanis* should likely be renamed to reflect its correct phylogenetic position within the Talaromyces clade. To avoid confusion with previously published literature, we decided to maintain the name *P. occitanis* in this work. Within the *Talaromyces* group, *P. occitanis* is more closely related to *Talaromyces cellulolyticus*. Of the predicted proteins, only 98 were found to be orphan genes. Analysis of the gene trees reconstructed in the phylome also revealed few species specific expansions in *P. occitanis,* which is consistent with the overall low duplication rates—i.e., the number of duplications per branch per gene—found in *Talaromyces* species.

We provide the first genome sequence for *P. occitanis* and a first annotation and comparative analysis for it. The genome sequence, annotation, and the extensive genome-wide phylogenetic analysis constitute important resources that will certainly pave the way for a better understanding of the evolution of this species and the processes in which it is involved. Our results suggest that *P. occitanis* contains a large number genes involved in pectin and xyloglucan degradation. Sequencing of a *P. occitanis* hyperpectinolytic strain obtained through mutagenesis, reveals a small set of point mutations likely to contain the one responsible for the phenotypic change. Our best candidate consisted in a non-synonymous mutation affecting a conserved residue of the Crlb-like transcription factor.

P. occitanis as a Producer of Carbohydrate Catabolic Enzymes

Hydrolyzing enzymes have been categorized within the Carbohydrate-Active enZymes database and are known as CAZy proteins. *P. occitanis* was obtained from soil and mutants were created in an attempt to enhance its natural capacity to produce enzymes able to degrade polysaccharides. The availability of the whole genome of *P. occitanis* now allows for the search of the genes responsible for the degradation of these compounds. We used dbCan (Yin et al., 2012) in order to assign CAZy domains to the proteins of the 29 considered species (**Supplementary Table 2**). 692 CAZy proteins are encoded in the *P. occitanis* genome. The two closest related species to *P. occitanis* have a similar number of CAZy proteins (*T. cellulolyticus*—694 and *T. verruculosus*—685), which is higher than that of the other *Talaromyces* species (*Talaromyces islandicus*–599). In fact, the first three species contain the highest number of CAZy proteins out of the entire set of *Eurotiomycetes* species considered in this study. Only the genomes of the three *Fusarium* species encode a higher number of CAZy proteins [*F. gramineraum* (873), *F. oxysporum* (837) and *F. verticillioides* (729)]. In relative terms, however, the genomes of the first three *Talaromyces* species, including *P. occitanis,* have the highest percentage of genes encoding CAZy proteins (ranging from 6.04 to 6.36%). *Fusarium* species, with larger genomes, have a smaller percentage of their genome encoding for CAZy proteins (5.07% on average).

CAZy proteins are subdivided into different families and we compared the number of proteins each species had grouped in each family (**Supplementary Table 3**). There do not seem to be

any major differences between the three *Talaromyces* species, which could indicate that the increase in CAZy proteins predates the divergence of these three species. When compared to the other species included in the analysis, with a particular focus on the *Fusarium* species that also have a large amount of CAZy proteins, we noticed that the increase in CAZy proteins was spread out among the different CAZy categories and not due to the expansion of a specific protein family.

Some CAZy families have been associated to the degradation of specific compounds (Espagne et al., 2008; Amselem et al., 2011; van den Brink and de Vries, 2011; Benoit et al., 2012). These families are not necessarily specific, some of them have been associated to more than one compound. According to this classification, *P. occitanis* has 158 proteins associated to pectin degradation (**Supplementary Table 4**). This value is only found increased in some *Fusarium* species, with *Nectria haematococca* having the highest number of pectin degrading proteins (217). Curiously, *P. occitanis* seems to have an increased capability to degrade xyloglucan as compared to the other species, at least in terms of the number of encoded proteins associated to the degradation of this compound. As before, this trend is shared with the two most closely related *Talaromyces* species, *T. verruculosus* and *T. cellulolyticus*. With an average of 68 proteins that belong to this category, it more than doubles the average number of proteins in the other species considered (average of 32 proteins).

A Mutation in CLrB as the Possible Cause for Hyperpectinolytic Activity in *P. occitanis* CT1

The two strains of *P. occitanis* were compared in order to search for the mutation that caused *P. occitanis* CT1 to be able to secrete large amounts of pectinases (**Supplementary Tables 5**, **6**). Seven reliable SNPs were detected comparing the two strains that were associated to proteins (see Materials and Methods, and **Table 1**). Four of these were located in putative promotor regions (i.e., 1,000 bp upstream from a protein-coding gene). One other SNP caused a synonymous mutation within a protein and two others were found to cause non-synonymous mutations (**Table 1**). Given the observed deregulation of pectinolytic enzymes observed in the mutant strain, we focused our attention in a non-synonymous SNPs affecting a transcription factor (ortholog to *Aspergillus nidulans* ClrB), which results in a Serine to Cysteine mutation at position 198 of the amino acid sequence. To broaden the taxonomic scope considered in the phylome, we reconstructed an additional phylogenetic tree after running a blast search against UniProt and using the same tree reconstruction pipeline used during the phylome reconstruction (**Figure 2**). The tree shows that this transcription factor is very conserved among *Pezizomycotina* species. The alignment showed that the mutation had occurred in a very conserved position in the alignment where nearly all the other *Pezizomycotina* species have a serine in position 198. The only exceptions are three sequences within *Sordariomycetes* that have a S → G mutation and a sequence in *A. terreus* that has a S → A mutation. None of the sequences found have the same mutation found in *P. occitanis* CT1.

Ayadi et al. (2011) had previously hypothesized that the hyperpectinolytic activity of CT1 could be the result of a mutation in a transcription factor, due to the fact that many different proteins appeared to be activated. In *Neurospora crassa* and *A. nidulans,* two zinc binuclear cluster transcription factors Clr-1, Clr-2, and ClrA, ClrB, respectively, were reported to be required for normal growth and enzymatic activity on cellulose or cellobiose, but not on xylan (Coradetti et al., 2012). Enzyme activity was abolished in *A. nidulans* Δ*clrB* mutant, while the Δ*clrA* mutant showed ∼50% of wt activity. Consistent with this, induction of major cellulase genes in the Δ*clrB* mutant was 1,000-fold reduced compared to the wt, while in the Δ*clrA* mutant, the average induction was 2-to 4-fold less. However, the role of these transcription factors in growth on pectin has not been tested so far (Coradetti et al., 2013).

Primary Structure of the FOXG_08883 Gene

To functionally test the role of the predicted candidate gene in regulation of pectinolytic enzymes, we chose *F. oxysporum* for two main reasons. First, the availability of an efficient transformation system, together with the biochemical and molecular biology tools which are not available in *P. occitanis* or *Penicillium expansum*; second, the interest in the identification of CWDE regulatory mechanisms and their relevance during plant infection. A blastp search of the *F. oxysporum* genome database as well as a phylome analysis identified the gene FOXG_08883 as the predicted ortholog of the *P. occitanis* PENO1_078870 gene. The amino acid sequence of the FOXG_08883 product shows significant similarity to transcription factors from related fungal species (**Figures 3A,B**) and encodes for a putative protein of 709 amino acids containing a characteristic GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domain and the fungal transcription factor regulatory middle homology region, suggesting a possible function as a transcription factor. The 5' upstream region of FOXG_08883 contained a number of motifs, some of which correspond to sequences present as repeats in the *F. oxysporum* genome, including two consecutive regions of 623 and 362 bp, respectively, which are present 226 and 214 times, respectively (**Supplementary Figure 1A**).

Construction of Mutants in the FOXG_08883 Gene

Targeted gene deletion followed by site-directed mutagenesis was performed to replace the wild type FOXG_08883 allele by the $^{Cys}160^{Ser}$ mutant allele in the *F. oxysporum* knock-out background. Targeted deletion of FOXG_08883 was initially achieved by replacing the complete ORF with the hygromycin resistance cassette (**Supplementary Figure 1B**). Transformants were analyzed by Southern analyses and PCR (**Supplementary Figures 1C,D**). Two transformants (named FOXG_08883Δ10 and FOXG_08883Δ11) lacked the wild type hybridization bands, suggesting gene deletion, but only FOXG_08883Δ11 showed amplification bands corresponding to promoter and terminator regions with primers located outside the construction, and failed to amplify the ORF. Generation

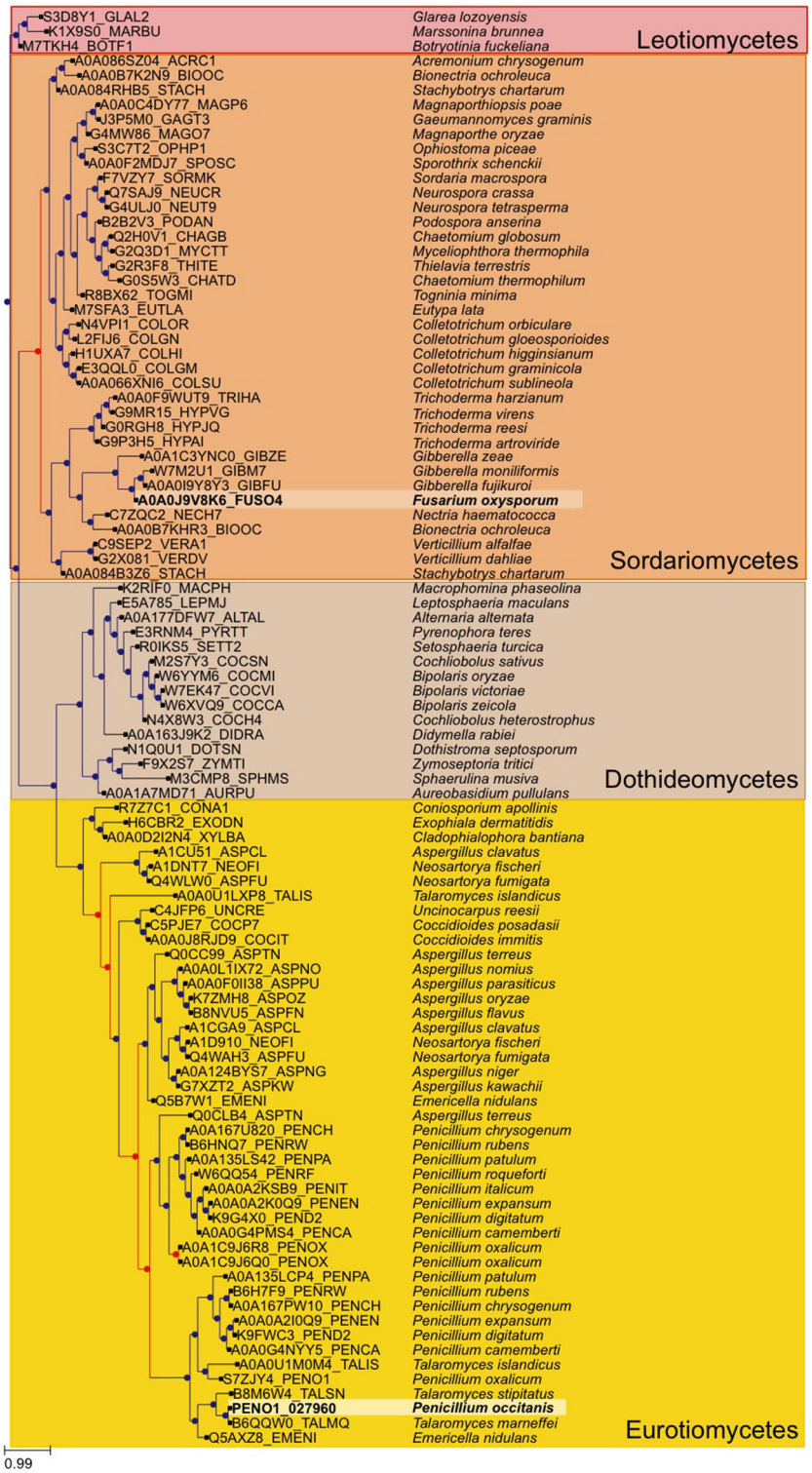

FIGURE 2 | Gene tree of the Crlb-like transcription factor found to have a mutation in the *P. occitanis* mutant. The tree was reconstructed by performing a blastp search against UniProt. The sequences were downloaded and the tree was reconstructed using the same pipeline used in the phylome. Sequences in bold and surrounded by a lighter square belong to the *P. occtanis* transcription factor and its ortholog in *F. oxysporum*. Duplication nodes are marked in red and speciation nodes are marked in blue according to the species overlap algorithm.

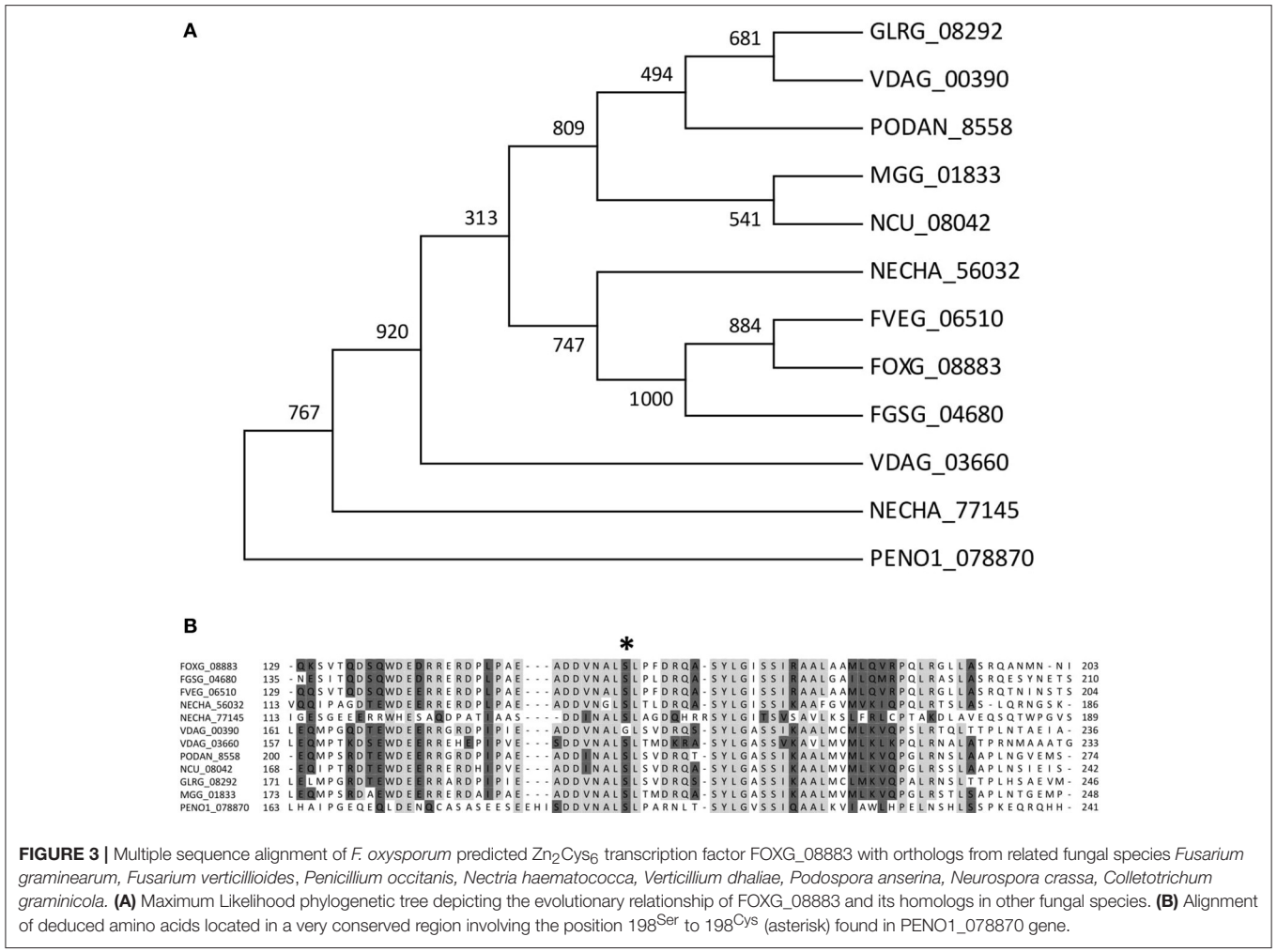

FIGURE 3 | Multiple sequence alignment of *F. oxysporum* predicted Zn_2Cys_6 transcription factor FOXG_08883 with orthologs from related fungal species *Fusarium graminearum, Fusarium verticillioides*, *Penicillium occitanis, Nectria haematococca, Verticillium dhaliae, Podospora anserina, Neurospora crassa, Colletotrichum graminicola.* **(A)** Maximum Likelihood phylogenetic tree depicting the evolutionary relationship of FOXG_08883 and its homologs in other fungal species. **(B)** Alignment of deduced amino acids located in a very conserved region involving the position 198^{Ser} to 198^{Cys} (asterisk) found in PENO1_078870 gene.

of site-directed mutants was performed by co-transformation of the FOXG_08883Δ11 strain with the phleomycin resistance cassette and a point-mutated FOXG_08883 allele in which cytosine^{599} had been replaced with a guanine and adenine^{600} with a thymine, thus originating a Ser by Cys change at position 160 of the encoded protein. Transformants carrying the mutated allele were confirmed by PCR with specific primers of the FOXG_08883 ORF (**Supplementary Figure 2A**) and sequencing of the resulting fragments. Southern blot analyses confirmed that nine transformants harbored the correctly mutated allele (**Supplementary Figure 2B**). Two mutants were selected for further studies: M50 with a unique integration and M22 with two copies of the mutated version.

Biochemical and Phenotypic Characterization of FOXG_08883 Mutants

To investigate the implication of FOX_08883 in regulation of polygalacturonase (*pg*), pectate lyase (*pl*) or xylanase (*xyl*) gene expression, RT-qPCR analysis was performed using RNA extracted from *F. oxysporum* germlings incubated in SM supplemented with 2.5% (w/v) tomato vascular tissue (TVT) (**Figure 4A**), 0.5% (w/v) pectin (**Figure 4B**) or 0.5% (w/v) pectin plus 0.5% (w/v) glucose (data not shown). Under TVT induction, the different mutants showed a 30–70% reduced expression, compared to the wild type strain, of *pg1* after 24 h and 50–80% of *pgx6* after 4 h. The decrease in expression was more severe in the case of *pl1,* which was 90% reduced after 4 h and 50% after 24 h. In contrast, the mutants showed increased expression for *xyl3*, whereas *xyl4* transcripts were unaffected in all conditions (data not shown). In the presence of pectin, neither the deletion nor the point-mutation mutants of FOXG_08883 were significantly affected in gene expression. As previously shown, glucose represses expression of all PG genes (Di Pietro and Roncero, 1998; Garcia-Maceira et al., 2000, 2001; Bravo-Ruiz et al., 2016). No difference in carbon catabolite repression was detected in both types of mutants (data not shown). In addition, PG and PL activities on PGA plates and in culture supernatants of pectin cultures were analyzed to determine the effect of FOXG_08883 deletion on secretion of pectinolytic enzymes. In no case significant differences between mutant and wild type strains were observed (**Figures 4C,D**).

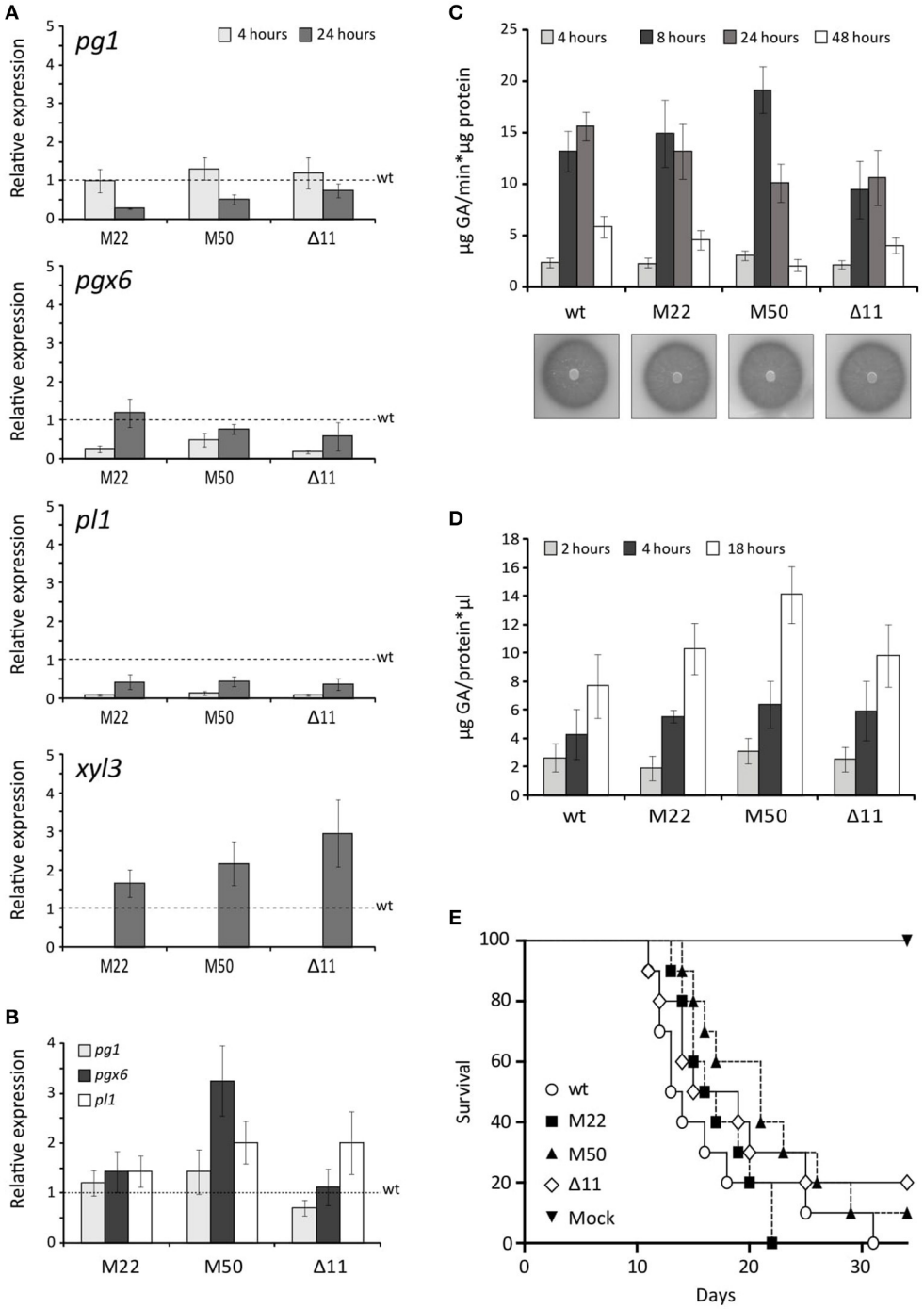

FIGURE 4 | Phenotypic characterisation of FOXG_08883 mutants. **(A)** Transcript levels of *pg1*, *pgx6*, *pl1* and *xyl3* genes were measured by RT-qPCR, in mutants and wild type strains (wt), grown for 4 and 24 h on 2.5% tomato vascular tissue (TVT). Bars represent standard errors calculated from three biological replicates each including three technical replicates **(B)** Transcript levels of *pg1*, *pgx6* and *pl1* genes were measured by qRT-PCR in *F. oxysporum* mutants compared wild type strain (wt), under 0.5% pectin-induced conditions for 4 h. Bars represent standard deviations calculated from three biological replicates each including three technical replicates **(C)** PG activity of the indicated strains was determined by halo formation on PGA-containing plates, and as total specific activity, from culture filtrates after 4, 8, 24, and 48 h growth on SM supplemented with 0.5% pectin, determined by Nelson-Somogyi method using 1% PGA as substrate. Enzyme activity is expressed as µg D-galacturonic acid released per min and µg of protein. Bars represent standard errors calculated from three biological replicates each including two technical replicates **(D)** Total PL activity in culture filtrates after 24 h growth on SM supplemented with 0.5% pectin, determined by measuring absorbance at 230 nm after incubation at 37°C for different times (2, 4, 18 h) in 50 mM Tris-HCl pH 8 buffer containing 1 mM of calcium chloride and using 2% PGA as substrate. Enzyme activity

(Continued)

FIGURE 4 | Continued
is expressed as μg of GA released per μl of sample and μg of protein. Bars represent standard errors calculated from three biological replicates each with two technical replicates **(E)** Incidence of *Fusarium* wilt of tomato plants (cultivar Monika) caused by the wild type strain (wt) and the different mutants. Groups of 10 plants were inoculated by immersing the roots into a suspension of 5×10^6 freshly obtained microconidia mL^{-1} from each strain and planted in minipots. Percentage survival was recorded daily. Data shown are from one representative experiment. Experiments were performed three times with similar results.

A *N. crassa* Δ*clr-1* mutant was found to grow poorly on cellobiose (Coradetti et al., 2012, 2013). By contrast, the *F. oxysporum* ΔFOXG_08883 mutant showed no difference in growth on solid or liquid minimal media containing either carboxymethylcellulose, cellobiose or cellulose as sole carbon source (**Supplementary Figure 3** and **Supplementary Table 7**). This points to differences in the regulatory mechanisms of cellulase genes between different *Pezizomycotina* species. Although our attempts to reproduce the phenotypic change in *F. oxysporum* by mutating the equivalent residue in the orthologous protein have failed, this mutation remains our best candidate. Indeed the two species are rather distantly related and, although *P. occitanis* only contains one copy of the gene, the phylogenetic analysis (**Figure 2**) uncovers a duplication preceding the divergence of *Talaromyces* and *Penicillium* species, followed by a loss of one of the duplicates in the former group. Thus, it is likely that the two orthologues may not fulfill fully equivalent functions.

Role of FOXG_08883 in Virulence on Tomato Plants

The role of the putative transcription factor encoded by FOXG_08883 in virulence was determined by inoculating 2-week-old tomato plant roots in microconidial suspensions of the wild type strain, the FOXG_08883Δ11 mutant and the two Δ11::FOXG_08883$^{160Ser>Cys}$ mutants (M22 and M50). Plants were scored for vascular wilt symptoms at different time intervals after inoculation. Severity of wilt symptoms in plants inoculated increased steadily throughout the experiment, leading to characteristic wilt symptoms 8 days after inoculation, and most plants were dead 30 days after inoculation, without significant differences between the mutants and the wild type strain (**Figure 4E**).

The availability, annotation and extensive phylogenomic analysis of the *P. occitanis* genome sequence represents an important resource for understanding the evolution and biology of this species, and sets the basis for the discovery of new genes for the degradation of complex polysaccharides, of high biotechnological interest.

AVAILABILITY OF DATA AND MATERIALS

All the materials described in the manuscript, including all relevant raw data, are freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality. The final annotation comprised 11,269 protein-coding genes. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NPFJ00000000 (*Penicillium occitanis* CT1), and NPFK00000000 (*Penicillium occitanis* CL100). The version described in this paper is version NPFJ01000000.

AUTHOR CONTRIBUTIONS

GB and AH performed experimental work, MM conducted bioinformatic analyses. MR, AG, and AD conceived the study. TG and MR coordinated the work, analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01627/full#supplementary-material

Supplementary Figure 1 | Strategy for targeted gene deletion **(A)** Physical structure of FOXG_08883 gene promoter and coding regions. Different domains found within the 5′UTR are boxed indicating their length and number of repeats found within *F. oxysporum* genome as well as relative location to the ATG codon. The presumed GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domains and the fungal transcription factor (TF) regulatory middle homology region are indicated within the coding region **(B)** Strategy for targeted replacement of the ORF using the split marker technique and the hygromycin cassette (Hyg^R) as selective marker. Primers used for amplification of both overlapping fragments or for confirmation of targeted gene-replacement are indicated with black arrows (sense) or light arrows (antisense). Primer names are simplified by their identification numbers. DNA fragments used as probes for Southern analyses with restriction sites *Hind* III and *Eco* RI are shown. Scale bar indicates 1 kb length **(C)** Southern analyses of wild type (wt) and targeted deletion strains (Δ), or ectopic transformants (Ect). gDNAs were treated with *Hind* III and *Eco* RI and hybridized with the FOXG_08883 ORF probe **(D)** PCR confirmation of targeted mutants using gDNAs as template and the indicated primers.

Supplementary Figure 2 | Strategy for generation of site-directed mutants **(A)** Co-transformation of the FOXG_08883Δ11 mutant with a FOXG_08883$^{160Ser>Cys}$ allele and the phleomycin cassette (Phl^R). DNA fragments used as probes for Southern analyses, primers used, and restriction sites for *Eco* RI are shown. Scale bar indicates 1 kb **(B)** Southern analysis of wild

type (wt), deletion mutant FOXG_08883Δ11, and different Phl^R transformants. gDNAs were treated with *Eco* RI and hybridized with the FOXG_08883 ORF as probe.

Supplementary Figure 3 | Fungal colonies from microconidia serial dilutions of the wild type (wt) and the indicated mutant strains grown for 3 days at 28°C on synthetic media (SM) containing the indicated carbon source: Sucrose, Cellobiose, or Carboxymethyl (CM)-cellullose.

Supplementary Table 1 | Genome characteristics for the assembly of the two strains.

Supplementary Table 2 | List of species included in the three phylomes.

Supplementary Table 3 | Number of homologs per species for each CAZY family.

Supplementary Table 4 | Number of proteins per species that degrades each compound.

Supplementary Table 5 | Mutations in PENOC when compared to the base genome PENO1, only mutations that affect a protein.

Supplementary Table 6 | Mutations in PENOC when compared to the base genome PENO1, only mutations that affect a protein.

Supplementary Table 7 | Mycelium dry weight (mg) after 120 h growth in SM containing 0.5% of the indicated carbon source, with 170 rpm at 28°C.

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Seed and Root Endophytic Fungi in a Range Expanding and a Related Plant Species

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Climate change is accelerating the spread of plants and their associated species to new ranges. The differences in range shift capacity of the various types of species may disrupt long-term co-evolved relationships especially those belowground, however, this may be less so for seed-borne endophytic microbes. We collected seeds and soil of the range-expanding *Centaurea stoebe* and the congeneric *Centaurea jacea* from three populations growing in Slovenia (native range of both *Centaurea* species) and the Netherlands (expanded range of *C. stoebe*, native range of *C. jacea*). We isolated and identified endophytic fungi directly from seeds, as well as from roots of the plants grown in Slovenian, Dutch or sterilized soil to compare fungal endophyte composition. Furthermore, we investigated whether *C. stoebe* hosts a reduced community composition of endophytes in the expanded range due to release from plant-species specific fungi while endophyte communities in *C. jacea* in both ranges are similar. We cultivated 46 unique and phylogenetically diverse endophytes. A majority of the seed endophytes resembled potential pathogens, while most root endophytes were not likely to be pathogenic. Only one endophyte was found in both roots and seeds, but was isolated from different plant species. Unexpectedly, seed endophyte diversity of southern *C. stoebe* populations was lower than of populations from the north, while the seed endophyte community composition of northern *C. stoebe* populations was significantly different southern *C. stoebe* as well as northern and southern *C. jacea* populations. Root endophyte diversity was considerably lower in *C. stoebe* than in *C. jacea* independent of plant and soil origin, but this difference disappeared when plants were grown in sterile soils. We conclude that the community composition of fungal endophytes not only differs between related plant species but also between populations of plants that expand their range compared to their native habitat. Our results suggest that fungal endophytes of two *Centaurea* species are not able to systemically infect plants. We highlight that endophytes remain poorly studied and further work should investigate the functional importance of endophytes.

Keywords: endophytes, fungi, range expanding plant species, cultivation, phylogeny, soil, seeds, soil sterilization

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INTRODUCTION

Ongoing anthropogenic global climate warming has enabled many plant species to expand their natural range (Walther et al., 2002; Parmesan, 2006) leading to an increase of non-native plant species in more northern, previously unsuitable, latitudes (Tamis et al., 2005). However, plants are more or less tightly linked to numerous associated organisms, which differ in their capacity to shift their range (Berg et al., 2010). Interactions between migrating plants and associated organisms have been studied extensively on introduced exotic plant species that have moved across continents. Some of these plant species become invasive, which is often attributed to a relaxation of plant interactions with specialized natural enemies, such as fungal pathogens (Keane and Crawley, 2002; Mitchell and Power, 2003). In the past decade a number of studies have suggested that such enemy release may also occur during intracontinental range shifts (Van Grunsven et al., 2007; Engelkes et al., 2008; Morriën et al., 2010), which has been demonstrated in several cases by comparing plant responses to soil from the original and new ranges (Van Grunsven et al., 2010; Dostálek et al., 2016). However, the plant holobiome (Mitter et al., 2016) consists of a much wider range of functionally diverse organisms and the holobiome concept most adequately matches to those organisms that are in intimate symbiotic relations with plants, such as endophytes. Little is known about how plant endophyte communities may respond to climate warming-induced range shifts within continents.

Endophytes are defined as organisms that asymptotically inhabit other organisms, without causing readily visible disease symptoms as pathogens do, or promoting plant performance as arbuscular mycorrhizal fungi (AMF) do (Stone and Bacon, 2000). Among the main groups of endophytes are fungi, hereafter simplified as 'endophytes.' Many, if not all plant species usually host several endophyte species simultaneously (Vandenkoornhuyse et al., 2002). In contrast to AMF, the phylogenetic diversity of endophytes is, enormous and spans the entire fungal kingdom, although most endophytes belong to the phylum Ascomycota (Jumpponen and Trappe, 1998; Arnold et al., 2000; Wehner et al., 2014; Glynou et al., 2016). Many fungal endophytes are highly plant host-specific, which explains their immense diversity (U'Ren et al., 2012; Wehner et al., 2014). Consequently, endophyte communities are suggested to resemble the phylogeny of their host plant (Johnston-Monje and Raizada, 2011) more than fungi that colonize the plant surface only.

In spite of the definition suggesting that endophytes are commensals rather than enemies or symbionts, endophytes can affect plant performance, especially under stress (Newsham, 2011). For instance, endophytes increase plant performance under thermal stress (Redman et al., 2002), induce plant resistance resulting in a reduction of root feeding nematodes (Martínez-Medina et al., 2017) and herbivores (Cosme et al., 2016). Under ambient conditions, endophytes might have both positive (Newsham, 2011) or negative effects on plant performance (Mayerhofer et al., 2012; Kia et al., 2017). Effects of endophytes are likely more important under stress. In addition to their role in plant performance, many endophytes can survive and grow as saprophytes in soils (Peay et al., 2016) and include species that are primary decomposers of infected plant material (Song et al., 2017). Therefore, the exact functions of most endophyte species remains largely unknown (Newsham, 2011), whereas their role in climate warming-induced plant range shifts has been completely unstudied.

Endophyte communities differ not only between plant species, but also between plant tissues. For instance, roots host communities of endophytes that largely differ from those in stems, leafs and shoots (Fisher and Petrini, 1992; Rodriguez et al., 2009), with only few endophytes being capable of systematically infecting the host plant (Johnston-Monje and Raizada, 2011). Further, plants interact with different communities of fungi during their life time and are most strongly affected in early (seed and seedling) growth stages (Gure et al., 2005; Müller et al., 2016). Fungi reduce survival of competing plants at early plant stages, while promoting adult plants resulting in higher plant diversity (Bennett and Cahill, 2016). Most plant-endophyte studies have been performed in agricultural settings, on grasses and on trees (Schardl et al., 2004; Rodriguez et al., 2009), while the effects of fungal endophytes in wild plants have rarely been studied (Rodriguez et al., 2009; Herrera Paredes and Lebeis, 2016) as most studies have. The tight connection of endophytes and their hosts has resulted in specific coevolutionary adaptations. Many endophytes can disperse 'vertically,' i.e., via seeds produced by their hosts, and mutualistic seedborne endophytes can promote germination (Ernst et al., 2003; Schardl et al., 2004). However, many pathogenic organisms that use seeds as vectors for dispersal may suppress seed germination (Elmer, 2001; Schardl et al., 2004). As belowground organisms have a limited dispersal capacity (Berg et al., 2010), this might be an important strategy to spread along with their plant host, which, in case of pathogens, can reduce the success of range expanding plant species.

To study endophytes in relation to climate warming-induced range expansion of a wild plant species we isolated endophytes from the seeds of the range expanding plant species *Centaurea stoebe* and the congeneric native *Centaurea jacea*. Both plants are native in Slovenia, whereas *C. jacea* is also native in the Netherlands, which is the expanded range of *C. stoebe*. We grew the seeds in sterilized soil, as well as in sterilized soil inoculated with soils collected from Slovenia and the Netherlands, and isolated fungal endophytes from the roots of the adult plants. We tested the hypotheses that (1) seed endophytic community will resemble the core root endophytic community as seed endophytes could quickly infect germinating roots; (2) endophyte community isolated from the roots or seeds of *C. jacea* will differ from endophyte community from *C. stoebe* as plant pathogens generally are species specific; and (3) southern and northern populations of *C. jacea* will host equal number and similar communities of endophyte taxa while northern populations of the range expander *C. stoebe* will host less and distinct endophyte taxa than southern populations due to the (partial) release from plant-species specific fungi during range shift.

MATERIALS AND METHODS

Plant Species

Climate change such as global climate warming threatens many plant species (Thomas et al., 2004) but also enables plants to expand their naturalized habitat to previously unsuitable climate zones (Hooftman et al., 2006; Van der Putten, 2012). Among them is *C. stoebe* L, a perennial in the family Asteraceae that co-occurs with the common *C. jacea* L. in riverine habitats in the Netherlands. *C. stoebe* originates from Central and South-Eastern Europe (original range) and has expanded its range to higher latitudes in the past century. It has been established in the Netherlands (new range) since 1950 (Sparrius, 2014) but it is still considered a rare species in the novel range, as it occurs only in a few locations.

Seed Collection and Germination

In total more than 1000 seeds of more than 50 *C. jacea* and *C. stoebe* plants were collected from three randomly chosen populations in the Netherlands and Slovenia (Supplementary Table S1). From each population, 100 seeds were surface-sterilized in 5% household bleach (Dunne bleek, OKE, the Netherlands) for 3 min followed by washing with sterile demineralized H2O. Seeds were germinated on glass beads with sterile demineralized H2O in a growth cabinet at a 20/10°C; 16/8 h light/dark day/night regime under 60% humidity.

Soil Collection

Soil was collected from three different locations within a riverine area in Slovenia (35 kg; N45° 58′- 46° 09′; E014° 32′- E014° 45′) and the Netherlands (500 kg; N51° 51′; E5° 53′; Supplementary Table S2). The higher amount of soil needed from the Netherlands was because we only inoculated 10% alive soil (either from Slovenia or the Netherlands to 90% sterile soils (see below for further details). The soil was collected from 5 to 20 cm layer below the soil surface and transported cool (4°C) to the laboratory. In the laboratory, soil was sieved through a 5 mm × 5 mm mesh to remove larger stones, insect larvae, and earthworms. Then, the individual soil samples were formed by pooling three soil subsamples from the same riverine area. All pooled soils were sterilized by autoclaving (high pressure saturated steam at 121°C for 20–40 min) three times with 24 h interval. The live soils were kept in the dark climate room (4°C) before the use in the experiment.

Greenhouse Experiment

To create soil treatments 36 1-L pots were filled with a mix of live and sterilized Dutch soil (1:9; NL soil treatment); 36 pots were filled with a mix of live Slovenian and sterilized Dutch soil (1:9; SLO soil treatment); 36 pots were filled with a mix of sterilized Slovenian and Dutch soils (1:19; STERILE soil treatment). All pots received the same amount of soil (950 g) calculated based on dry weight of different soils. Germinated plant seedlings of similar size were individually planted in each pot. The pots were randomly placed on movable carts in a greenhouse at 21/15°C; 16/8 h light/dark; 60% humidity. Soil moisture was kept constant throughout the experiment by adjusting the pot weight to 1 kg with sterile demineralized H_2O every second day. The carts were rotated weekly to avoid effects of variable conditions in the greenhouse. Rarely germinating weeds (in the live soil treatments) were instantly removed. No nutrients were added to the pots.

Eleven weeks after planting seedlings, shoots of all plants were clipped. Roots were carefully removed from the soil and thoroughly rinsed. Then, five randomly collected root fragments of approximately 5 cm length each were cut into pieces of 0.5 cm and stored in 2 mL centrifuge tubes filled with sterilized demineralized H_2O.

Isolation of Fungal Endophytes from Seeds

To cultivate fungi, three seeds of all populations from both plant species (Supplementary Table S1) were placed on 1.6% H_2O-agar pH 6.7 containing 50 μg/ml streptomycin in 10 cm Petri dishes. The seeds were first surface sterilized using two sterilization protocols; (1) rinsing with sterile demineralized H_2O for 5 min; (2) thoroughly sterilizing by soaking seeds in 5% household bleach solution for 5 min followed by incubation in 70% ethanol for 3 min and washing with sterile demineralized H_2O. For each plant species, five replicates per population with were initiated. The resulting cultures of endophytes were transferred to 0.5x potato-dextrose agar pH 6.7 (PDA; Oxoid) (Larone, 1987).

We also used another procedure aiming at isolating oomycetes. For that, five rinsed and thoroughly surface sterilized seeds (as explained before) of all populations from both plant species (Supplementary Table S1) were placed in 6 cm Petri dishes filled with a mix of sterile pond water filtered through cheesecloth and sterile demineralized H_2O (1:1) containing grass leaves (*Agrostis capillaris*, 2–3 cm) to bait zoospore forming oomycetes (Pettitt et al., 2002). After incubation overnight at room temperature, grass leafs were transferred on 1.6% H_2O-agar pH 6.7 containing 50 μg/ml streptomycin in a 6 cm diameter Petri dish. Growing cultures were transferred to a 6 cm diameter Petri dish containing 0.5x potato-dextrose agar pH 6.7 (PDA; Oxoid). To reduce the number of potentially duplicated isolates for both fungi and oomycetes, only one culture isolated from the same plant replicate was kept in case they were morphologically indistinguishable (Bosshard, 2011).

Isolation of Fungal Endophytes from Roots

To surface sterilize the roots, the collected root pieces were thoroughly washed in sterile demineralized H_2O, transferred to new sterilized centrifuge tubes filled with 70% ethanol and incubated for 7 min. Root pieces were washed again in sterile demineralized H_2O, and then placed in sterile demineralized H_2O in new centrifuge tubes, followed by surface drying on sterile tissue paper under sterile conditions in a flow cabinet. Three root pieces were placed on 1.6% H_2O-agar pH 6.7 containing 50 μg/ml streptomycin in 10 cm diameter Petri dishes. Five replicates were initiated and stored at room temperature.

The remaining root pieces were divided into three and placed into a 6 cm diameter Petri dishes filled with a mix of sterile pond water filtered through cheesecloth and sterile demineralized H_2O (1:1) together with three grass leafs (*Agrostis capillaris*, 2–3 cm). The Petri dishes were placed at room temperature, incubated overnight. Grass leaves were transferred onto a 10 cm Petri dish containing 1.6% H_2O-agar pH 6.7 containing 50 μg/ml streptomycin. All H_2O-agar containing Petri dishes were checked for fungal and oomycete growth and newly formed colonies were transferred to Petri dishes containing 0.5x potato-dextrose agar pH 6.7 (PDA; Oxoid) (Larone, 1987). To reduce the number of potentially duplicated isolates, only one culture isolated from the same plant replicate was kept in case they were morphologically indistinguishable (Bosshard, 2011).

Molecular Work

DNA was extracted from all cultures using the Zymo Research Fungal/Bacterial miniprep kit according to the manufacturer's instructions. The ITS region of all cultures was PCR amplified in 25 μl volume containing 3.125 μL 2 mM dNTPs, 1 μL 25 mM $MgCl_2$, 2.5 μL 10x buffer with $MgCl_2$, 0.125 μL 5 U [μL]-1 FastStart Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 15.25 μL ddH_2O, 1 μL 10 μM of both primers ITS1 and ITS4 (White et al., 1990) and 1 μL template DNA. PCR conditions were composed of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and elongation at 72°C for 60 s with a final elongation at 72°C for 10 min. Amplified products were send for sequencing (LGC Genomics, Berlin, Germany).

Sequence Analyses

Obtained chromatograms from all cultures were manually curated in Chromas Lite v 2.1[1]. Curated sequences were aligned using MAFFT (Katoh and Standley, 2013) and visualized in Seaview v4.6.1 (Gouy et al., 2010).

The resulting 46 consensus sequences were subjected to BLASTn searched against the NCBI nucleotide database[2]. For all consensus sequences, two best matches of known fungal species (three in case different species showed identical matches to best Blast matches of cultivated taxa, or unknown sequences), were aligned using MAFFT and visualized in Seaview. This resulted in an alignment containing 130 sequences.

Maximum likelihood (ML) and Bayesian analyses were performed to assess the phylogenetic relatedness of all cultivated fungi. Maximum likelihood analyses were run in RAxML v8. (Stamatakis, 2014) using the GTR+gamma model with eight rate categories. Rapid hill-climbing tree search algorithm with 1000 bootstrap replicates were used to build and assess the most stable shape of phylogenetic relationships. The phylogenetic tree was visualized in FigTree (Rambaut, 2007) and labeling of the final branches optimized in Gimp.

[1]http://chromas-lite.software.informer.com/2.1/

[2]http://www.ncbi.nlm.nih.gov/Blast.cgi

Data Depository

All sequence data has been submitted to GenBank under the accession numbers MF687671–MF687716.

Data Analyses

For seed endophyte data the total number of unique cultures was calculated per Petri dish ($n = 24$, one Petri dish per one treatment combination) and this data were used for the analyses. For root endophyte data the total number of unique cultures (cultures with distinct sequences) was calculated per pot ($n = 108$, three pots per one treatment combination). Therefore, to avoid pseudoreplication we first averaged the data per treatment combination and used this data for further analyses. To test the effect of plant species and seed origin on the number of seed endophyte cultures that were identified as being taxonomically different, we used general linear model with plant species (*C. jacea* and *C. stoebe*), seed origin (*North* – collected in The Netherlands and *South* – collected in Slovenia) and sterilization treatment (non-sterilized and sterilized) as fixed factors. Differences in the number of root cultures between plant species, seed and soil origins were analyzed using general linear model with plant species (*C. jacea* and *C. stoebe*), seed origin (*North* and *South*) and type of soil inoculum (Dutch soil *-NL*, Slovenian soil *-SLO* and *Sterile soil*) as fixed factors. In both analyses, populations were treated as true replicates.

To test whether the seed and root endophyte community composition was affected by plant species, seed origin and type of soil inoculum we used detrended correspondence analyses (DCA) and canonical correspondence analyses (CCA). For this we used presence-absence data of endophyte cultures. Detrending by segments was used in the DCA. Populations were treated as true replicates. For seed endophyte community analyses, all endophytes were included in the analyses. For root endophyte community analyses, the endophytes with more than two occurrences were included in the analyses because there were a large number of endophytes with one occurrence only.

Multivariate analyses were performed using CANOCO, version 5.03 (Šmilauer and Lepš, 2014) and all other analyses were executed using R, version 3.2.3 (R Core Team, 2015). To fulfill the assumptions of normality and homogeneity of variances total number of root endophyte cultures was log-transformed.

RESULTS

Taxonomic Diversity of Endophytes

After removing morphologically and phylogenetically identical species from the respective plant populations grown in southern and northern soils, we obtained 91 distinct seed and 72 unique root endophytes. Many of those shared identical sequences resulting in 46 unique sequences (Supplementary Table S3).

All cultures except two basidiomycetes resembled fungi of the Ascomycota (**Figure 1** and Supplementary Table S3). The 46 unique sequences were phylogenetically diverse and placed in 19

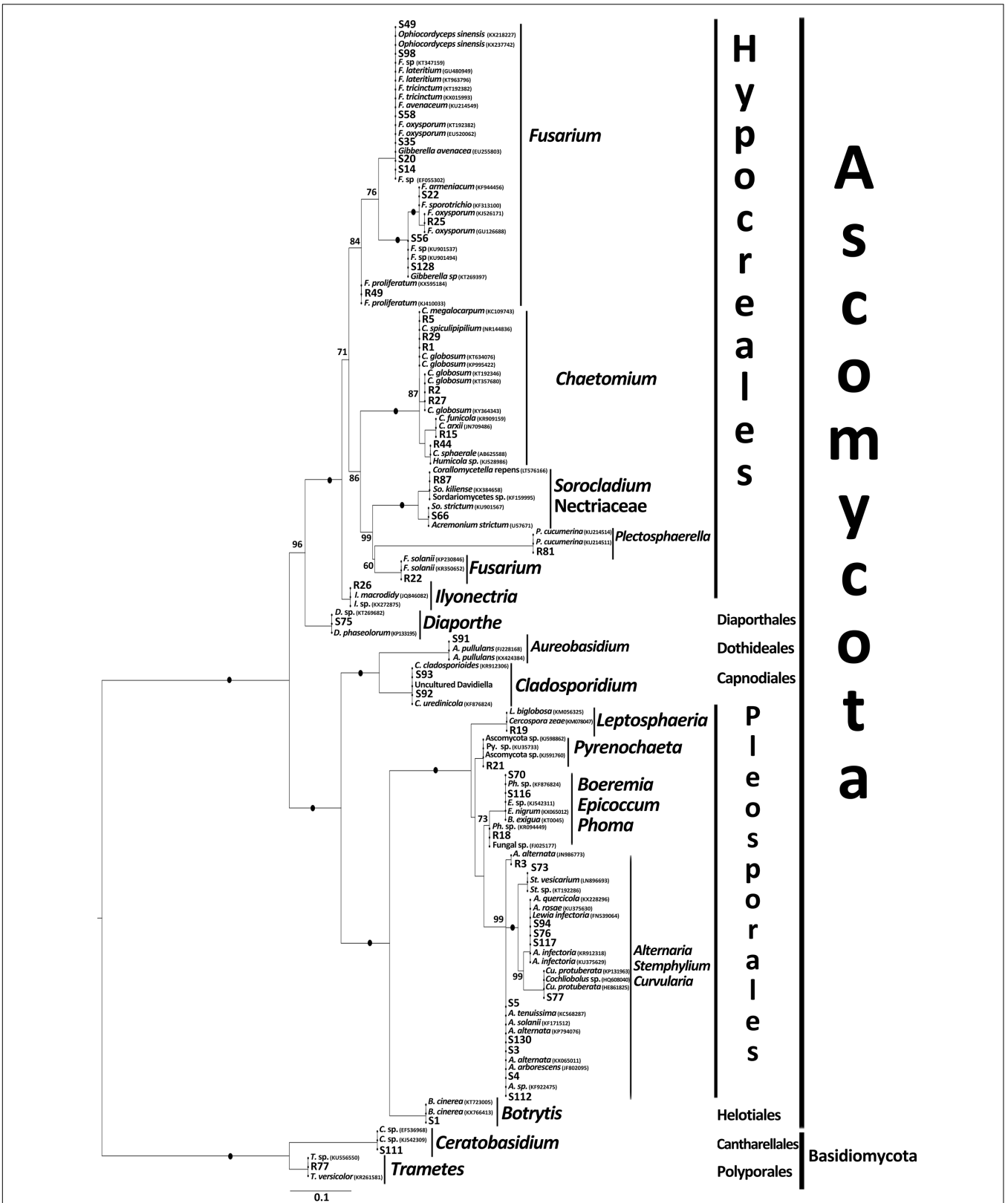

FIGURE 1 | Maximum likelihood phylogenetic tree of all cultivated fungal endophytes (bold) and their best blast matches showing their phylogenetic affinities. For additional details see Supplementary Table S3.

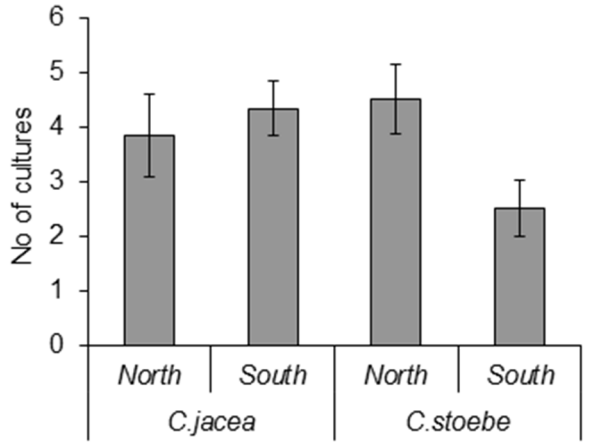

FIGURE 2 | Numbers of different fungal endophyte taxa isolated from southern and northern seeds of *Centaurea jacea*, which is native in both ranges, and the range expander *Centaurea stoebe*, which is native in the south.

genera, 15 families, 10 orders, and 4 fungal classes (**Figure 1** and Supplementary Table S3).

Comparison between the Species Identities and Community Composition of Seed and Root Endophytes

The surface sterilization we used was highly efficient as on average less than one different culture (0.67) was obtained from inside roots, while sterilized seeds harbored more cultures (3.79) per population. The seed endophyte community yielded 28 unique cultures, whereas the root endophyte community yielded 17 unique cultures. There was no overlap in endophyte composition of roots and seeds, besides one culture with perfect match to *Fusarium oxysporum* f. sp. *cumini* that was collected from southern *C. stoebe* seeds, as well as from southern *C. jacea* roots (Supplementary Table S3).

Plant Species, Seed Origin, and Sterilization Effects on the Diversity and Community Composition of Seed Endophytes

Potential pathogens dominated the community composition of seed endophytes, with especially *Fusarium* and *Alternaria* species representing more than 50% of the different isolates (Supplementary Table S3).

Seed endophyte taxa richness was significantly affected by the interaction between plant species and seed origin ($F_{1,16} = 5.23$, $P = 0.036$; **Figure 2**). In particular, the number of cultures isolated from the *C. stoebe* seeds collected in the southern range was lower than in the northern range whereas the number of cultures isolated from the *C. jacea* seeds did not differ between origins (**Figure 2**).

The community composition of seed endophytes was affected by the two-way interactions between plant species and seed origin ($F = 1.5$; $P = 0.001$; 18.3% explained variation, adjusted explained variation 6.0%; **Figure 3**). Seed sterilization did not affect the community composition of endophytes ($F = 0.6$; $P = 0.997$).

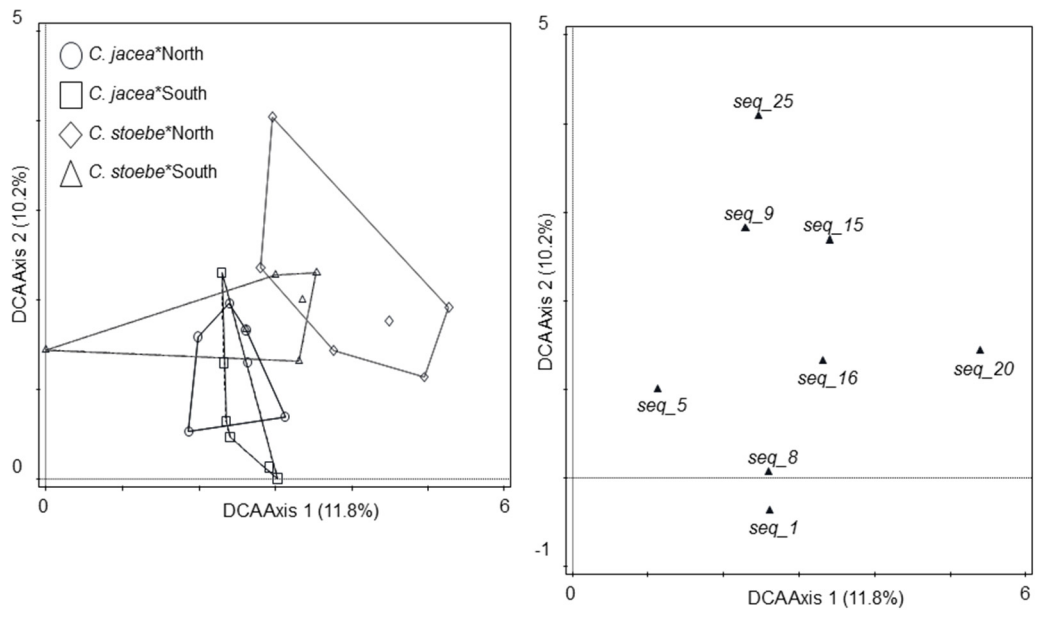

FIGURE 3 | Ordination diagrams of detrended correspondence analysis (DCA) of endophyte community isolated from seeds of *C. jacea* collected in The Netherlands ("North," circles) and in Slovenia ("South," squares), as well as seeds of *C. stoebe* collected in The Netherlands ("North," diamonds) and in Slovenia ("South," open triangles, left panel). All endophyte cultures with more than 10% fit are shown (right panel). Percentages of total explained variation by DCA axes are given in parentheses.

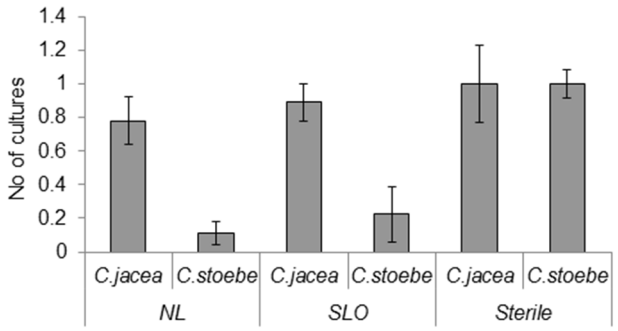

FIGURE 4 | Numbers of different fungal endophyte taxa isolated from roots from the common *C. jacea* and the range expander *C. stoebe* grown in northern (NL), southern (SLO), and sterile soils (Sterile) expander *C. stoebe*.

Plant Species, Seed Origin, and Soil Origin Effects on the Diversity and Community Composition of Root Endophytes

The most common (present in more than 10% of the samples) root endophytes were *Chaetomium* spp. (Supplementary Table S3). Range-expanding *C. stoebe* hosted a lower diversity of root endophytes than *C. jacea* plants in NL and Slo soils whereas in sterilized soil there was no difference in the diversity of root endophytes between the two plants species ($F_{2,24} = 7.71$, $P = 0.0026$; **Figure 4**). Root endophyte community composition was affected by a combination of plant species, soil and seed origin when singleton endophytes were not included in the analyses (by three-way interaction; $F = 2.4$; $P = 0.005$, 61.1% explained variation; 35.2% adjusted explained variation; **Figure 5**).

DISCUSSION

We here show that there is no overlap of root-inhabiting and seed-inhabiting fungal endophytes in neither the range expanding plant species *C. stoebe* nor its common congener *C. jacea*. This strongly suggests that seeds might not serve as vehicles for (pathogenic) root endophytes to spread to new ranges.

High Diversity of Mainly Ascomycete Fungal Endophytes in Seeds and Roots

Almost all endophytes cultivated from both seeds and roots belong to Ascomycota, which is in line with many previous studies on endophytes in different parts of the plant (Jumpponen and Trappe, 1998; Arnold et al., 2000; Rodriguez et al., 2009). Interestingly, most seed endophytes, in contrast to root endophytes, most closely resembled potential pathogens. The overall function of endophytes is being debated (Newsham, 2011; Mayerhofer et al., 2012) and remains largely unknown (Vandenkoornhuyse et al., 2002). We assigned potential functions based on sequence identity without experimentally testing the vast amount of cultures. This approach is well-accepted to get an overall understanding of likely functions (Nguyen et al., 2016). Therefore, our results suggest that endophytes differ in their functioning between infected plant tissues with most endophytes inhabiting seeds likely being negative while root endophytes being neutral or positive for plant performance.

No Overlap between Seed and Root Endophytes

In line with potential functional differences between seed and root endophytes, seed and roots hosted a fundamentally different community of endophytes. Only one sequence resembling *F. oxysporum* f. sp. *cumini* was shared between an endophyte isolated from a seed and another one isolated from the root of southern plants. The presence of this fungi is not surprising as *F. oxysporum* f. sp. *cumini* generally has the ability to infect and damage host roots and shoots (Özer and Bayraktar, 2015). However, differentiating *F. oxysporum* subspecies and especially races is difficult and relies on other markers than the one we used here (ITS) as a general fungal barcode (Gordon and Martyn, 1997; Lievens et al., 2008). Considering that even races within *F. oxysporum* f. sp. *cumini* are differentially impacting plant performance (Özer and Bayraktar, 2015) and the fact that the sequence-identical cultures we obtained were originating from cultures isolated from two different plant species, ecological function of both cultures could be dissimilar. This further shows that root and seed endophyte communities are fundamentally different, suggesting that most endophytes have a restricted localization in the plant tissues in at least in the *Centaurea* species studied here (Rodriguez et al., 2009). The restriction of belowground endophytic fungi to root tissue also reduces the possibility that these belowground taxa spread (Berg et al., 2010). This would give the plant a competitive advantage in case they escape their more specialized pathogens – some of which are considered as being endophytic (Saikkonen et al., 1998).

Seed Endophytes Differ in *C. stoebe* Plants in the Expanded Range

Species specific seed endophytes seem to be generally plant pathogenic, which could control plant growth and in turn population dynamics, independent whether the plant is native or not (Blaney and Kotanen, 2001). Interestingly, we found the lowest diversity of (potentially pathogenic) fungi in the native range of *C. stoebe* compared to all other plant seed populations including its expanded range, while endophyte diversity in seeds of *C. stoebe* in the expanded range was highest. Fungi that are more generalist and less pathogenic might therefore infect seeds in the expanded range that have less negative impact on plant growth, which is in line with fungi having more negative effect on plant seeds in the native compared to the invaded range (Halbritter et al., 2012). These pathogenic endophytes could therefore be among the "enemies" that drive range expansion according to the enemy release hypothesis, which is among the key hypotheses to explain the success of plants coming to a new range (Keane and Crawley, 2002; Reinhart et al., 2003). These results indicate that *C. stoebe* cannot only expand, but can

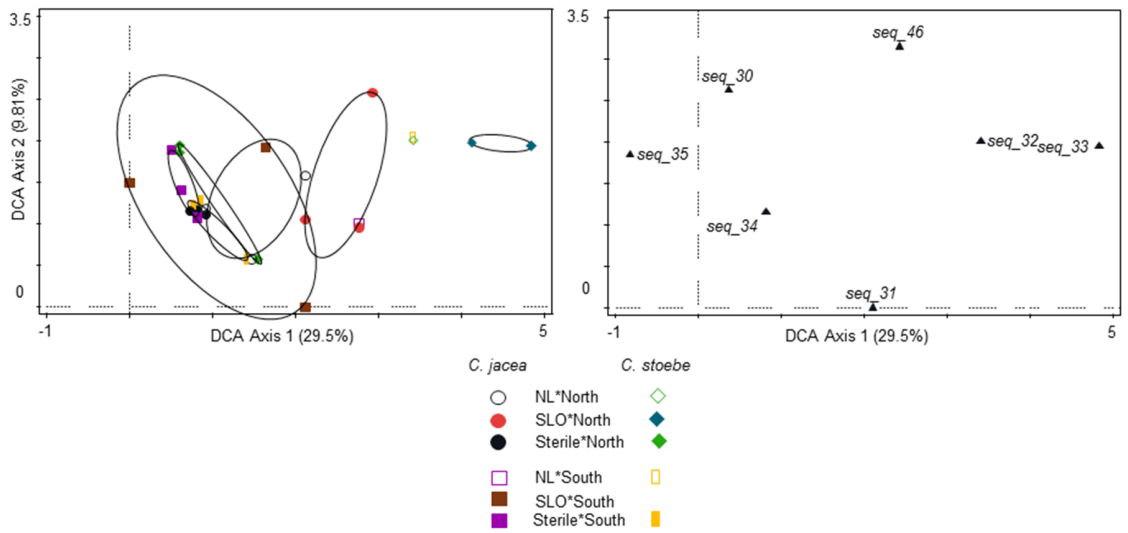

FIGURE 5 | Ordination diagrams of DCA of endophyte community isolated from roots of northern *C. jacea* plants grown in northern (open black circles), southern (filled red circles), and sterilized soils (filled black circles); from roots of southern *C. jacea* plants grown in northern (open pink squares), southern (brown filled squares), and sterile soils (pink filled squares); from roots of northern *C. stoebe* plants grown in northern (open green diamonds), southern (filled blue diamonds), and sterile soils (filled green diamonds); from roots of southern *C. stoebe* plants grown in northern (open yellow rectangles) and sterile soils (filled yellow rectangles). All endophyte cultures with are shown (right panel). Percentages of total explained variation by DCA axes are given in parentheses. The overlapping symbols have been shifted by 0.1 unit to improve the visibility.

only perform well in new ranges indirectly by "escaping" their associated endophytes. While endophytes are directly impacted by climate change (Giauque and Hawkes, 2013) and soil biota are known to affect the performance of range expanding plants (Van Nuland et al., 2017), we lack an integrated understanding on the importance of endophytes in climate induced range expansions. Functional studies on a range of endophytes and their hosts in both ranges are needed to confirm that this hypothesis equally holds for seed endophytes.

Root Endophytes

In contrast to seed endophytes, root endophytes were mostly assigned as being non-pathogenic. Still, and in line with the seed endophytes, the diversity of root endophytes was much lower in *C. stoebe* than in *C. jacea* when plants were grown in Dutch or Slovenian soils. This suggests that compared with *C. jacea*, the range expanding plant *C. stoebe* is more antagonistic toward soil organisms as previously shown for fungi and root feeding nematodes (Wilschut et al., 2016), but nematodes might not always be reduced (Morriën et al., 2012; Viketoft and van der Putten, 2015). *C. stoebe* is known to produce a wide range of secondary metabolites that are more negative toward soil organisms in a new range, making it a noxious invader, especially in the United States, where it threatens natural systems (Ortega and Pearson, 2005; Ridenour et al., 2008). Production of secondary metabolites might be the key underlying factor that acts against plant-specialized soil pathogenic organisms and act as a novel weapon to change the microbial community structure to their own favor (Callaway et al., 2008; Verhoeven et al., 2009). This would also lead to a reduced infection potential of fungal endophytes as observed here.

In line with the reduction of seed endophytes in southern seeds of *C. stoebe*, no endophytes were cultured when those seeds where grown in southern soils. This suggests that southern seeds of *C. stoebe* seem particularly strong in defending themselves especially in their native soil habitat. This allows *C. stoebe* to defend against specialized native pathogens and, when expanding, benefit from more general, less harmful interactions, which is supported by a profound increase in infection in sterilized soils. Northern plants which escaped specialized pathogen pressure decades ago host higher endophyte diversities suggesting a trade-off toward losing costly-to-produce chemical defenses in favor of growth, which also allows them to benefit from more generalist mutualistic interactions including AMF (Bunn et al., 2015) and potentially endophytes as suggested here. Furthermore, range expanding plants could benefit in plant communities by accumulating pathogens that are deleterious for native plants (Mangla and Callaway, 2008). This suggests that range expanding plants benefit initially mostly from a release of specialist pathogens, while later from other mechanisms including increase of mutualists.

The profound reduction of endophytes in *C. stoebe* compared with *C. jacea* that is lost in sterile soil suggests that the native rhizosphere microbiome represents an intimate component of the plants ability to cope with incoming organisms. This is in line with more reduced mechanistic studies that show that invasive microbes are less likely to establish when a more diverse naïve microbiome is present (Mallon et al., 2015; Wei et al., 2015). This result further suggests that experiments done in sterile soils under non-sterile conditions can miss patterns that are actually present under more natural conditions. We therefore propose to avoid

using experimental setups with entirely sterilized soils and always inoculate at least a fraction of natural soil to reduce the random effects of invasive microorganisms.

CONCLUSION

We conclude that there is no overlap in the taxonomic composition of endophytes in seeds and roots in the range expanding (*C. stoebe*) and its common congener (*C. jacea*), suggesting that root-inhabiting organisms, including plant pathogens, cannot spread along with the plant to infect roots in the new range. We further show that the range expanding plant hosts a reduced diversity of endophytes in roots, but that this difference is not present when soils are sterilized. This suggests that range expanding plants only in combination with a diverse microbiome obtained from soils have an increased defense against specialized soilborne organisms including pathogens, which provides them with a competitive advantage to establish in plant communities. Overall the distribution of endophytes in different plant species, especially their presence in different plant parts remains little studied. Moreover, there are no studies focusing on endophytes in range expanding plant species. As these can adapt distinct ecological roles their functional importance to affect range expanding plant species remains to be elucidated.

AUTHOR CONTRIBUTIONS

SG, OK, and WP designed the study; BV, MC, FH, and SG conducted the experimental work, SG and OK analyzed data; SG, MC, OK, and WP drafted the manuscript supplemented with comments from FH.

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Fungal Diversity in Tomato Rhizosphere Soil under Conventional and Desert Farming Systems

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This study examined fungal diversity and composition in conventional (CM) and desert farming (DE) systems in Oman. Fungal diversity in the rhizosphere of tomato was assessed using 454-pyrosequencing and culture-based techniques. Both techniques produced variable results in terms of fungal diversity, with 25% of the fungal classes shared between the two techniques. In addition, pyrosequencing recovered more taxa compared to direct plating. These findings could be attributed to the ability of pyrosequencing to recover taxa that cannot grow or are slow growing on culture media. Both techniques showed that fungal diversity in the conventional farm was comparable to that in the desert farm. However, the composition of fungal classes and taxa in the two farming systems were different. Pyrosequencing revealed that *Microsporidetes* and *Dothideomycetes* are the two most common fungal classes in CM and DE, respectively. However, the culture-based technique revealed that Eurotiomycetes was the most abundant class in both farming systems and some classes, such as *Microsporidetes*, were not detected by the culture-based technique. Although some plant pathogens (e.g., *Pythium* or *Fusarium*) were detected in the rhizosphere of tomato, the majority of fungal species in the rhizosphere of tomato were saprophytes. Our study shows that the cultivation system may have an impact on fungal diversity. The factors which affected fungal diversity in both farms are discussed.

Keywords: farming system, fungal community, pathogen, saprophytes, desert fungi

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INTRODUCTION

Soil is a precious and complex natural resource that represents a huge reservoir of biodiversity with several billion prokaryotic and eukaryotic microorganisms. These microbes significantly share biomass and ecosystem functions in both natural and managed agricultural soils (Sidorenko et al., 1978). Microbial diversity is directly or indirectly affected by cultivation techniques, management practices, crop rotation, soil tillage, animal grazing, plant species and climatic changes (Acosta-Martínez et al., 2014; Chen et al., 2017; Gangireddygari et al., 2017; Liu et al., 2017; Yao et al., 2017). Variations in soil temperature, precipitation and soil pH also influence soil fungal diversity. Fungi are the dominant eukaryotes among soil microbial communities where they play crucial and key roles in terrestrial ecosystems (Abed et al., 2013; Peay et al., 2013; Acosta-Martínez et al., 2014).

Oman is situated at an arid region in the eastern part of the Arabian Peninsula and in summer, the temperature can rise up to 50°C. Most farms in Oman use traditional methods to promote biodiversity by cultivating several crops in the same field. The majority of farms in the northern

part of the country grow date palms, while rest occupies citrus, vegetable crops such as tomatoes and cucumbers and other crops (Kazeeroni and Al-Sadi, 2016). Tomato is the major vegetable crop produced in Oman with the total production of over 70,000 tons in 2014 (FAO, 2015). Most tomato production in Oman is in commercial farms in the main agricultural area, the Batinah region. However, some of the farms that are located in desert areas also produce crops including tomatoes.

Microbial abundance, diversity and activity largely have implications on sustainable productivity of agricultural land and production systems. Information on the microbial communities associated with rhizospheres and their complex interrelationship is essential in the selection of sustainable crop rotations and management practices (Lenc et al., 2015; Chen et al., 2017). Direct culture of microorganisms and molecular methods are widely used to analyze soil microbes (Al-Sadi et al., 2015; Thomson et al., 2015; Kazeeroni and Al-Sadi, 2016). With the advent of next generation sequencing technologies, 454 pyrosequencing is used nowadays for assessing fungal diversity because of its high sensitivity (Esmaeili Taheri et al., 2015; Kazeeroni and Al-Sadi, 2016).

Although several studies addressed tomatoes, the information about the occurrence and the organization of fungal organisms in the tomato rhizosphere is currently limited. Furthermore, studies on fungal diversity in desert farming systems remain rare. Considering the different ways that can change the farming systems underlying soils and soil microbes, it is essential to understand the fungal diversity and their functions in soils under different managements. In this study, we examined soil fungal composition and diversity using pyrosequencing and culture-based techniques in two different tomato-farming systems: commercial vs. desert. Our main objective was to study how the changes of soil fungal communities vary with the different farming techniques. Knowledge in these areas will help predict how fungal communities vary under varying cultivation systems.

MATERIALS AND METHODS

Collection of Samples

Soil samples were collected from conventional and desert farms in Barka and Thumrait, Oman during June 2014 and the information on the details of the locations and weather conditions of the soils samples are mentioned in **Table 1**. Each soil was collected along random directions from three different lots of each tomato plant, approximately about 1 kg from each sample, taken from 10 to 12 cm depth near the active growing roots. The soil samples were kept in sterile plastic bags and brought to the laboratory. All samples were thoroughly homogenized before stored at 10°C.

Soil Analysis

Soil samples were air-dried and sieved. Soil texture, pH and electrical conductivity (EC) were determined using standard methods (Gee and Bauder, 1986; Zhang et al., 2005). Determination of potassium (K) and phosphorus (P) were done using a flame photometric method (Sheerwood 450 flame photometer) and Inductively Coupled Plasma (Perkin Elmer, United States), respectively. Organic and inorganic carbon levels were determined using Total Organic Carbon analyzer (TOC-V, Shimadzu, Japan). Total nitrogen (N) was estimated by Kjeldahl distillation method using Kjeltec Analyser (FOSS TECATOR, Sweden). Differences among soils were examined using SAS (SAS Institute Inc., United States).

Direct Plating

This method was performed for isolating fungi from soil samples. Soil samples (0.1–0.15 g) were plated onto rose Bengal-amended 2.5% potato dextrose agar (Oxoid, England) plates using three replicates for each sample. Incubation was at 25°C for 3–7 days. Fungal colonies present on the incubation plates were subcultured for identification.

Identification of Fungi

Fungal isolates were identified based on morphological characteristics under light microscope and sequences data. Fungal isolates were grown on PDA for 3–7 days. Then fungal isolates were preliminarily assigned to different genera based on the size and shape of spores and mycelia.

To confirm the identity of fungi, DNA was extracted from freeze dried mycelium using the protocol of Lee and Taylor (1990). The ITS region was amplified using the primer pair ITS1 and ITS4 (White et al., 1990) as explained by Al-Sadi et al. (2011). Additional loci (β-tubulin, Calmodulin, RNA polymerase II second largest subunit, Translation elongation factor 1-alpha) were used to identify the species of *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium* using the primers and conditions detailed in literature (Carbone and Kohn, 1999; Samson et al., 2014). Purification and sequencing of PCR products were carried out at Macrogen, Korea. Sequences were aligned and improved using MEGA v.6 (Tamura et al., 2013). A maximum likelihood analysis was performed by using raxmlGUI v.1.3 (Silvestro and Michalak, 2012) for the isolates that belong to the kingdom fungi using the ITS region. The optimal ML tree search was conducted with 1000 separate runs, using the default algorithm. Bootstrap 50% majority-rule consensus trees were generated and the final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTRGAMMA substitution model. ITS sequences generated from the analysis were deposited in GenBank (**Table 2**).

Pyrosequencing Analyses

DNA was extracted from 3 to 5 replicates from each soil sample according to the protocol of Volossiouk et al. (1995). A two-step process was used for the amplification of samples. Firstly, the forward (i5 and ITS1F) and reverse (i7 and ITS2aR) primers were constructed as described previously (White et al., 1990; Gardes and Bruns, 1993; Kazeeroni and Al-Sadi, 2016; Al-Balushi et al., 2017). The reaction mixtures and conditions for the first and the second PCRs were as per Al-Balushi et al. (2017). Checking of sequences was done using RDP ver 9 (Cole et al., 2009). Analysis and taxonomic classification was done using a distributed BLASTn.NET algorithm (Dowd et al., 2005) based on a 97% cut off. Fungi were classified based on trimmed taxa.

TABLE 1 | Physicochemical properties of soil samples.

Sample name	Soil texture	pH	EC (mS)	%TIC	%TOC	%N	P (mg kg^{-1})	K (mg kg^{-1})
CM	Sandy	8.0 a	1.28 b	5.27 a	3.464 a	0.056 a	5.076 a	61.876 a
DE	Loamy sand	7.8 a	7.72 a	4.13 a	2.768 a	0.020 b	3.272 b	45.639 b

EC, electrical conductivity; TIC, total inorganic carbon; TOC, total organic carbon; N, nitrogen; P, phosphorus; and K; potassium. Values with the same letter in the same column are not significantly different from each other at $P < 0.05$ (Tukey's Studentized Range test, SAS).

TABLE 2 | ITS GenBank accession numbers of fungal isolates detected in this study.

Fungal isolates	Accession number
Aspergillus pachycristatus	KY814690
Aspergillus quadrilineatus	KY814680
Aspergillus quadrilineatus	KY814684
Aspergillus quadrilineatus	KY814689
Aspergillus rugulosus	KY814676
Aspergillus rugulosus	KY814688
Aspergillus rugulosus	KY814687
Cephaliophora sp.	KY814682
Chaetomium sp.	KY814677
Cladosporium tenuissimum	KY814674
Fusarium chlamydosporum	KY814673
Fusarium chlamydosporum	KY814685
Fusarium nygamai	KY814686
Fusarium solani	KY814675
Fusarium solani	KY814679
Fusarium solani	KY814691
Mortierella sp.	KY814683
Penicillium corylophilum	KY814681
Pythium aphanidermatum	KY814678

The relative abundance for individual taxa was then determined after checking the percentage of sequences assigned to each fungal phylogenetic level.

RESULTS

Soil Analysis

Soils differed in their properties (**Table 1**). The CM soil was sandy, while the soil from DE was loamy sandy. The pH was found to be alkaline in DE (7.8) and CM (8), while EC was significantly higher in DE (7.72) compared to CM (1.27) ($P < 0.05$; **Table 1**). The total inorganic carbon (TIC) and total organic carbon (TOC) concentrations were not significantly different between CM and DE ($P > 0.05$). The available N, P, and K concentration were significantly higher in the CM farming system compared to DE ($P < 0.05$; **Table 1**).

Phylogenetic Analysis

The ITS alignment was used to represent the fungal species recovered from direct plating technique. The alignment comprised 68 strains (including the outgroup taxon *Allomyces reticulatus* and 18 isolates recovered in this study), and the manually adjusted dataset comprised 959 characters including gaps. A best scoring RAxML tree resulted with the value of Likelihood: −11745.862498 (**Supplementary Figure S1**). Based on the phylogenetic tree, 18 isolates from the present study belonged to *Ascomycota* phylum (classes *Dothideomycetes, Eurotiomycetes, Pezizomycetes* and *Sordariomycetes*), while the subdivision *Mucoromycotina* belonged to the phylum *Zygomycota*. Fungal classes were separated from each other with a very high bootstrap support (94–100%). Some of the isolates could not be matched with appropriate reference strains in GenBank, suggesting that some isolates could be new species or the sequence of their corresponding species are not available in GenBank.

Evaluation of Fungal Diversity by Culture-Based Technique

Ascomycota was the most abundant phylum, present in both farming systems and *Oomycota* and *Zygomycota* were the other constituents. The phylum *Oomycota* was present only in CM while *Zygomycota* was present only in DM. In *Ascomycota*, soil samples from both farms presented a high relative abundance of *Eurotiomycetes* at class level (42.85% in CM, 40% in DE). This was followed by *Sordariomycetes* (42.85%) and *Dothideomycetes* (7.1%) in CM while *Sordariomycetes* and *Pezizomycetes* were found in the same level of abundance in the DE farming system (20%). *Dothideomycetes* and *Oomycetes* were unique classes in CM while *Zygomycetes* and *Pezizomycetes* were unique classes in DE (**Figure 1**). Totally eight genera were recovered from both farming systems and these were dominated by *Aspergillus* (**Figure 2**). A total of 12 fungal species were isolated from both farming systems. The most common species across two farming systems was *Aspergillus quadrilineatus*. *Cephaliophora* sp., *Mortierella* sp. and *Penicillium corylophilum* were only present in DE while *Chaetomium* sp., *Cladosporium tenuissimum*, *Aspergillus pachycristatus*, *A. rugulosus*, *F. nygamai*, *F. solani* and *Pythium aphanidermatum* were unique in CM. The Shannon values were 2.0 for soil from CM compared to 1.6 from DE (**Table 3**).

Evaluation of Fungal Diversity by Pyrosequencing Technique

Using a 97.0% similarity barcoding threshold, pyrosequencing showed that the majority of fungal taxa in CM was in the *Microsporidia* (60.26%), followed by *Ascomycota* (28.5%), *Chytridiomycota* (3.51%), *Basidiomycota* (0.77%) and *Zygomycota* (0.01%). All the *Microsporidia* belonged to a single class *Microsporidetes*. *Ascomycota* was distributed over classes *Leotiomycetes* (4.5%), *Dothideomycetes* (3%) and

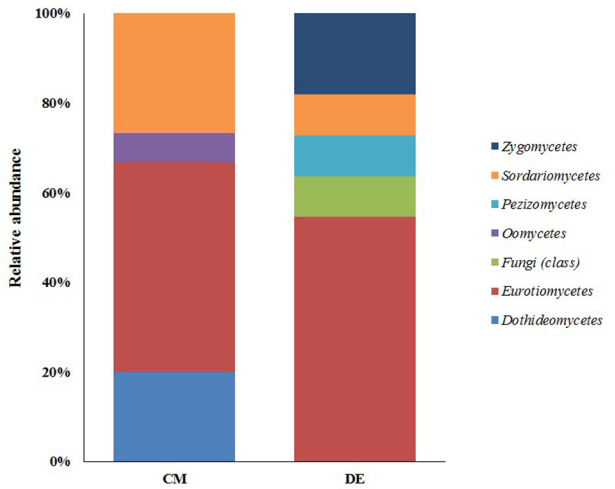

FIGURE 1 | Class-level relative abundance of fungal communities in commercial farming (CM) and desert farming (DE) systems using culture-based technique.

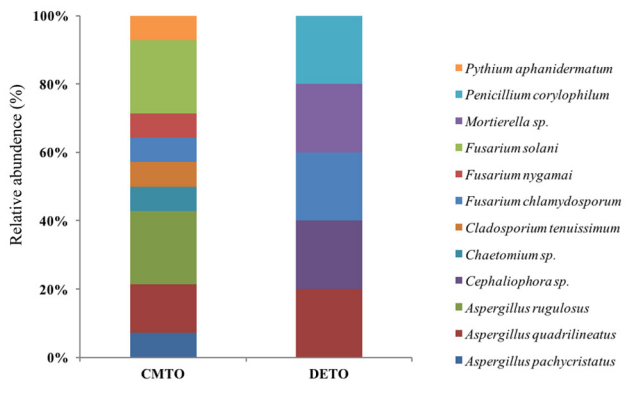

FIGURE 2 | Species-level relative abundance of fungal communities in commercial farming (CM) and desert farming (DE) systems using culture-based technique.

TABLE 3 | Shannon-Wiener index of commercial farm (CM) and desert farm (DE) as determined by direct plating technique.

	Direct plating		Pyrosequencing	
	CM	DE	CM	DE
No. of phyla	2	2	5	3
No. of classes	4	4	8	9
No. of families	5	4	9	11
No. of genera	5	5	10	11
No. of species	9	5	15	11
Shannon Wiener index	2.0	1.6	1.4	1.9

Sordariomycetes (1.5%). In DE over 95% of all OTUs belonged to the phylum *Ascomycota,* which was distributed in four classes; *Dothideomycetes* (38.07%), *Eurotiomycetes* (24.07%), *Leotiomycetes* (5.77%) and *Sordariomycetes* (4.04%). Another

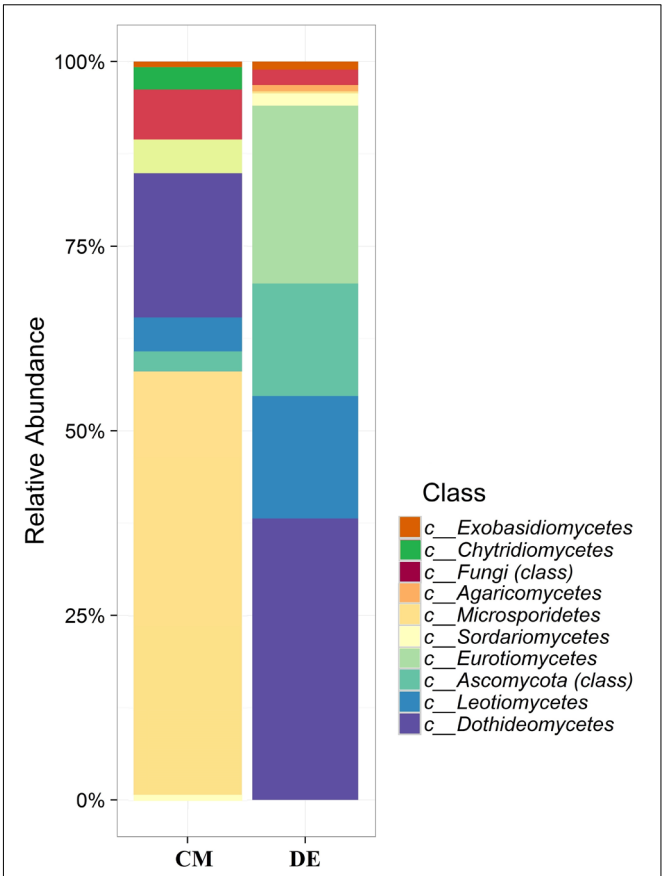

FIGURE 3 | Class-level relative abundance of fungal communities in commercial farming (CM) and desert farming (DE) systems using pyrosequencing technique.

23.73% could not be assigned to any classes and kept as Ascomycota *incertae sedis. Zygomycota* and *Chytridiomycota* were absent in the DE and *Microsporidia* contribution is in lesser amount (0.3%). The distribution of classes based on pyrosequencing is illustrated in **Figure 3**. The pyrosequencing approach yielded a total of 15 species in CM, with a Shannon value of 1.4 (**Figure 4** and **Table 3**). *Systenostrema alba* was the most dominant, comprising 60% of the total species in CM, followed by *Rhizina undulata* (17.56%) *Mortierella* sp. (4.17%) and *Oidium aloysiae* (4.04%). Pyrosequencing detected 11 fungal species in DE, with a Shannon value of 1.9 (**Table 3**). *Cladosporium* sp. (27%) and *Emericella nidulans* were the most abundant taxa, followed by *Trichocladium asperum* (12.8%), *Phoma gardeniae* (11%) and *Symbiotaphrina kochii* (10.94%).

Direct Plating vs. Pyrosequencing

Direct plating and pyrosequencing methods were used in this study for estimating fungal abundance and diversity in two different farming systems of tomato. The water molds (*Oomycetes*) were not picked up by pyrosequencing and only detected by the culture-based method. On the other hand, *Basidiomycota*, *Chytridiomycota* and unicellular parasites

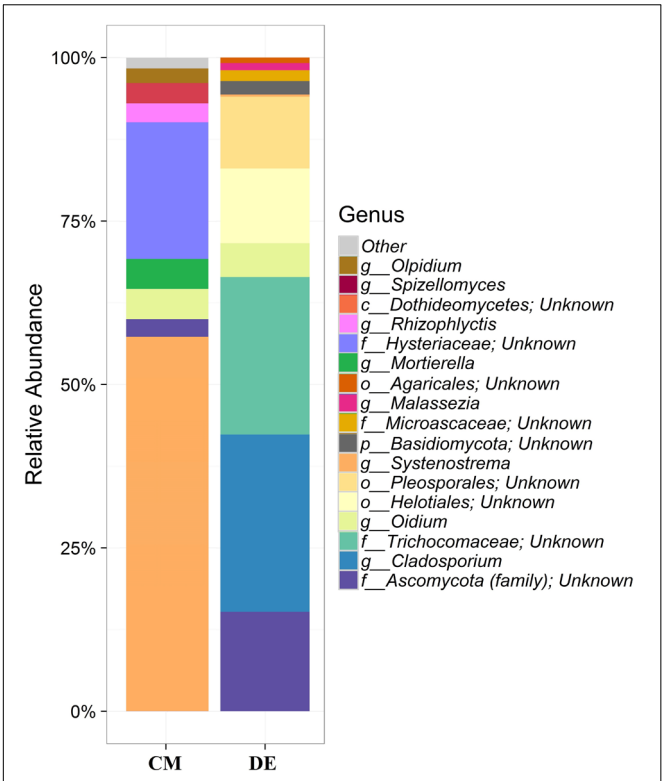

FIGURE 4 | Generic-level relative abundance of fungal communities in commercial farming (CM) and desert farming (DE) systems using pyrosequencing technique.

Microsporidias were unable to be recovered using direct plating. Pyrosequencing detected more species compared to direct plating technique (**Table 3**).

DISCUSSION

Several studies using molecular techniques and cultivation-based methods have described the fungal communities present in different farming systems (Al-Sadi et al., 2015; Van Geel et al., 2015; Kazeeroni and Al-Sadi, 2016). These studies have shown that fungal communities present in each system vary with the soil physiochemical properties and the cropping systems (Huang et al., 2015; Thomson et al., 2015). Our results demonstrate that CM and DE soil are highly diverse in soil microbiota. In general, the fungal diversity in the CM farming system was high compared to the DE system. The presence of relatively high percentage of TOC and TIC in both farming systems may be favorable for the growth of most fungi. In addition, N, P and K levels are relatively high in CM soil and this is probably because of the addition of a certain amount of organic and chemical fertilizers to the soil. Thus, the application of fertilizers by growers could have contributed to creating differences in the available minerals in soils between the two farming systems and soil types (Grüter et al., 2017; Wang et al., 2017). Grantina et al. (2011) reported positive impact of the available potassium in soil on the total number of cultivable filamentous fungi (CFF) and on the fungal diversity. In another investigation, a negative impact of phosphorus was observed on species richness of fungi in soil (Huang et al., 2005). As suggested by Gyaneshwar et al. (2002), this could be due to variations in the number of phosphate solubilizing microorganisms in soil.

Ascomycota is the largest and widespread phylum of fungi and is abundant in soil and composts (Abed et al., 2013; De Gannes et al., 2013; Kazeeroni and Al-Sadi, 2016). They are considered important decomposers and causal agents of several soil-borne diseases. In the present study based on the culture-based technique, *Ascomycota* was dominant in both farming systems. *Eurotiomycetes* was identified as the dominant class in both CM and DE, mainly because it contains two of the most common fungal genera viz., *Penicillium* and *Aspergillus* in most of the ecosystems (Godinho et al., 2015; Yee et al., 2016). Many saprophytic fungi were detected in DE; whereas plant pathogenic fungi viz., *Cladosporium* sp., *Fusarium* spp. and *Pythium* sp. were detected in CM soil. In the present study, *Fusarium* that causes wilt disease in tomato was detected in CM soil samples. The prevalence of *Fusarium* in CM soil might be due to the potential ability of chlamydospores of *Fusarium* to survive in the soil for many years under harsh environmental conditions. Chellemi et al. (2012) demonstrated that repeated tomato cultivation increased the incidence of *Fusarium* wilt caused by *F. oxysporum* f. sp. *lycopersici* by 20% or more.

The mycoparasite, *Chaetomium* sp. was detected in the CE soil. However, other fungal biocontrol agents like *Trichoderma* spp. and *Gliocladium virens* were not detected in both the farming systems. The existence of these organisms in soil is crucial for suppression of damping off and Fusarial wilt diseases of tomato (Blaya et al., 2013; Guzmán-Valle et al., 2014). Hence, the soil health in both the farming systems has to be improved by incorporation of organic amendments and application of biopesticides. Some of the fungal isolates could not be identified to the species level, possibly because they are new species or they need more genes to be sequenced. Future studies may address the identity of these isolates.

Pyrosequencing revealed that 95% of the taxa in DE soil belonged to the *Ascomycota*, whereas 60% of the taxa present in CM soil belonged to the phylum *Microsporidia* (a group of spore-forming unicellular parasites) and the genus *Systenostrema.* This is in agreement with our previous findings that *Microsporidia* are one of the dominant phyla in soil of farming systems in Oman (Kazeeroni and Al-Sadi, 2016). *Microsporidia* are obligate, spore-forming, fungi-related, intracellular parasites that infect many vertebrates and invertebrates. Several species of microsporidia have been described as biocontrol agents and pathogens of several beneficial insects (Bjornson and Oi, 2014). For example, *Nosema pyrausta* is effective in controlling European corn borer (*Ostrinia nubilalis*) (Lewis et al., 2009). A formulation of *Paranosema locustae* is commercially available for biological control of rangeland grasshoppers (Bjornson and Oi, 2014). Several studies reported that *Microsporidia* are natural intracellular parasites of the nematodes including *Caenorhabditis* (Kaya et al., 1988; Troemel et al., 2008; Zhang et al., 2016). The *Microsporidia*, *Nematocida parisii* was reported as a natural intracellular

pathogen of *Caenorhabditis elegans* (Troemel et al., 2008). Zhang et al. (2016) described six new species in the *Nematocida* genus that are capable of infecting *Caenorhabditis elegans*.

In general, fungal species diversity was higher with the pyrosequencing than the culture-based method. These dissimilarities are to be expected and are not surprising since many of the fungi are not cultivable. Some studies showed that approximately 1% of the total microbes could be detected by culture-based methods (Sugiyama et al., 2010). On the contrary, some fungi can easily be cultured even they are present in small quantities. In addition, the high temperature in the Omani desert, which sometimes exceeds 50°C in summer, could have affected fungal diversity in soil by killing or suppressing several fungal species that are heat sensitive (Abed et al., 2013; Classen et al., 2015; Costa et al., 2015). This in turn could have affected the number of fungal species recovered from soil by culture-dependent methods. Also the absence of *Chytridiomycota* and *Basidiomycota* in culture-based method could have been because they either need specific media or their presence was limited as evidenced by pyrosequencing analysis (only 3.51% and less than 0.77% of the total soil population, respectively) (Gleason et al., 2007; Yee et al., 2016). Therefore, the detection of the precise diversity of fungi in a habitat using culture-based techniques is still challengeable. Pyrosequencing recovered more species that were not revealed by the culture-based method, implying that this approach will speed up the detection of very rare fungal species (Huang et al., 2015; Liu et al., 2017). However, pyrosequencing still has some limitations in describing fungal diversity. Future studies should investigate if using other media in addition to PDA and also other genes for pyrosequencing could help recover more fungal species and reduce the existing gap between pyrosequencing and culture based techniques.

CONCLUSION

This study provided evidence that farming systems strongly influence the composition of soil fungal communities. It is surprising to note that a few soil fungi that were detected by direct culturing method could not be detected by pyrosequencing. More research is required by using different soil DNA extraction procedures. Culturing fungi by using multiple nutrient media might result in the isolation of additional fungi from soil. Viability of fungal communities in soil needs to be considered when assessing their diversity in a farming system. One of the major drawbacks in PCR-based methods is their inability to discriminate between nucleic acids from viable and dead cells. The DNA extracted from dead cells can also serve as a template in PCR amplification. To overcome such issues, viability PCR using propidium monoazide (PMA) that differentiate nucleic acids from live and dead cells (Cangelosi and Meschke, 2014) has to be tested.

AUTHOR CONTRIBUTIONS

AA-S, EK, SM, and HA-M planned the experiment. EK and HA-M conducted the experiment, AA-S, EK, SM, and VR analyzed data, EK, AA-S, SM, VR, and HA-M wrote the manuscript. All authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01462/full#supplementary-material

FIGURE S1 | Phylogenetic analysis of fungi recovered from direct plating based on ITS data set. The tree is rooted with *Allomyces reticulatus* (*Blastocladiomycota*). RAxML bootstrap values higher than 50% are given above or below the nodes. The isolates from present study are in bold.

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REVIEW
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Engineering Mycorrhizal Symbioses to Alter Plant Metabolism and Improve Crop Health

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Creating sustainable bioeconomies for the 21st century relies on optimizing the use of biological resources to improve agricultural productivity and create new products. Arbuscular mycorrhizae (phylum Glomeromycota) form symbiotic relationships with over 80% of vascular plants. In return for carbon, these fungi improve plant health and tolerance to environmental stress. This symbiosis is over 400 million years old and there are currently over 200 known arbuscular mycorrhizae, with dozens of new species described annually. Metagenomic sequencing of native soil communities, from species-rich meadows to mangroves, suggests biologically diverse habitats support a variety of mycorrhizal species with potential agricultural, medical, and biotechnological applications. This review looks at the effect of mycorrhizae on plant metabolism and how we can harness this symbiosis to improve crop health. I will first describe the mechanisms that underlie this symbiosis and what physiological, metabolic, and environmental factors trigger these plant-fungal relationships. These include mycorrhizal manipulation of host genetic expression, host mitochondrial and plastid proliferation, and increased production of terpenoids and jasmonic acid by the host plant. I will then discuss the effects of mycorrhizae on plant root and foliar secondary metabolism. I subsequently outline how mycorrhizae induce three key benefits in crops: defense against pathogen and herbivore attack, drought resistance, and heavy metal tolerance. I conclude with an overview of current efforts to harness mycorrhizal diversity to improve crop health through customized inoculum. I argue future research should embrace synthetic biology to create mycorrhizal chasses with improved symbiotic abilities and potentially novel functions to improve plant health. As the effects of climate change and anthropogenic disturbance increase, the global diversity of arbuscular mycorrhizal fungi should be monitored and protected to ensure this important agricultural and biotechnological resource for the future.

Keywords: fungal diversity, endosymbiosis, agriculture, synthetic biology, microbial-plant communication, bioprotectants

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INTRODUCTION

Mutualistic symbiosis is the reciprocally beneficial relationship between two organisms and can have a profound effect on organism fitness, ecology, and evolution (Wade, 2007; Gilbert et al., 2015; Kiers and West, 2015). Symbioses abound across all forms of marine and terrestrial life, from whales (Cetacea) and barnacles (*Coronula diadema*) to fig wasps (*Courtella wardi*) and fig trees (*Ficus carica*) (Jousselin et al., 2003; Nogata and Matsumura, 2006). Some symbioses can

also form among three partners, as seen in the exchange of nutrients among three-toed sloths (*Bradypus* spp.), pyralid moths (*Cryptoses* spp.), and algae (*Trichophilus* spp.) (Pauli et al., 2014). However, some of the most intriguing forms of symbioses happen in the microbial world (Keeling and Palmer, 2008). Bacteria and fungi are capable of invading host organisms, altering genetic and metabolic processes along the way (McFall-Ngai et al., 2013). To date, bacterial symbioses have received the most attention due to their complex relationships across many species across all trophic levels, from algae to plants and humans (McFall-Ngai and Ruby, 1991). Fungal symbioses have received less attention despite their key role in terrestrial nutrient exchange and recycling systems (Gadd, 2006; Emery et al., 2015). For example, some fungi (*Neocallimastix* spp.) colonize the guts of cows, helping them digest plant matter (Davies et al., 1993). Other species (ascomycete and basidiomycetes) form symbiotic relationships with cyanobacteria to form lichen, a composite organism (holobiont) that degrades organic and inorganic materials and serve as an important food-source for herbivores (Spribille et al., 2016).

Microbial symbioses have important ecological roles and can also be manipulated to increase the sustainability and resilience of global agricultural systems. To date, the agricultural use of naturally occurring symbioses has focused on promoting symbiosis between nitrogen-fixing bacteria (rhizobia) and legume plants to decrease inorganic nitrogen application on arable fields, which can lead to eutrophication and decline of soil microbial diversity over time (Matson et al., 1997). Arbuscular mycorrhizal fungi (AMF) form an equally important symbiosis with plants that is currently underexploited. This symbiosis is over 400 million years old and predates the symbiotic relationship between nitrogen-fixing bacteria and legumes. In the phylum Glomeromycota there are currently 200 known species from 10 families and many more are likely to be discovered in terrestrial landscapes rich in plant diversity and/or in extreme environments (Bever et al., 2001; Öpik et al., 2010; Ohsowski et al., 2012). In exchange for carbon, mycorrhizae provide plants with Phosphorus (P), minerals, and other nutrients to over 80% of vascular plants (Parniske, 2008). Major crops such as wheat and maize form symbioses with mycorrhizal fungi. The only crops that do not form this symbiosis are from the Brassicaceae and Papaveraceae families (Fester and Sawers, 2011). A growing body of greenhouse and field-scale experiments has shown the positive effect of mycorrhizal inoculation on crop productivity and resilience to environmental stress. In this review, I will provide an overview of our current knowledge of how mycorrhizal symbiosis effects plant metabolism and crop health and provide new insight into how synthetic biology could revolutionize how we harness this symbiosis in the future.

MYCORRHIZAL INFECTION AND HOST METABOLIC RESPONSE

Arbuscular mycorrhizal symbiosis dramatically alters plant primary and secondary metabolism in affected roots (**Figure 1**). Upon infection, cells with arbuscules gradually emit enzymes that degrade or stop the suppression of plant cell wall materials (e.g., lignin) and suppress salicylic acid production (which decreases AMF symbiosis) (Smith and Gianinazzi-Pearson, 1988). A shared, permeable membrane is created between the arbuscule and host plant which allows for the exchange of nutrients. This membrane is comprised of three layers: the plant-derived periarbuscular membrane (PAM), the periarbuscular space (PAS) composed of plant and fungal-derived elements, and the fungal plasma membrane (Parniske, 2008). It contains enzymes capable of generating energy gradients for active, bi-directional transport of nutrients and compounds (Smith and Gianinazzi-Pearson, 1988). Infection causes specific physiological changes in host cells. The number of mitochondria increase threefold and migrate toward the arbuscule, the nucleus increases in size, and nuclear chromatin decondenses (allowing for increased transcriptional activity) (Gianinazzi-Pearson, 1996; Lohse et al., 2005). Plastids also increase in number and stromules become more abundant; they can move toward arbuscules, forming a net-like structure over the fungus (Buee et al., 2000; Lohse et al., 2005).

These physiological changes trigger metabolic changes in root cortex cells. Increased numbers of mitochondria and plastids lead to increased energy production (from the TCA cycle) and production of plastid metabolites (fatty acids, amino acids, carotenoids, and terpenoids) respectively (Lohse et al., 2005; Jung et al., 2012). In the cytosol, sugar levels increase due to increased photosynthesis in the above-ground leaves, which favors high efflux rates between the arbuscule and host cell (Smith and Gianinazzi-Pearson, 1988; Berger et al., 2007; Gaude et al., 2015). Levels of jasmonic acid (derived from linoleic acid produced in the plastids) also increase and trigger the production of phytoalexins (defensive compounds). Most of these defensive compounds are nitrogen-rich alkaloids produced by plastids. As these metabolic changes occur, phosphorus is transferred from the mycorrhiza to the host cell in exchange for fatty acids, amino acids, and sugars (fructose and glucose) (Smith and Gianinazzi-Pearson, 1988). The production of anti-fungal compounds (e.g., gallic acid) by the host plant decreases (Gaude et al., 2015).

Foliar secondary metabolism also changes dramatically. Defensive compounds in foliar tissues increase. These compounds include rutin, *p*-hydroxybenzoic acid, antioxidants (flavonoids), and terpenoids (Copetta et al., 2006; Toussaint, 2007; Geneva et al., 2010; Zubek et al., 2015; Kapoor et al., 2016). Earlier studies suggested plant secondary metabolism increased due to increased access to nutrients (specifically, P) provided by the mycorrhizal-endosymbiont (Gupta et al., 2002; Smith et al., 2003). However, several recent experimental studies suggest that increased production of specific secondary compounds does not correlate with increased plant P content (Copetta et al., 2006; Toussaint, 2007). Instead, hormonal changes induced by AMF infection may trigger these metabolic changes. In addition, changes in root chemistry may also lead to the storage of these compounds in foliar tissues. For example, the increased production of glandular trichomes in *Ocimum basilicum* L. when inoculated by *Gigaspora rosea* as noted by Copetta et al. (2006) could be due to increased production of chloroplasts in infected roots. Potentially, sequestering additional volatiles in leaves

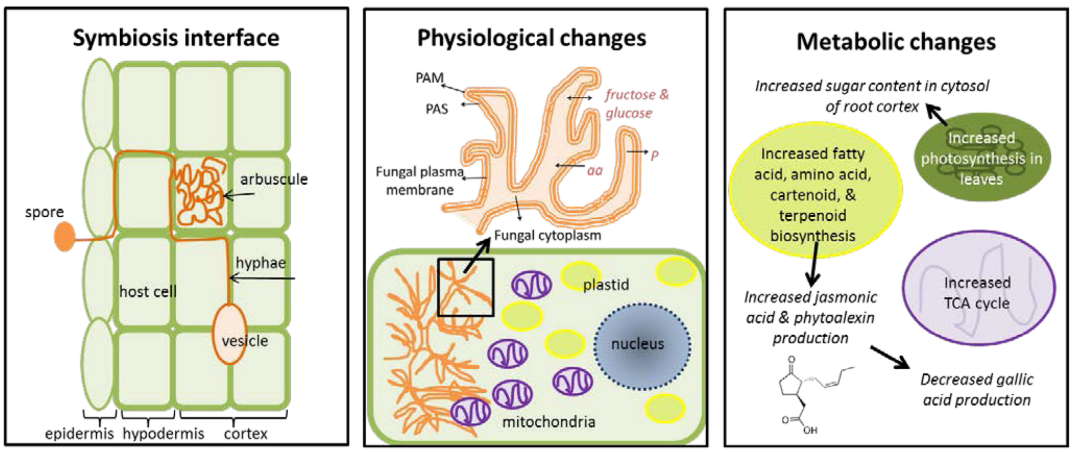

FIGURE 1 | Overview of physiological and metabolic changes induced by mycorrhizal symbiosis with host plants. PAM, plant-derived periarbuscular membrane; PAS, the periarbuscular space; aa, amino acids; P, phosphorus.

protects the mycorrhizae living within the hosts' roots from these bioactive compounds. Additionally, plants may circulate these compounds to where they are most needed.

Arbuscular mycorrhizal fungi symbiosis causes both global (species-independent) and local (species-specific) changes in plant metabolism. Global changes include increased production of amino acids (glutamic, aspartic, and asparagine acid); fatty acids (palmitic and oleic); secondary metabolites (phenyl alcohols, a linolenic acid, apocarotenoids, isoflavonoids); plant hormones (oxylipin, cytokinins, and jasmonic acid); activation of the oxylipin pathway; and increased sugar metabolism (Fernández et al., 2014; Gaude et al., 2015; Rivero et al., 2015). In contrast, levels of specific secondary compounds increase according to plant species identity. For example, Schweiger et al. (2014) found that inoculating *Plantago lanceolata*, *P. major*, *Veronica chamaedrys*, *Medicago truncatula*, and *Poa annua* with *Rhizophagus irregularis* caused 18–45% of each species core metabolomes and increased species-specific compounds (e.g., sorbitol in *P. lanceolata*). Different mycorrhizal fungi can also produce different metabolic effects. For example, repeated pot experiments have shown that *Funneliformis mosseae* causes more metabolic changes than *R. irregularis* (Rivero et al., 2015).

IMPACT ON CROP HEALTH

Arbuscular mycorrhizal fungi symbiosis can boost plant defenses against pathogens. Previous studies have reported mycorrhizal-induced protection against fungal (*Alternaria, Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, and *Verticillium)*, bacterial (*Ralstonia solanacearum* and *Pseudomonas syringae*), nematode (*Pratylenchus* and *Meloidogyne*), and insect (*Otiorhynchus sulcatus*) damage (Garcia-Garrido and Ocampo, 1989; de la Peña et al., 2006; Fritz et al., 2006; Pozo and Azcón-Aguilar, 2007; Jung et al., 2012). A recent meta-analysis by Veresoglou and Rillig (2012) suggest inoculation of crops with mycorrhiza reduces fungal infections by 30–42% and nematode infestations by 44–57%. This protection results from passive and active activation of plant secondary metabolism by AMF. Passively, AMF infection causes host plants to produce and store highly potent defensive compounds (alkaloids and terpenoids). These are stored in trichomes and vacuoles and can be released at will (Wink, 1993; Champagne and Boutry, 2016). More actively, external and internal fungal hyphae may sense pathogen effectors and other secondary compounds in the surrounding environment (soil and apoplast, respectively) and 'warn' host cells by producing lipo-chitooligosaccharides (LCOs) and short chito-oligosaccharides (Cos) (Kosuta et al., 2003; Maillet et al., 2011; Bonfante and Genre, 2015; Zipfel and Oldroyd, 2017). These messages may transmit through the host plant from cell to cell through the plasmodesmata.

Drought tolerance also increases under inoculation with AMF. This may be due to increased production and accumulation of the sugar trehalose in affected plant cells. Trehalose forms a gel-like substance that attaches to cellular compartments and stabilizes lipid bilayers (Müller et al., 1995; Richards et al., 2002; Lunn et al., 2014). During desiccation organelles remain intact and can spring back to life under favorable environmental conditions (Wingler, 2002). For example, Adams et al. (1990) showed that *Selaginella lepidophylla*'s ability to withstand long-term desiccation was due to high levels of trehalose which formed 12.5% of plant body mass. As many high plants do not produce trehalose, this sugar may potentially be supplied by fungal endosymbionts. Trehalose has been detected in the roots of trees and vascular plants inoculated with ectomycorrhizal and arbuscular mycorrhiza (Müller et al., 1995). Increased trehalose production alters plant carbohydrate metabolism by decreasing sugar and starch levels (Wagner et al., 1986). Providing this benefit to host plants thus comes at a cost to mycorrhizae and emphasizes the mutualism of this symbiosis.

Arbuscular mycorrhizal fungi can also increase tolerance to heavy metals in crops. The reasons for this are still unclear. Potentially, the chitin and melanin found in the cell walls of

AMF hyphae may bind metals in the surrounding soil and/or in the host plant (Morley and Gadd, 1995; Ruscitti et al., 2011; Eisenman and Casadevall, 2012). Melanin in particular is well-known for its ability to protect fungi from a variety of harsh environmental conditions, including nuclear radiation (Zhdanova et al., 2000). Non-mycorrhizal fungi (primarily *Aspergillus*, *Phanerochaete chrys,* and *Trichoderma*) have been shown to absorb and incorporate into their cell walls up to 90% of metal ions from soil contaminated with cadmium (Mohsenzadeh and Shahrokhi, 2014), silver (Aksu, 2001), uranium (Wang and Zhou, 2005), and lead (Jianlong et al., 2001). Fungi also use chelating proteins (e.g., phytochelatins and metallothioneins) and metabolites (e.g., oxalate) to deactivate the toxicity of metals (Tomsett, 1993; Sayer and Gadd, 1997). This absorption often triggers changes in fungal metabolism and can cause fungi to change color (e.g., orange and black) as new compounds are produced (Baldrian, 2003). In ectomycorrhiza, Blaudez et al. (2000) have shown that metal ions are deposited throughout the cell wall (50%), cytoplasm (30%), and vacuole (20%). This highlights that multiple mechanisms may be used by mycorrhiza to immobilize metal toxins. The hyphae of ectomycorrhizae exposed to heavy metals also seem to proliferate (Darlington and Rauser, 1988) suggesting they may confer some fitness advantage to fungi. Mycorrhizae may also alter host metabolism to respond to metal toxicity. Shabani et al. (2016) have shown that inoculation of *Festuca arundinacea* with *Funneliformis mosseae* increased the transcription of host metallothioneins and ABC transporters (which aid in the excretion of toxins) in nickel-contaminated soil.

INOCULUM, SYNTHETIC BIOLOGY AND THE DEVELOPMENT OF FUNGAL CHASSES

Over the past 20 years most attempts to harness AMF diversity to improve crop health have focused on increasing fungal diversity by encouraging plant species diversity (in native habitats like grasslands). In addition, a number of initiatives have sought to create AMF inoculum optimized to increase plant growth. This inoculum often comprises of soil taken from (presumably, fungal-rich) habitats and transferred to arable fields. Although the species-composition of native mycorrhizal communities may vary globally depending on local vegetation, elevation, climate, and soil chemistry, most AMF have low host-specificity (Lee et al., 2013; Veresoglou and Rillig, 2014; Peay et al., 2016). Since AMF readily form symbiosis with multiple plant species, mycorrhizae isolated from one location have the potentially to successfully colonize plants at other sites. Stahl et al.'s (1988) research established that native strains of AMF from non-disturbed sagebrush grasslands increased the biomass and tissue phosphorus content of vegetation planted on reclaimed coal pits in Wyoming. Native soil inocula rich in mycorrhizae have increased the productivity and vegetation cover of American prairies (Richter and Stutz, 2002), Belgian species-rich grasslands (Torrez et al., 2016), and land reclaimed from Mercury mining in California (Emam, 2016). Some mycorrhizae have more beneficial traits than others and in the future inoculum containing these species could be developed. For example, three AMF with hyphae 10x longer than usual mycorrhiza are *Acaulospora laevis* (10.55 cm), *Glomus calospora* (12.3 cm), and *Glomus tenue* (14.2 cm) (Smith and Gianinazzi-Pearson, 1988). Longer hyphae could increase fungal phosphorus uptake and transfer to plant hosts, increasing the production of beneficial phytoalexins and plant growth. In addition, metagenomic research on the microbial communities of biodiverse and/or extreme environments suggests there are many other AMF species which could be exploited further. For example, three new species of AMF (*Diversispora omaniana*, *Septoglomus nakheelum,* and *Rhizophagus arabicus* spp. nov.) were identified in 2014 from environmental samples in the Arabian desert and could be propagated as inoculum for crops grown in arid regions in other parts of the world (Symanczik et al., 2014).

Synthetic biology could draw upon arbuscular mycorrhizal diversity to increase the effect of AMF on plant health (**Figure 2**). Synthetic biology can increase the expression of native host genes by altering transcription rates or by inserting new genes from foreign organisms (Khalil and Collins, 2010). In addition, specific traits could be selected for and expressed in modified fungal chasses. To date, synthetic biology initiatives have focused on using filamentous fungi to produce high value compounds (anti-tumor and antibiotics mainly) (Mattern et al., 2015; Xiao and Zhong, 2016). Currently, the biosynthetic pathways of 197 compounds linked to 779 nucleotide records from 174 fugal species are known and can be accessed via a public database[1] (Li et al., 2016). The majority of these genes (98%) come from the Ascomycota family and there is a strong bias toward *Aspergillus*.

Currently, there is little to no research on the use of mycorrhizae in synthetic biology, either to improve crop health or to perform advanced biological functions (e.g., mycoremediation). To initiate this process *R. irregularis* (formerly *Glomus intraradices*) could serve as an initial chassis because it is the only mycorrhizae with a fully sequenced genome (Franz Lang and Hijri, 2009; Tisserant et al., 2013). This extensive knowledge of this mycorrhizal host genome could overcome the challenges bioengineering fungi face including (1) the location of biosynthetic gene clusters (BGCs) on multiple loci and (2) the control of BGCs by shared *cis*-regulatory elements (van der Lee and Medema, 2016). Promoters and other regulators of gene expression (e.g., transcription factors) currently developed for use in *Aspergillus niger* and *Penicillium chrysogenum* could be trialed out in these mycorrhizae (Polli et al., 2016; Wanka et al., 2016). Potential genetic targets in *R. irregularis* for manipulation are listed in **Table 1**.

Potential applications of synthetically modified mycorrhizae include increased phosphorus uptake, increased production of economically valuable terpenoids (e.g., antibiotic monoterpenes such as carvacrol) in host plants, and potentially even nitrogen-fixation. The latter could be achieved by modifying AMF metabolism or by engineering N-fixing bacteria to engage in

[1] http://mibig.secondarymetabolites.org/

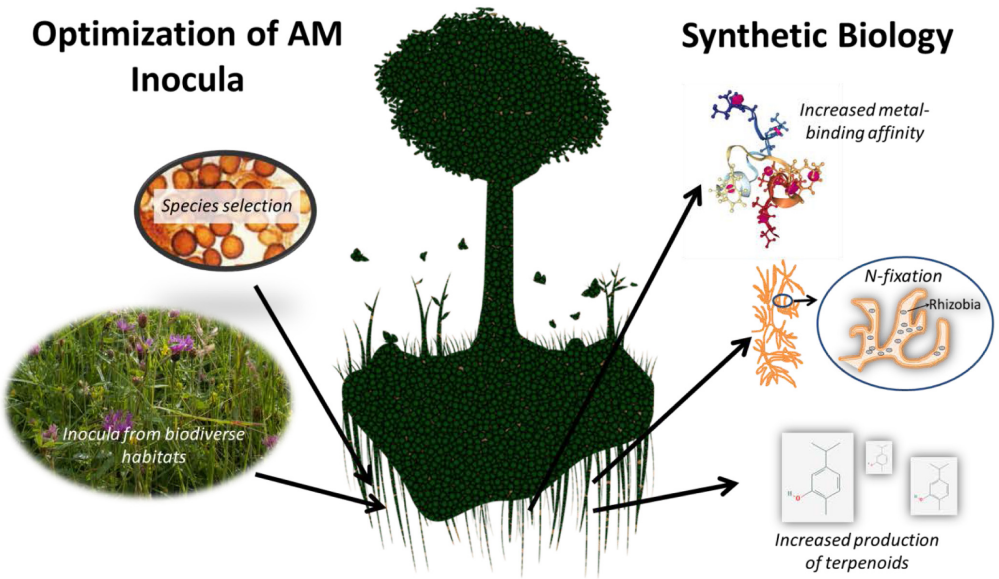

FIGURE 2 | Mycorrhizal symbiosis can be harnessed for agriculture by optimizing soil inocula and through synthetic biology. Image credits (left to right): spores of *Glomus* spp. (IVAM); tree and root image (modified) (CC0 Public Domain); metallothionein from sea urchin (*Strongylocentrotus purpuratus*) (PDB ID 1QJK; Riek et al., 1999); carvacrol (PubChem). All other images belong to the author.

TABLE 1 | Genetic targets in *Rhizophagus irregularis*.

Potential Use	UniProt Entry	Protein name	Gene name	Length	Mass (Da)
Drought tolerance	A4QMP6	Trehalase (EC 3.2.1.28) (Alpha-trehalose glucohydrolase)	NTH1	740	86,029
Drought tolerance	A4QMP8	Trehalose-6-phosphatase (Fragment)	TPS2	179	19,765
Heavy metal tolerance	B0AZW1	Metallothionein 1	ntMT1	71	7,202
Nitrogen uptake	D7P896	Nitrate transporter (Fragment)		329	35,823
Nutrient exchange	C8YXI2	Aquaporin 1	AQP1	253	27,190
Phosphorus uptake	G0Z6L2	Phosphate transporter (Fragment)	PT	81	8,573
Phosphorus uptake	Q8X1F6	Phosphate transporter		521	58,478
Plant defense	Q9C0Q8	Chitin synthase (EC 2.4.1.16) (Fragment)	CHS	205	23,115
Symbiosis	B5U322	Germinating spore putative ATP-sulfurylase (Fragment)		82	9,191
Symbiosis	C7EXJ7	Elongation factor 1-alpha (Fragment)	EF1-alpha	255	27,925
Symbiosis	Q9UV76	MYC2 (Fragment)	myc2	286	33,156
Symbiosis	Q2V9G7	Elongation factor 1-alpha (Fragment)	EF1-alpha	306	33,228
Symbiosis	Q659Q9	Elongation factor 1-alpha (Fragment)	tef1a	110	12,346
Symbiosis	Q9UV77	MYC1 (Fragment)	myc1	370	41,872

The table lists the potential agricultural use of the gene, the UniProt entry, gene name, and protein name, length, and mass.

symbiosis with specific AMF strains (Manchanda and Garg, 2007). *Burkholderia* spp. engage with *Gigaspora* and *Scutellospora* with as many as 250,000 bacteria per spore (Bianciotto et al., 2000; Artursson et al., 2006), indicating that mycorrhiza have a natural capacity to engage in symbiosis with bacteria. In addition, introducing metallothioneins from other fungi, bacteria, or even higher eukaryotes such as sea urchins into mycorrhiza could improve their ability to protect host plants from metal-contaminated soils. As mycorrhizae do not sexually reproduce, there is little chance these genetic changes would enter into native mycorrhizal gene pools (Pawlowska, 2005). These steps could usher in a revolution in the use of mycorrhiza in synthetic biology.

CONCLUSION

Arbuscular mycorrhizal symbioses with plants hold immense promise for the development of more sustainable agricultural systems (Gosling et al., 2006; Garg and Chandel, 2010). Fungi are already extensively used in biotechnology to produce antibiotics, anti-cancer drugs, pigments, bioethanol, and biomaterials (Bennett, 1998; Adrio and Demain, 2003; Cragg et al., 2015; Haneef et al., 2017). To date, arbuscular mycorrhizae have received less attention, despite their dramatic effect on plant metabolism and host resilience to environmental stresses. This fusion of plant and fungal endophyte increases the production of specific plant secondary metabolism products (e.g., fatty

acids and terpenoids) and redirects the products of plant primary metabolism (e.g., fructose and glucose) to the fungal partner. This symbiosis also appears to increase the tolerance of crops to pathogen, water, and heavy metal stresses through a variety of mechanisms. Advances in metagenomic sequencing will allow us to promote native AMF diversity while boosting crop fitness (Lumini et al., 2011). The tools and techniques provided by synthetic biology may also lead to new innovations in how these symbioses function and the benefits provided to host plants. Future research should focus on identifying key mycorrhizal genes that affect plant growth and begin experimenting with genetic modification of potential chasses AMF, specifically *R. irregularis*. Rising climate change and anthropogenic disturbance of native ecosystems may harm the diversity and functioning of AMF across the world (Fitter et al., 2000; Antoninka et al., 2009; Classen et al., 2015; French et al., 2017). Conservation efforts must now extend below the soil if we are to ensure the preservation of this resource for the future (Bodelier, 2011).

AUTHOR CONTRIBUTIONS

KF conceived and wrote the manuscript.

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Fungal Communities in Rhizosphere Soil under Conservation Tillage Shift in Response to Plant Growth

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Conservation tillage is an extensively used agricultural practice in northern China that alters soil texture and nutrient conditions, causing changes in the soil microbial community. However, how conservation tillage affects rhizosphere and bulk soil fungal communities during plant growth remains unclear. The present study investigated the effect of long-term (6 years) conservation (chisel plow, zero) and conventional (plow) tillage during wheat growth on the rhizosphere fungal community, using high-throughput sequencing of the internal transcribed spacer (ITS) gene and quantitative PCR. During tillering, fungal alpha diversity in both rhizosphere and bulk soil were significantly higher under zero tillage compared to other methods. Although tillage had no significant effect during the flowering stage, fungal alpha diversity at this stage was significantly different between rhizosphere and bulk soils, with bulk soil presenting the highest diversity. This was also reflected in the phylogenetic structure of the communities, as rhizosphere soil communities underwent a greater shift from tillering to flowering compared to bulk soil communities. In general, less variation in community structure was observed under zero tillage compared to plow and chisel plow treatments. Changes in the relative abundance of the fungal orders Capnodiales, Pleosporales, and Xylariales contributed the highest to the dissimilarities observed. Structural equation models revealed that the soil fungal communities under the three tillage regimes were likely influenced by the changes in soil properties associated with plant growth. This study suggested that: (1) differences in nutrient resources between rhizosphere and bulk soils can select for different types of fungi thereby increasing community variation during plant growth; (2) tillage can alter fungal communities' variability, with zero tillage promoting more stable communities. This work suggests that long-term changes in tillage regimes may result in unique soil fungal ecology, which might influence other aspects of soil functioning (e.g., decomposition).

Keywords: conservation tillage, rhizosphere soil, fungal community, plant growth, structural equation model

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INTRODUCTION

The rhizosphere, which is the volume of soil surrounding the plant root, is influenced by root activities such as exudation of reactive carbon compounds and uptake of mobile nutrients and water (George et al., 2006; Hartmann et al., 2008). Roots have evolved to adapt to their surrounding environment by optimizing their functional architecture to use resources in heterogeneous

soils (Hinsinger et al., 2005; Pierret et al., 2007). Thus, the co-evolution of rhizosphere and plant roots play a major role in soil physical, chemical, and biological processes that sustain biodiversity, provide soil carbon sequestration, and cycle nutrients in natural and agricultural systems (Hinsinger et al., 2009; Lambers et al., 2009). The plant-rhizosphere system also affects the biomass and activity of soil microorganisms that is generally enhanced due to root exudates (Raaijmakers et al., 2009). Different soil types harbor particular indigenous microorganisms that control the influence of plant root activity on rhizosphere microbial communities (Singh et al., 2007; Berg and Smalla, 2009). Plant species releasing root exudates are thought to select for rhizosphere microbial populations that respond with chemotaxis and fast growth (Hartmann et al., 2009). Plant growth also affects the composition of the rhizosphere microbial community, as root exudates change during the plant's life cycle and seasonal environment responses (Baetz and Martinoia, 2014). However, most studies have focused on the rhizosphere bacterial communities, and only a few have focused on fungal communities in the plant rhizosphere.

Soil fungal communities play essential roles in biogeochemical cycles, organic matter decomposition, plant growth, and disease development and control (Raaijmakers et al., 2009). Rhizosphere fungi are closely linked to plant health and growth, owing to their roles in antagonizing pathogens, decomposing plant residues, and providing nutrients (Ehrmann and Ritz, 2014). Variation in the fungal community of the rhizosphere is suggested to be plant-dependent because roots release several organic compounds that contribute to a unique rhizosphere nutrient pool, which is accessible to soil microorganisms (Klaubauf et al., 2010; Jiang et al., 2012; Han et al., 2016). Soil physical and chemical properties are known to be significantly correlated with changes in the rhizosphere fungal community (Schappe et al., 2017). Soil texture highly affects the organic carbon content and consequently determines plant rhizosphere microbial communities (Singh et al., 2007; Wang et al., 2009). Soil enzyme activity, particularly the hydrolysis of immobilized complex biomolecules as a strategy for nutrient acquisition, is strongly associated with rhizosphere fungal communities (Welc et al., 2014). Thus, improving the knowledge on rhizosphere fungal communities is expected to result in a better understanding of their roles in soil ecosystems.

Conservation tillage is extensively used in the dryland regions of northern China, which account for ∼56% of the nation's total land areas. In these regions, crop production is constrained by multiple factors, including adverse weather and topography, limited water resources, and exhaustion of available soil water and nutrients due to conventional agricultural practices (Wang et al., 2007). Conservation tillage can improve the soil structure, conserve soil water, increase soil nutrient levels, substantially increase the efficiency of water-use for crops, and increase crop yields (Zhang et al., 2012). Our previous study indicated that conservation tillage affects crop residue decomposition, thereby altering soil organic carbon (SOC) content and leading to changes in the distribution patterns of the soil fungal community (Wang et al., 2016a). Using indirect techniques, a few studies have shown that conservation tillage modifies the rhizosphere environment, changing substrate utilization and arbuscular mycorrhizal (AM) fungal communities (Lupwayi et al., 1998; Mirás-Avalos et al., 2011). However, these studies could not clearly and accurately delineate the differences between the rhizosphere fungal community of soils subject to conservation and conventional tillage. In particular, information on how conservation tillage influences the rhizosphere fungal community responses to winter wheat growth is quite limited.

The present study used quantitative PCR (qPCR) and Illumina MiSeq (Illumina, San Diego, CA, USA) deep sequencing to characterize fungal communities within the rhizosphere and bulk soil of a cropland with a wheat–maize rotation. To further understand the dynamics of fungal communities during plant growth, winter wheat root systems were sampled at two growth stages. We assumed that tillage influenced rhizosphere ecology, altering the rhizosphere fungal community as a consequence of changes in the surrounding soil environment in response to plant growth. The present investigation aimed to determine: (1) how rhizosphere and bulk soil fungal communities are affected by the growing roots of winter wheat; (2) if long-term tillage influences the patterns of rhizosphere and bulk soil fungal communities' response to plant growth.

MATERIALS AND METHODS

Study Site

This study was performed at Northwest A&F University, Yanglin, Shaanxi, China (34°17′N, 108°04′E), which is 521 m above sea level. The mean annual precipitation in this region is 633 mm, and the average annual temperature is 13.2°C. The experimental area was located in the Guanzhong Plain, which belongs to the drylands of northern China (Wang et al., 2016b), where a long-term trial began in 2009. Before this year, the experimental area was managed using rotary cultivation. Winter wheat (cv. Shaanmai 139) was sown over the residues of maize (cv. Shaandan 609) on October 18, 2014 using wheat drills. Urea fertilizer, with nitrogen (N) content >46%, and calcium phosphate fertilizer, with 16% phosphorous (P), were applied to all treatments (750 kg·ha^{-1}) at the time of soil preparation.

Tillage Treatment and Soil Sampling

Experimental treatments combined three tillage methods and residue retention in wheat–maize rotation croplands. The main characteristics of conservation (chisel plow and zero) and conventional (plow) tillage treatments are described in Wang et al. (2016a).

Sampling was conducted at the tillering (vegetative) stage (November 21, 2014, after 34 days of snow) and at the flowering (reproductive) stage (May 2, 2015, after 196 days of snow). Rhizosphere soil was sampled from randomly selected wheat plants showing similar characteristics; their roots were then vigorously shaken to remove the soil not tightly adhering to them (Smalla et al., 2001). Bulk soil samples were collected away from plant roots, at 0–20 cm depth, using a standard soil corer. All

samples were sieved through a 2-mm mesh to eliminate large rocks and roots. Each composite soil sample (rhizosphere or bulk soil) was homogenized and stored at 4°C for less than 24 h until DNA extraction.

Soil Physicochemical Analysis

The physical and chemical properties of the soil were analyzed in the laboratory. SOC, total N, and texture were measured as previously described (Zhao et al., 2014). Soil moisture was measured gravimetrically. Urease and invertase activities were assayed in soil samples (5 g), after adding an appropriate substrate and incubating the soil and substrate mixture for 24 h at 37°C and at the optimal pH for each enzyme, as described by Gu et al. (2009).

DNA Extraction, PCR Amplification, and Illumina Sequencing

Microbial DNA was extracted from fresh soil (3 replicates × 1 g) using the E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Inc., Norcross, GA, USA), according to the manufacturer's instructions. The concentration and quality of the extracted DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The fungal internal transcribed spacer-1 (ITS-1) region was amplified from each sample using primers ITS1F (5′-ACTTGGTCATTTAGAGGA AGTAA-3′) and ITS2 (5′-BGCTGCGTTCTTCATCGATGC-3′) (Mukherjee et al., 2014), which provide a comprehensive coverage with the highest taxonomical accuracy for fungal sequences (Mello et al., 2011). The reverse primer contained a 6-bp error-correcting barcode unique to each sample. The PCR protocol used to amplify ITS-1 has been described previously (Mukherjee et al., 2014), and sequencing was performed on an Illumina MiSeq PE300 instrument obtained from Majorbio BioPharm Technology Co., Ltd. (Shanghai, China).

The software FLASH was used to merge paired sequence reads generated from the original DNA fragments (Caporaso et al., 2010). The sequences were further analyzed using USEARCH v 5.2.32 to filter and denoise the data by clustering sequences with <3% dissimilarity. The QIIME pipeline was used to select ITS-1 operational taxonomic units (OTUs) by combining reads of clustered OTUs with 97% similarity (Edgar, 2010). The ITS-1 sequences obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession SRP080901.

Quantitative PCR

The relative abundances of the fungal 18S rDNA gene copies were measured by qPCR using the fungi-specific primer pair FR1 (5′-ANCCATTCAATCGGTANT-3′) and FF390 (5′-CGATAACGA ACGAGACCT-3′) (Chemidlin Prevost-Boure et al., 2011). The 20-μL qPCR mixture contained 10 μL EvaGreen 2 × qPCR MasterMix (Applied Biological Materials Inc., Richmond, BC, Canada), 0.3 μM each primer (final concentration), and fungal, environmental, or standard DNA templates (10–20 ng μL^{-1} per reaction). The qPCR experiments were performed in a Bio-Rad C1000/CFX96 Thermocycler (Bio-Rad, Hercules, CA, USA) using an initial denaturation at 95°C for 10 min followed by 29 cycles at 95°C for 15 s, 50°C for 30 s, and 70°C for 60 s. The temperature was then increased from 65°C to 95°C at 0.5°C s^{-1} to perform a melting curve analysis of the PCR products. All qPCRs were run in triplicate for each DNA template. The abundances of fungal 18S rDNA genes were quantified using standard curves generated from 10-fold serial dilutions of cloned full-length copies of the 18S rDNA gene. Amplification efficiency ranged from 91 to 97%, and the R^2 values of the standard curves ranged from 0.996 to 0.999.

Statistical and Bioinformatics Analyses

Alpha diversity was estimated using the Shannon and the Simpson diversity indices. Estimation of the beta diversity and phylogenetic community comparisons were performed using weighted and unweighted UniFrac distance matrices. Taxonomic compositions were determined based on the relative abundances of dominant orders within Ascomycota, Basidiomycota, and Zygomycota. Correlations between the soil bacterial community structure and soil characteristics were determined using Mantel tests with 999 permutations.

Both the ANOVA and the Spearman's rank correlations between abundant phyla and soil properties were performed in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The principal coordinate analysis (PCoA) based on the Bray distance, the non-metric multidimensional scaling (NMDS) based on weighted and unweighted UniFrac distances, PERMANOVA, SIMPER, and Mantel tests were performed using the "vegan" package in the R v 3.20 statistical environment (Oksanen et al., 2013). All tests considered $P < 0.05$ as the significance threshold.

Structural Equation Model

Two path analyses were conducted to measure the direct and indirect effects of tillage, plant growth, and rhizosphere on soil fungal communities. In path analysis, a structural equation model (SEM) is designed to feature variables and to hypothesize causal relationships among these variables in a path diagram (Fanin and Bertrand, 2016). Here, we considered plant growth as the result of dry matter accumulation associated with yield–trait relationships of wheat in China (Meng et al., 2013). Considering that plant roots release a great variety of compounds that distinguish the soil surrounding the roots from bulk soils, we used the carbon (C) partitioned below the ground to represent the rhizosphere effects in the SEM (Jones et al., 2009). For tillage treatments, the fractal characterization of soil aggregation and fragmentation were thought to quantify the soil disturbance caused by tillage (Perfect and Blevins, 1997). The degree of soil disturbance distinguished the three tillage treatments (plow tillage > chisel plow tillage > zero tillage); differences in net fixed carbon distinguished rhizosphere from bulk soil (rhizosphere > bulk); dry matter accumulation weight was considered the winter wheat growth from the tillering to the flowering stage (flowering > tillering).

The adequacy of the model was assessed by χ^2 tests ($P > 0.05$) and by the calculation of the root mean square error of approximation (RMSEA) (values <0.05). These statistical tests were performed in R using the "lavaan" package.

RESULTS

Plant Growth Shaped Fungal Alpha Diversity and Abundance in Rhizosphere Soil

Shannon and Simpson indices were used as estimates of fungal alpha diversity. The ANOVA results showed that tillage treatments (For Shannon/Simpson indices: $F = 8.476/4.820$, $P = 0.005/0.029$) had a stronger effect on the bulk soil fungal alpha diversity than plant growth ($F = 1.070/0.802$, $P = 0.321/0.388$). In contrast, fungal alpha diversity in the rhizosphere soil was significantly influenced by both plant growth ($F = 7.395/8.707$, $P = 0.019/0.012$) and tillage ($F = 7.131/6.864$, $P = 0.009/0.010$). In addition, soil fungal alpha diversity was significantly influenced by tillage in the tillering stage and by rhizosphere or bulk soil in the flowering stage (Table S1). Different variation patterns were found among the three tillage treatments. Plow tillage showed a larger difference in fungal alpha diversity between rhizosphere and bulk soils than conservation (chisel plow and zero) tillage (**Table 1**). Fungal alpha diversity was significantly correlated with SOC, invertase activity, soil texture, and moisture in zero tillage, whereas in plow tillage changes in fungal alpha diversity were significantly correlated to soil invertase, urease, and soil texture (Table S1).

Soil fungal abundance was significantly different between rhizosphere and bulk soils in the tillering ($F = 9.246$, $P = 0.010$) and flowering ($F = 8.745$, $P = 0.012$) stages. In the rhizosphere soil, fungal abundance significantly increased in chisel plow tillage, as opposed to that registered in plow and zero tillage (**Table 1**). Spearman correlation showed that soil invertase ($R = 0.601$, $P = 0.001$) and urease ($R = 0.754$, $P = 0.009$) activities and total nitrogen (TN; $R = 0.636$, $P = 0.028$) were significantly related to fungal abundance in chisel plow tillage, but only soil invertase correlated with fungal abundance in zero tillage ($R = 0.648$, $P = 0.017$) and plow tillage ($R = 0.580$, $P = 0.048$).

Effect of Plant Growth on Rhizosphere Soil Fungal Beta Diversity

Overall, 1,564,488 quality sequences and a mean of 16,826 sequences per sample were obtained across all soil samples. The phylogenetic analysis of community membership and composition was performed using unweighted and weighted UniFrac distances, respectively, and the changes in the fungal phylogenetic structure according to the plant growth stage were evaluated by NMDS analyses (**Figures 1A,B**). Changes in the phylogenetic structure (membership and composition) of fungal communities in the tillering and flowering stages were generally much stronger in the rhizosphere (PERMANOVA: $R^2 = 0.147/0.304$, $P = 0.001/0.001$) than in bulk soil (PERMANOVA: $R^2 = 0.082/0.081$, $P = 0.049/0.038$). However, bulk soil fungal phylogenetic structure was significantly influenced by tillage (PERMANOVA: $R^2 = 0.238/0.328$, $P = 0.001/0.001$). Phylogenetic composition (PERMANOVA: $R^2 = 0.549$, $P = 0.001$; **Figure 1B**) had more clear clusters than phylogenetic membership (PERMANOVA: $R^2 = 0.309$, $P = 0.001$; **Figure 1A**). Rhizosphere and bulk soil fungal phylogenetic structure also varied according to plant growth among the three tillage treatments (**Figure 1**). Significantly lower variations between rhizosphere and bulk soil fungal phylogenetic membership and plant growth were found in zero tillage compared to plow and chisel plow tillage (**Figure 1A**). In addition, zero tillage led to lower variation in the fungal phylogenetic composition between tillering and flowering stages compared to the other tillage treatments (**Figure 1B**). Soil properties had significantly different relationships with fungal phylogenetic membership and composition among the three tillage treatments (**Table 2**). Soil physical structure (texture and moisture; Mantel test: $P < 0.05$) and invertase activity (Mantel test: $P < 0.05$) were the main factors determining the variation of fungal phylogenetic structure (membership and composition; **Table 2**). Additionally, SOC (Mantel test: $P < 0.05$) and total nitrogen (Mantel test:

TABLE 1 | Soil physicochemical properties, qPCR, and alpha-diversity ranks according to tillage and temporal-spatial treatments.

Tillage	Treatment	Soil texture	Soil moisture	TN	SOC	Urease	Invertase	qPCR	Alpha-diversity	
		(%)	(%)	(g/kg)	(g/kg)	(mg/g)	(mg/g)	(log_{10})	Shannon	Simpson
PT	TB	0.043 NS b	15.8 B a	0.760 NS ns	7.164 B ab	7.802 NS b	2.880 B d	6.708 A ns	4.017 B ab	0.049 A bc
	FB	0.013 B c	14.2 NS ab	0.785 NS ns	7.500 NS a	8.116 B b	5.307 C c	6.513 NS ns	4.260 NS a	0.037 NS c
	TR	0.087 NS a	15.6 B a	0.797 B ns	6.564 B ab	16.908 NS a	13.295 NS a	6.757 NS ns	3.677 B bc	0.068 A ab
	FR	0.095 B a	11.8 NS b	0.795 C ns	6.219 C b	11.633 AB b	11.069 B b	6.800 NS ns	3.483 NS b	0.083 NS a
CPT	TB	0.043 NS b	17.0 A a	0.809 NS c	9.293 A ab	8.225 NS b	3.743 A d	6.445 B c	3.903 B ns	0.058 A ab
	FB	0.037 A b	14.9 NS b	1.059 NS bc	8.345 NS b	9.420 AB b	8.796 A c	6.549 NS bc	4.153 NS ns	0.046 NS b
	TR	0.098 NS a	17.3 A a	1.345 A ab	8.327 A b	12.986 NS a	13.924 NS b	6.932 NS a	4.070 AB ns	0.054 AB ab
	FR	0.117 A a	11.4 NS c	1.483 A a	10.401 A a	9.333 B b	15.338 A a	6.702 NS ab	3.767 NS ns	0.068 NS a
ZT	TB	0.023 NS c	16.4 AB ab	1.037 NS bc	9.159 A ns	8.744 NS bc	3.456 A c	6.485 AB ns	4.550 A a	0.027 B b
	FB	0.020 B c	14.6 NS b	0.987 NS c	8.432 NS ns	11.522 A c	7.523 B b	6.517 NS ns	4.313 NS a	0.038 NS b
	TR	0.070 NS a	17.8 A a	1.256 A a	8.749 A ns	15.150 NS a	13.458 NS a	6.627 NS ns	4.247 A a	0.038 B b
	FR	0.054 C b	11.8 NS c	1.197 B ab	8.364 B ns	14.744 A ab	12.560 B a	6.613 NS ns	3.873 NS b	0.060 NS a

[a] *Values are mean of three soil samples. Soil texture, Sand/(Clay+Silt); SOC, soil organic carbon; TN, total nitrogen.*

[b] *PT, Plow tillage; ZT, Zero tillage; CPT, Chisel plough tillage; TB, tillering bulk soil; FB, flowering bulk soil; TR, tillering rhizosphere; FR, flowering rhizosphere.*

[c] *Different letters indicate significant differences (ANOVA, $P < 0.05$, Tukey's HSD post-hoc analysis) among tillage (capital letter) and temporal-spatial treatments (small letter).*

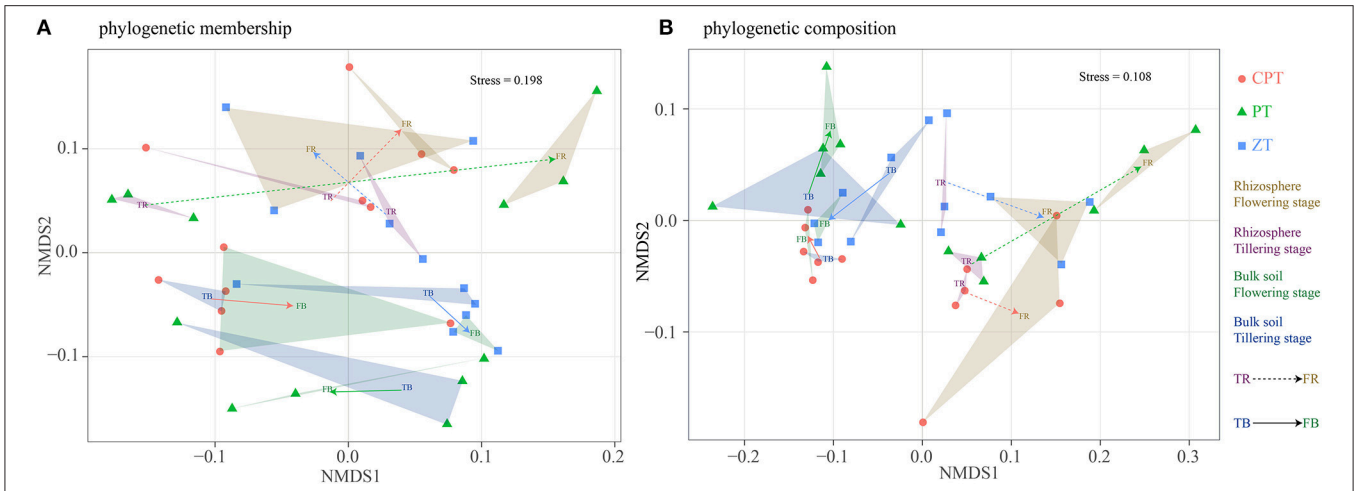

FIGURE 1 | Fungal community structure indicated by non-metric multi-dimensional scaling (NMDS) plots of weighted **(A)** and unweighted **(B)** pairwise UniFrac distances. PT = Plow tillage (*circle*); ZT = Zero tillage (*square*); CPT = Chisel plow tillage (*triangle*).

TABLE 2 | Mantel test results showing the significant correlation between soil properties and fungal beta diversity.

Beta diversity	Tillage[b]	Soil texture[a]	Soil moisture	TN	SOC	Urease	Invertase
Phylogenetic membership	PT	0.370*	0.503**	−0.069^{ns}	0.287*	0.136^{ns}	0.349*
	CPT	0.532**	0.365**	0.252*	0.107^{ns}	−0.039^{ns}	0.466**
	ZT	0.167^{ns}	0.444**	0.137^{ns}	0.023^{ns}	0.137^{ns}	0.226*
Phylogenetic composition	PT	0.572**	0.525**	−0.022^{ns}	0.356*	0.020^{ns}	0.455**
	CPT	0.769**	0.458**	0.400*	0.183^{ns}	0.131^{ns}	0.547**
	ZT	0.307*	0.228^{ns}	0.205^{ns}	−0.069^{ns}	−0.036^{ns}	0.294*
Taxonomic composition	PT	0.297*	0.696**	0.030^{ns}	0.299*	−0.118^{ns}	0.153^{ns}
	CPT	0.613**	0.673*	0.322*	0.359*	0.037^{ns}	0.420*
	ZT	0.052^{ns}	0.211^{ns}	0.005^{ns}	−0.050^{ns}	−0.132^{ns}	0.126^{ns}

[a] *Values are mean of three soil samples. Soil texture, Sand/(Clay+Silt); SOC, soil organic carbon; TN, total nitrogen.*
[b] *PT, Plow tillage; ZT, Zero tillage; CPT, Chisel plough tillage.*
*$0.01 < P < 0.05$; **$P < 0.01$; ns, no significant $P > 0.05$.*

$P < 0.05$) influenced fungal phylogenetic structure in chisel plow and plow tillage, respectively (**Table 2**).

Among all sequences, 82.2% were classified in bulk soil and 69.8% in rhizosphere soil. The dominant fungal phyla across all soil samples were Ascomycota (average 68.7%), Zygomycota (average 13.3%), and Basidiomycota (average 4.1%); at the order level, the fungal communities of all soil samples were dominated by Sordariales, Pleosporales, Hypocreales, Pezizales, Capnodiales, Xylariales, Microascales, Mortierellales, Mucorales, and Tremellales (**Figure 2A**). The PCoA based on the Bray distance conducted to assess the dynamics of rhizosphere and bulk soil fungal taxonomic composition over plant growth stages revealed that the composition of rhizosphere fungal communities differed significantly from bulk soil communities (PERMANOVA: $R^2 = 0.325$, $P = 0.001$) in both tillering and flowering stages (**Figure 2B**). Bulk soil samples were clearly clustered and significantly separated according to tillage treatments (PERMANOVA: $R^2 = 0.308$, $P = 0.001$), and plant growth stages rather than tillage treatment significantly influenced (PERMANOVA: $R^2 = 0.502$, $P = 0.001$ vs. $R^2 = 0.122$, $P = 0.041$; **Figure 2B**) fungal taxonomic composition in the rhizosphere soil. In addition, zero tillage samples were considerably closer to each other than chisel plow and plow tillage samples (**Figure 2B**). The relative abundance of fungal orders varied in the rhizome and bulk soil samples along plant growth, but showed different patterns among the three tillage treatments (**Figure 2A**, Table S2). Soil physical properties (texture/moisture) and invertase activity was significantly related to fungal taxonomic composition under plow tillage (**Table 2**); SOC and soil texture were the main factors distinguishing fungal communities under chisel plow and zero tillage (**Table 2**). Changes in the relative abundance of fungal orders (Capnodiales, Pleosporales, and Xylariales) contributed to the dissimilarities observed in the patterns of rhizosphere and bulk soil fungal taxonomic composition along plant growth (**Table 3**, Table S2).

Structural Equation Models

We hypothesized that changes in soil properties caused by tillage and rhizosphere would have a different effect on soil fungal diversity, abundance, and composition between plant growth

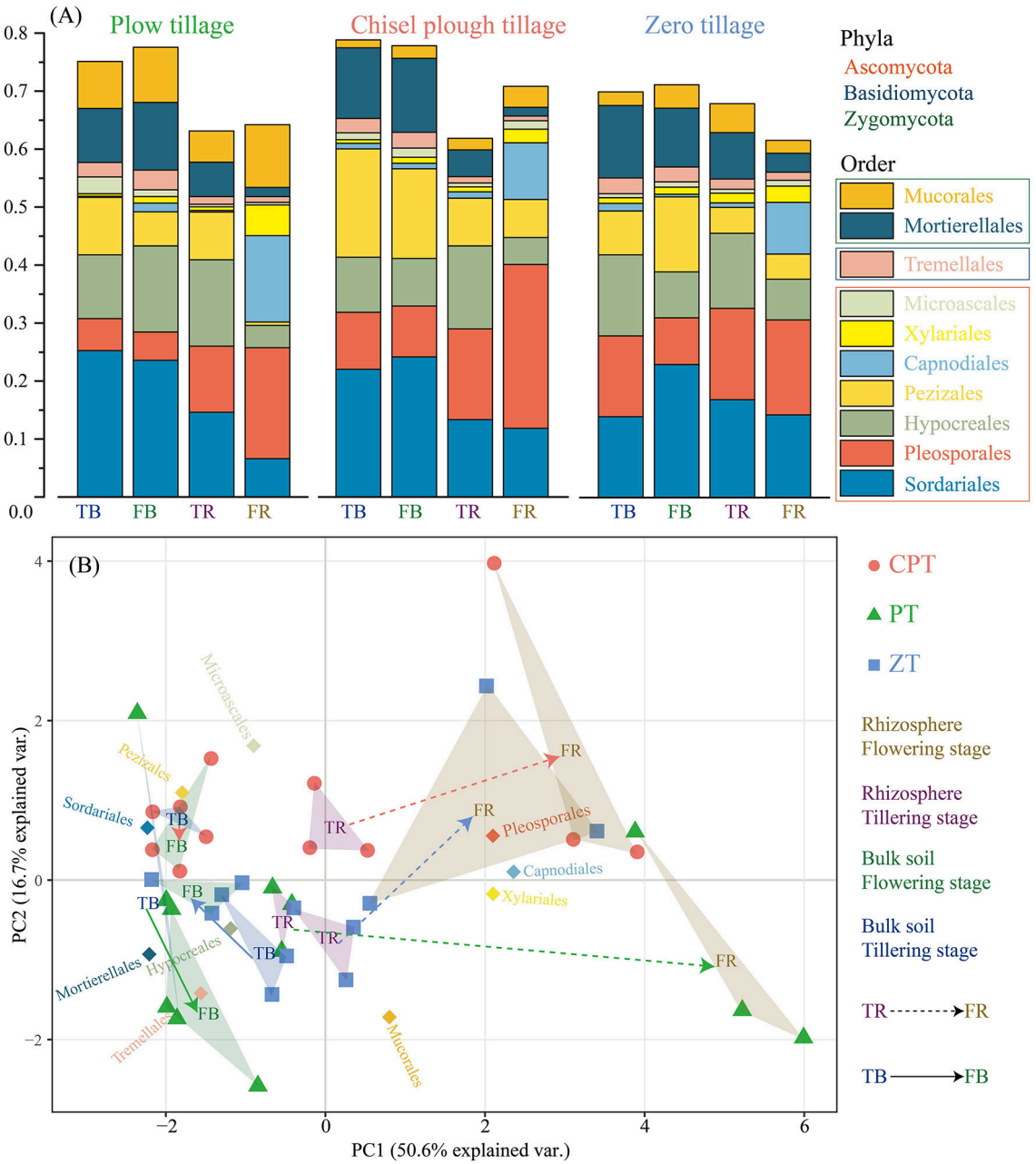

FIGURE 2 | (A) Relative abundance of the dominant fungal orders in all soil samples combined and in each tillage treatment. **(B)** Principal Coordinate Analysis (PCoA) of abundant fungal orders.

stages. The SEM analyses were performed separately for plant growth stages and indicated that rhizosphere and tillage directly induced more changes in soil properties in the flowering stage (SOC: 29.2%, invertase: 80.5%, soil texture: 69.0%, TN: 42.4%, soil moisture: 60.1%) than in the tillering stage (urease: 76.3%, invertase: 98.4%, TN: 61.3%, soil moisture: 36.9%; **Figure 3**). Soil invertase activity was the main determinant of the soil fungal community in the tillering stage, whereas soil texture and invertase significantly influenced fungal diversity, composition, and abundance in the flowering stage (**Figure 3**).

A comparison of the three tillage treatments showed that the soil fungal community was differently influenced by changes in soil properties associated with plant growth (**Figure 4**). Soil enzyme activities and physical properties lead to changes in fungal communities in chisel plow (invertase: 94.2%, soil moisture: 77.2%) and plow tillage (urease: 68.0%, invertase: 88.2%, soil texture: 79.1%, soil moisture: 45.4%), whereas soil total nutrients (SOC: 29.8%, TN: 61.1%), and physical properties (soil texture: 89.1%, soil moisture: 62.3%) influenced fungal communities in zero tillage soils (**Figure 4**). Compared to plow

TABLE 3 | SIMPER analysis results showing the dominant fungal orders that contributed to the dissimilarity between rhizosphere and bulk soil and growth stages among tillage treatments.

Stages compared	PT[a]		CPT		ZT	
	Order	%	Order	%	Order	%
BT-BF[b]	**All**	**11.98**	**All**	**6.78**	**All**	**13.09**
	Mucorales	14.61	*Pezizales*	18.40	*Pezizales*	20.54
	Pezizales	14.33	*Mucorales*	15.04	*Sordariales*	17.38
	Capnodiales	13.45	*Mortierellales*	13.71	*Pleosporales*	14.91
RT-RF	**All**	**32.28**	**All**	**20.84**	**All**	**16.59**
	Capnodiales	23.53	*Capnodiales*	22.40	*Capnodiales*	28.17
	Pezizales	15.30	*Hypocreales*	17.54	*Mortierellales*	15.34
	Hypocreales	13.88	*Pleosporales*	14.49	*Hypocreales*	12.84
TB-TR	**All**	**13.51**	**All**	**15.8**	**All**	**9.88**
	Sordariales	18.78	*Pezizales*	20.53	*Pezizales*	21.75
	Pleosporales	17.19	*Mortierellales*	18.47	*Mucorales*	16.35
	Microascales	13.42	*Sordariales*	14.49	*Mortierellales*	15.06
FB-FR	**All**	**35.57**	**All**	**27.75**	**All**	**21.63**
	Capnodiales	15.83	*Pleosporales*	17.91	*Capnodiales*	23.60
	Sordariales	14.33	*Mortierellales*	17.91	*Pezizales*	15.26
	Pleosporales	13.19	*Capnodiales*	16.38	*Mortierellales*	15.09

[a] *PT, plow tillage; CPT, chisel plough tillage; ZT, zero tillage.*
[b] *BT, bulk-tillering stage; RT, rhizosphere-tillering stage; BF, bulk-flowering stage; RF, rhizosphere-flowering stage.*

tillage, fungal diversity (1.2%) and abundance (23.9%) had a lower and non-significant influence on the variation noted in chisel plow and zero tillage, respectively (**Figure 4**).

DISCUSSION

Influence of Plant Growth on Rhizosphere Fungal Community

The present study investigated the influence of plant growth on rhizosphere and bulk fungal communities under three tillage treatments. Larger variations in fungal alpha diversity between rhizosphere and bulk soils were identified at the flowering stage than at the tillering stage. This was due to the opposite changes in soil fungal alpha diversity in rhizosphere and bulk soils along plant growth (**Table 1**); notably, fungal alpha diversity was more strongly influenced by plant growth in the rhizosphere than in bulk soils. Soil enzyme activities and physical structure, as major factors affecting microbial communities, were highly correlated with both Shannon and Simpson indices, which can be explained by rhizosphere fungal alpha diversity being mostly shaped by plant growth stages (Nannipieri et al., 2003; Bell et al., 2009).

Trends in the changes of fungal phylogenetic structure (membership and composition) were consistent with variations in the fungal alpha diversity under the three tillage treatments. Fungal phylogenetic structure is more sensitive to plant growth in the rhizosphere than in bulk soils because the latter is highly stable (Houlden et al., 2008). In addition, plant growth has a greater impact on fungal phylogenetic composition than on phylogenetic membership because similar plant species select similar plant-driven microbial species by influencing the available nutrients in the surrounding soil (Hartmann et al., 2009). Changes in fungal phylogenetic membership and composition showed a significant relationship with soil invertase activities (**Table 2**). Higher soil invertase activity along roots depends on the root system, implying that the rhizosphere soil environment was strongly affected by plant root activities, thus causing larger variations in the fungal phylogenetic structure between tillering and flowering stages in rhizosphere soils (Welc et al., 2014; Razavi et al., 2016). In contrast, lower soil invertase activity in bulk soils reflects the weak influence of root activity on bulk soil fungal microbial community, and therefore management (i.e., tillage) is the main factor determining bulk soil environment during plant growth (Niu et al., 2015; Banerjee et al., 2016).

Compared to the tillering stage, fungal taxonomic composition significantly differed between rhizosphere and bulk soils at the flowering stage, under the three tillage treatments. Due to the significant increase in the relative abundance of Capnodiales, Pleosporales, and Xylariales, fungal taxonomic composition was more affected by plant growth stage in the rhizosphere than in bulk soil. Fungi within these orders (phylum Ascomycota) grow quickly and become dominant at the flowering stage because they are able to immediately use carbon resources released by roots (Hannula et al., 2012). Because the dominant Sordariales and Mortierellales are considered to be primary straw residue decomposers in arable soils, and the fungal taxonomic composition in bulk soils was likely driven by residue decomposition processes in crop residuals (Ma et al., 2013). The taxonomic composition distinguished tillering from flowering stages due to fungal proportion changes in the early stages of residue decomposition. Bastian et al. (2009) showed that the microbial succession on fresh organic residue incorporated in soil was dominated by copiotrophs and r-strategists in the early stages of decomposition.

According to the SEM, soil and rhizosphere fungal communities were more differentiated at the flowering than at the tillering stage due to the influence of soil properties. In particular, SEM revealed that increasing variations in the fungal community between rhizosphere and bulk soils during plant growth were caused by changes in soil nutrient status due to an increase in root exudates (Houlden et al., 2008). Consequently, different fungi thereby increasing the variations in fungal communities between rhizosphere and bulk soils during plant growth.

Tillage Treatments Shaped Rhizosphere Fungal Communities

We found higher variations in both Shannon and Simpson indices under conventional (plow) tillage than under conservation (chisel plow, zero) tillage. In addition, fungal alpha diversity was significantly and uniquely correlated to soil properties under each tillage treatment. Zero tillage had the highest Shannon diversity value associated with lower variations

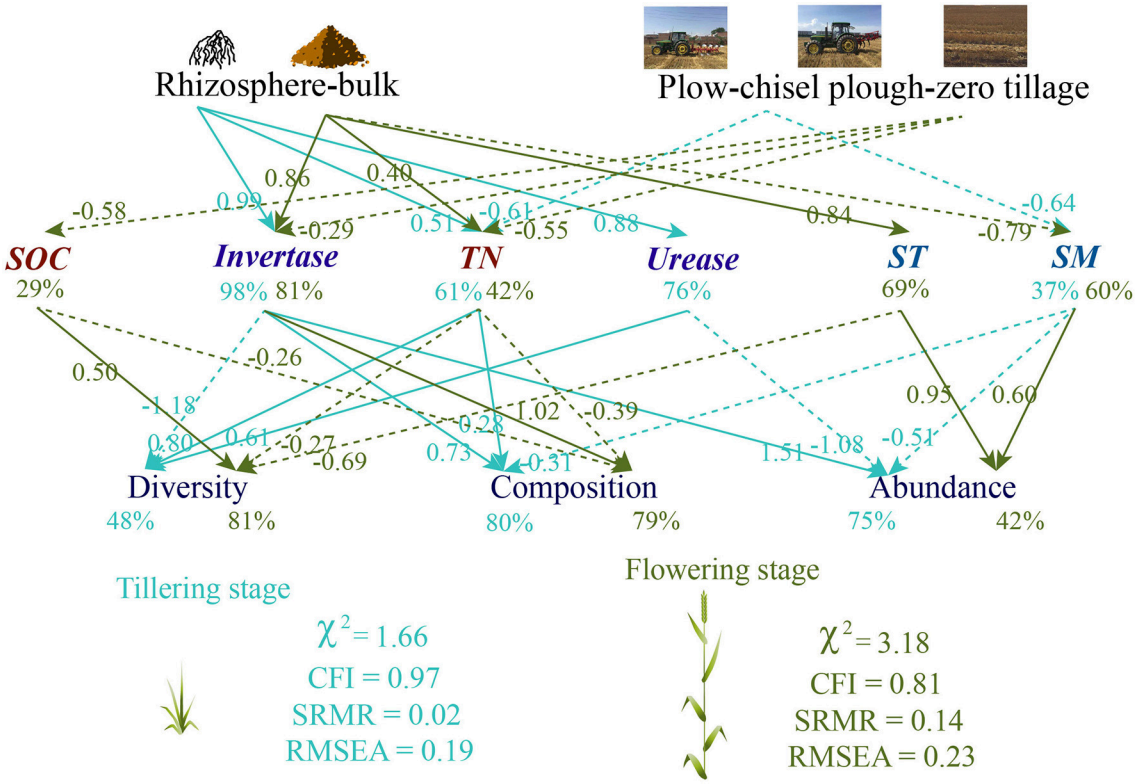

FIGURE 3 | Path diagrams of the structural equation models for the relationship that tillage and plant growthon soil fungal diversity, abundance, and composition.

in SOC and soil invertase activity, likely due to the build-up of labile C pools under conservation tillage (Chen et al., 2009). Relatively stable SOC and invertase activity indicated that zero tillage was preserving soil nutrient status, promoting fungal diversity (Plaza et al., 2013).

Although plant growth increased the distinction of fungal phylogenetic structure between rhizosphere and bulk soils among the three tillage treatments, the least variability in phylogenetic structures were observed under zero tillage. These results can be explained by lower variations of soil physical structure, which is significantly correlated with changes in the fungal phylogenetic membership and composition under zero tillage. Soil physical structure is important in terms of moisture retention, organic C storage, and cation exchange; all of which have been demonstrated to shape microbial communities (Chodak et al., 2016). In contrast to zero tillage, plow tillage, which is a conventional management practice that disturbs soil density, results in relatively low nutrient levels (here we observed low SOC and invertase activity), leading to a fungal phylogenetic structure that is more sensitive to plant growth and root activity (Yin et al., 2010; Wang et al., 2016a).

Rhizosphere fungal taxonomic composition was most balanced under conservation tillage at the flowering stage because the relative abundance of the major fungal orders (Capnodiales and Pleosporales) increased in similar proportion to plant growth. Capnodiales and Pleosporales, which belong to the class Dothideomycetes, have different strategies for breaking down cellulose in root-surrounding soils (Ohm et al., 2012), suggesting that plow tillage may alter nutrient use between the tillering and flowering stages in the rhizosphere more so than conservation tillage. At the tillering stage, zero tillage resulted in the greatest similarities between rhizosphere and bulk soil had more similar proportions of with respect to dominant fungal orders, which is mostly due to Sordariales preferring fine soils and decomposing residuals in environments with diverse nutrients, such as those associated with decomposing plant residues (Klaubauf et al., 2010; Ma et al., 2013; Wang et al., 2016a).

The three different SEMs based on the three tillage treatments revealed that changes in soil properties may directly influenced soil fungal community due to plant growth and root activity. Root activity and plant growth caused lower variations in soil texture under zero tillage, explaining the more stable soil fungal community, while soil invertase activity, strongly correlated to root exudates, was the most important soil variable determining the fungal community under plow and chisel plow tillage. Different patterns of direct and indirect effects on soil fungal community can be considered in relation to the biogeochemical processes occurring under the three tillage treatments, implying that long-term tillage may lead to a unique soil fungal ecology in response to biotic and abiotic factors (Fierer et al., 2009; Eisenhauer et al., 2015). Consequently, zero tillage, which

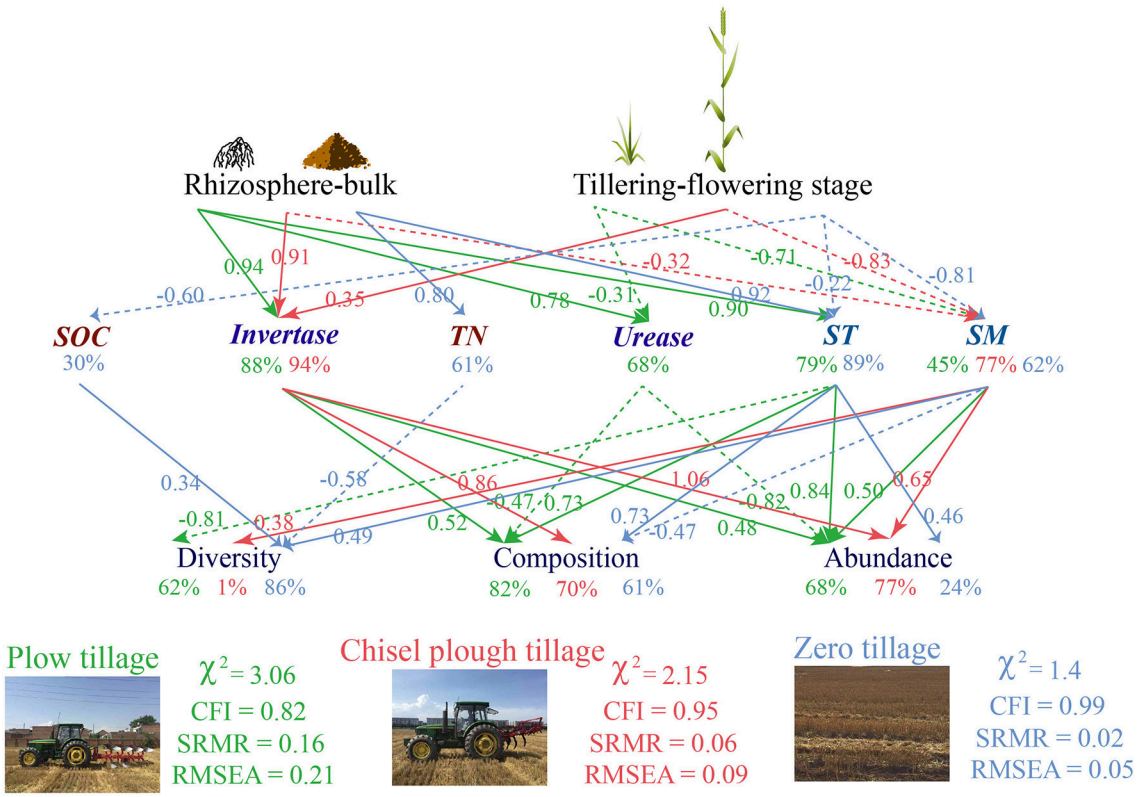

FIGURE 4 | Path diagrams of the structural equation models for soil fungal community influenced by soil properties under the three tillage treatments in response to rhizosphere and plant growth.

produces less disturbance, establishes nutrient-rich conditions that increase the stability of soil fungal community responses to root activity and straw decomposition.

CONCLUSION

In this study, we explored the changes in rhizosphere and bulk soil fungal communities during plant growth under three tillage treatments. Tillage is considered an anthropic agriculture management practice that can alter soil biota causing direct and indirect effects on crop growth and soil nutrient transformation (Brussaard et al., 2007). The results of the present study revealed that plant growth increased the discrimination of fungal communities between bulk and rhizosphere soil, mostly due to increases in relative abundance of Sordariales and Mortierellales in bulk soil, as they decompose crop residues, and increases in Capnodiales and Pleosporales in rhizosphere soil, which can grow quickly by using carbon components from plant roots in rhizosphere soil. This study also revealed that the changes were not consistent between tillage, therefore, tillage may contribute to unique soil fungal ecology. In this study, tillage altered the variability in fungal communities, with zero tillage promoting more stable communities, likely because the practice preserves soil physical structure and nutrient status. Future research should focus on the differences in carbon resources between rhizosphere and bulk soils under the three tillage practices, quantifying the relationship between organic carbon source utilization and fungal communities.

AUTHOR CONTRIBUTIONS

ZW contributed to design of the experiments, data analysis and manuscript writing; TL contributed to experimentation; XW, YaL, JH, YuL, and JD contributed to data interpretation and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01301/full#supplementary-material

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frontiers in Microbiology

ORIGINAL RESEARCH
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Endophytic Fungi *Piriformospora indica* Mediated Protection of Host from Arsenic Toxicity

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Complex intercellular interaction is a common theme in plant-pathogen/symbiont relationship. Cellular physiology of both the partners is affected by abiotic stress. However, little is known about the degree of protection each offers to the other from different types of environmental stress. Our current study focused on the changes in response to toxic arsenic in the presence of an endophytic fungus *Piriformospora indica* that colonizes the paddy roots. The primary impact of arsenic was observed in the form of hyper-colonization of fungus in the host root and resulted in the recovery of its overall biomass, root damage, and chlorophyll due to arsenic toxicity. Further, fungal colonization leads to balance the redox status of the cell by adjusting the antioxidative enzyme system which in turn protects photosynthetic machinery of the plant from arsenic stress. We observed that fungus has ability to immobilize soluble arsenic and interestingly, it was also observed that fungal colonization restricts most of arsenic in the colonized root while a small fraction of it translocated to shoot of colonized plants. Our study suggests that *P. indica* protects the paddy (*Oryza sativa*) from arsenic toxicity by three different mechanisms viz. reducing the availability of free arsenic in the plant environment, bio-transformation of the toxic arsenic salts into insoluble particulate matter and modulating the antioxidative system of the host cell.

Keywords: arsenic, arsenic toxicity, abiotic stress tolerance, endophytic fungi, symbiosis, plant microbe interactions, bioremediation, hyper-colonization

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INTRODUCTION

Cellular metabolism shapes the phenotypes of cells/organism. Kinetic changes in cellular metabolism provide flexibility and robustness for adaptability in response to environmental cues. The changes are much more evident when cells are challenged either with toxic doses of chemicals or in response to infectious virus, fungi, and bacteria. The interaction becomes much more complex when both the biotic and abiotic factors act in collusion. Heavy anthropological and geological activities are a major contributor of toxic products in nature. (Nordstrom, 2002; Jang et al., 2007). The toxicants, in turn, pose a serious risk to health and environment when they end up at the top of the food chain after being taken up by the plants. Toxicity of the harmful chemical largely depends on the geological and biological activities that determine the free availability of the toxic ions. Metal and metalloid toxicity is a serious environmental and health issue. While technological

advancement is the need of the hour, new innovations are a must. Mechanistic understanding of bioremediation of heavy metals and metalloids can provide new tools and resources for the proper management of environmental toxicants. One should understand the process of detoxification of elements as these elements cannot be degraded. Major mechanisms of detoxification of the elements are only possible by compartmentalization and biotransformation into an inactive form of element or safe excretion by the cell or organism (Nies et al., 1989; Nies, 1999). Agriculture land contaminated with arsenic is one of the major problems of developing countries including India, Bangladesh, and China; where overexploitation of groundwater containing arsenic, is in practice for cultivation. Irrigation practices with groundwater increases surface arsenic and accumulation in crops that is finally consumed by animals and humans leading to several health complications such as skin lesions, neurological impairment, and cancer (Smedley and Kinniburgh, 2002; Huq et al., 2006; Ratnaike, 2006; Wei et al., 2013; Ramos-Chavez et al., 2015; Singh et al., 2015; Wiwanitkit, 2015; Wu et al., 2015). In the plants arsenic mainly interferes with photosynthesis and reduces transpiration efficiency. Impact and accumulation of arsenic on the plants generally associated with the genotype which decide the sensitivity and response of arsenic on the metabolism of plant (Finnegan and Chen, 2012).

Plants modulate the biochemical and molecular response upon arsenic exposure to minimize the toxic effects on the cell metabolism viz. expression pattern of phosphate and hexose transporters, antioxidative enzyme system, antioxidant metabolite pools, glutathione metabolism, phytochelatins (PC), and vacuolar PC-As transporters (Finnegan and Chen, 2012; Tripathi P. et al., 2012; Tripathi R. D. et al., 2012; Chen et al., 2017). However, these biochemical and molecular changes varies with the species to species of economically important crops which demarcate the tolerant and sensitive variety with broad range of arsenic bioaccumulation (Rai et al., 2011). Bioaccumulation restriction of toxic arsenic might be the solution to prevent it from entering the food chain. Several microorganisms have been used for bioremediation of such toxic metals and metalloids. However, their utilization is limited to the specific niche from where these microbes were isolated or their axenic cultures not available such as in the case of mycorrhiza. Introduction into other niche destabilizes the micro-environment and biogeochemical cycles, therefore, an alternative strategy is needed to deal with metal bioremediation.

Previous work suggest that the specific fungal strains in the soil reduces the negative effects of arsenic on the plants however it is unknown that how this phenomenon of tolerance against arsenic is mediated by fungi (Srivastava et al., 2012; Tripathi et al., 2013; Spagnoletti and Lavado, 2015). We hypothesized that the fungal induced tolerance of host cell against arsenic might be an innovative solution to the problem. We used *Piriformospora indica* a root colonizing endophytic fungus which attributes several beneficial traits to their host with a wide range of biotic and abiotic stress tolerance, growth and biomass yield promotion (Kumar et al., 2009, 2011; Yadav et al., 2010; Jogawat et al., 2013; Johri et al., 2015) to induce arsenic resistance by reprogramming the metabolism of rice plant and reduce arsenic load in the host.

MATERIALS AND METHODS

Plant, fungal culture, and growth conditions: Rice (*Oryza sativa* L. IR64) seeds were surface sterilized for 2 min in 70% ethanol followed by 10 min in a NaClO solution (0.75% Cl). Seeds were finally washed six times with sterile water and further dH_2O at 60°C for 5 min to eliminate naturally occurring microbes that may have been in or on the rice seed as described previously (Kumar et al., 2009). Seeds were germinated on water-agar plates (0.8% Bacto Agar; Difco, Detroit, MI) at 37°C in the dark. *P. indica* was cultured on *Aspergillus* minimal media for 8 days (Hill and Kafer, 2001; Yadav et al., 2010). For colonization, radicle of seedling plants were transferred into sterile spore suspension (10^6 spores per ml) for 2 min and further transferred to water agar plate for 1 day, while in case of control plants autoclaved spore suspension was used. This procedure of inoculation of spore gives a similar and uniform level of colonization in root of every inoculated plant. Further, seedlings were transferred to closed lid jars for hydroponic culture. Rice plants were grown in a growth chamber under controlled temperature and humidity (32°C/70%). One-quarter strength of hydroponic solution (Kamachi et al., 1991) was changed weekly with 10 ml fresh solution containing arsenic (100 μM sodium arsenate; Ahsan et al., 2008) respectively in each jar and removed solution was kept for arsenic analysis.

In order to study bioprotection offered by *P. indica* against arsenic toxicity all plants were initially grown for 3 days and subsequently following types of sets were used. (Set-1), rice plants were grown till 25 days without any fungus were used as a control; (Set-2), rice plants were inoculated only with *P. indica* at day zero and grown till 25 days; (Set-3), rice plants treated with sodium arsenate at day zero and grown till 25 days; (Set-4), rice plants were colonized with *P. indica* and at day 3 treated with sodium arsenate and grown for total 25 days (Delayed treatment); (Set-5), rice plants inoculated with *P. indica* and sodium arsenate at day zero and grown for total 25 days (Simultaneous treatment); (Set-6), *P. indica* was inoculated at day 3 after treatment with sodium arsenate (this time point is considered as day zero) and grown for next 25 days (Early treatment). For this experiment, control plants and treatment were set accordingly. Plants were harvested at different time periods and carefully washed and rinsed in de-ionized autoclaved water and weighed. Plant samples were stored in water for 1 h to study colonization, however for superoxide accumulation, arsenic uptake, and enzyme assays study fresh samples were used. Growth promoting effect of fungus was checked by measuring the dry weight. To measure dry weight, plant materials after harvesting were kept at 100°C for 72 h in a hot air oven. All experiments were done independently in triplicates and for each case 15 seedlings were used per jar in triplicates.

Fungal Growth Condition and Arsenic Treatment

Fungus was grown in minimal media with different concentration of sodium arsenite (As III) and sodium arsenate (As V) at 0, 1, 1.5, 2, 2.5 mM. After 1 week of growth mycelia was filtered and washed 10 times with autoclaved MQ water

and fresh biomass was taken after drying the excess moisture on blotting paper.

Fungal Colonization Analysis

To study colonization, 10 root samples were selected randomly from the rice root. Samples were softened in 10% KOH solution for 15 min and acidified with 1N HCl for 10 min and finally stained with 0.02% Trypan blue overnight (Kumar et al., 2009; Jogawat et al., 2013). Samples were destained with 50% Lacto-phenol for 1–2 h prior to observation under light microscope (Zeiss Microscope, Germany). The distribution of chlamydospores within the root was taken as an index for studying colonization. Percent colonization was calculated for the inoculated plants according to the method described previously (Mcgonigle et al., 1990). To check the effect of arsenic toxicity on the colonization pattern of endophyte, root samples examined for the presence of *P. indica* within the root tissues of plant stained with Calcofluor White stain. Samples were fixed prior to observation under a confocal microscope (Leica TCS CS2 confocal Microscope). For real-time PCR comparison, 0.15 g of root tissues of the each sample was used to isolate total genomic DNA by the CTAB method. PCR reactions were carried out with 1 μg of genomic DNA as template and specific primers using sybr green. Specific primers for EF-1-alpha (*tef*) gene (AJ249912) of *P. indica* PitefFOR (5′–TCGTCGCTGTCA ACAAGATG-3′) and PitefREV (5′–GAGGGCTCGAGCATG TTGT-3′) and actin gene (AB047313.1) of rice plant OsActinF (5′-GCCGTCCTCTCTCTGTATGC-3′) and OsActinR (5′-GAC GAAGGATAGCATGGGGG- 3′) were used.

Antioxidant Enzyme Activities in Rice Plants

In order to know the impact of colonization of *P. indica* on antioxidative system of plant during arsenic stress, antioxidative enzyme activities were also checked in the presence, absence, and delayed treatment of arsenic. For this purpose, all the experiments and conditions were kept same as described in the previous section. Protein isolation was done as described previously with some modifications (Kumar et al., 2009), fresh root and shoot tissue were homogenized at 4°C in an ice-chilled mortar with liquid N_2in QB buffer [without DTT (1,4-Dithiothreitol) for SOD, CAT, and GST assay] with 50 mg polyvinyl pyrrolidone (PVP) per gram tissue (for GR assay). Crude homogenates were centrifuged at 15,000 × g for 15 min at 4°C and the supernatant fractions were kept frozen at −20°C. Protein contents were determined by Bradford method using BSA as standard (Bradford, 1976).

SOD Assay

In this case, activity was monitored according to the method described previously (Roth and Gilbert, 1984). One milliliter of reaction mixture contains 50 mM sodium phosphate buffer (pH 7.8), 100 μM EDTA, with 20 μl of enzyme extract, and 10 mM of pyrogallol. The enzyme activity (U/mg Protein) was calculated by scanning the reaction mixture for 120 s (60 s interval) at 420 nm.

CAT Assay

Catalase activity was assayed by measuring the initial rate of H_2O_2 disappearance using the method described previously (Beers and Sizer, 1952). One milliliter of catalase assay reaction mixture contains 0.05 mM sodium phosphate buffer (pH 7.0) with 20 μl of enzyme extract and 1 mM of H_2O_2. The decrease in H_2O_2 was followed by a decline in optical density at 240 nm, and the activity (U/mg protein) was calculated using the extinction coefficient of 40 mM cm^{-1} for H_2O_2.

GST Assay

For the measurement of GST activity, 1 ml of reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.5) with 20 μl of enzyme extract and 2% CDNB (1-chloro-2,4-dinitrobenzene). The enzyme activity (U/mg protein) was calculated by scanning the reaction mixture for 180 s (60 s interval) at 340 nm. GST activity was monitored as described previously (Habig et al., 1974).

GR Assay

The activity (U/mg protein) was determined by the oxidation of NADPH at 340 nm with an extinction coefficient of 6.2 mM cm^{-1}, as described previously (Nordhoff et al., 1997). The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH and 0.5 mM glutathione (oxidized form, GSSG), and 10 μl of enzyme extract (total reaction mixture 1 ml). The reaction was initiated by the addition of NADPH at 25°C.

Estimation of H_2O_2 Production

The H_2O_2 content from rice seedlings was measured as described earlier (Junglee et al., 2014). One gram tissue was extracted with 5 ml of TCA (0.1%, w/v) at 48°C and homogenate was centrifuged at 12,000 g for 15 min. To 0.5 ml supernatant, 0.5 ml of 0.05 M sodium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide solution were added. The absorption of the mixture was measured at 390 nm using UV-visible spectrophotometer (Thermo Fisher Scientific). The H_2O_2 content was determined using an extinction coefficient (ϵ) of 0.28 mM^{-1} cm^{-1} and expressed as mg g^{-1} fresh weight.

Estimation of Chlorophyll

Chlorophylls and carotenoids were isolated from leaves by homogenization in liquid nitrogen and subsequent threefold extraction with 80% acetone (v/v). After centrifugation for 5 min at 1,500 × g, the absorbance of the supernatant was measured at 663.6, 646.6, and 440.5 nm (Porra et al., 1989). Leaf samples were also extracted with 1% (w/v) HCl in methanol, and the anthocyanin contents were assayed spectrophotometrically. The relative amounts of anthocyanins were expressed by $[A_{530}–0.333A_{657}]$ m^{-2} (Mancinelli et al., 1975). Absorbance was measured with a UV-visible spectrophotometer (Thermo Fisher Scientific).

Estimation of Proline

Proline content in leaf tissues was determined by the ninhydrin method (Jogawat et al., 2013) using UV–vis spectrophotometer

(Thermo Fisher Scientific). It was calculated with using standard curve at 520 nm and expressed as mmol per gram fresh weight.

Dithizone Test for the Accumulation of Arsenic in Plant and Fungus

To analyze the accumulation of arsenic in plant dithizone test was performed. 3 mg of dithizone was dissolved in 6 ml of acetone; then 2 ml of distilled water and 1–2 drops of pure acetic acid were added. Plant samples were gently washed in distilled water and incubated in this solution for 1–24 h in the dark. After staining the shoots were observed for accumulated metal (Turnau and Wierzbicka, 2006).

Analysis of Zeta Potential of Fungal Cell Wall

P. indica Fungus was grown in minimal media with different concentration of As (III) and As (V). After 1 week of growth, mycelia was filtered and washed 10 times with autoclaved MQ water and three times with PBS. Mycelia was macerated in tissue grinder and again washed (three times) with autoclave MQ water. Finally, macerated mycelia was suspended in MQ water and analyzed for zeta potential using ZetaSizer 3.0 (Malvern). For adsorption studies of arsenic on the fungal cell wall, 1 g of cell wall was incubated in 100 ml of arsenic solution for 60 min. and cell wall material was washed with PBS three times. These samples were digested with acids for arsenic analysis. Adsorbed arsenic analysis was done by colorimetry in triplicates, independently (Pillai et al., 2000). Percent adsorption was calculated using total supplied arsenic and adsorbed arsenic on the cell wall.

Arsenic Estimation in Plant Materials

The concentration of arsenic ions in plant materials was determined by Atomic Fluorescence Spectrophotometer (AFS, PG Instruments AF-420) equipoise with an auto sampler, hydride generator, and high-intensity hollow cathode lamps. For this 500 mg of plant materials and 100 mg of fungal materials were acid digested at 80°C and further diluted in 1 N nitric acid and used as a sample for arsenic estimation. Independent triplicate was used to calculate arsenic in plant materials.

Transmission Electron Microscope, Scanning Electron Microscope, and EDAX Analysis (TEM, SEM, and EDAX Analysis)

Fungus was grown in minimal media with sodium arsenate (As V) at 0.1 and 0.5 mM concentration while control was grown without arsenic. After 3 days of growth, mycelia was filtered and washed 10 times with autoclaved MQ water. Further, fungal mycelium of control and treated were washed with 1XPBS (pH 7.2) and fixed in 2.5% glutaraldehyde prepared in phosphate buffer (pH 7.4) for 2 h at 4°C. Cells were washed three times with 0.1 mM phosphate buffer and post fixed in 1% Osmium tetroxide for 4 h. Fixed cells were washed with phosphate buffer, dehydrated in acetone series (15–100%), and embedded in Araldite-DDSA mixture (Ladd Research Industries, USA, Burlington). After baking at 60°C, block was cut (60–80 nm thick) by an ultramicrotome (Leica EM UC7) and sections were stained with Uranyl acetate and Lead citrate. Analysis of sections was done under FEI Tecnai G2 spirit twin transmission electron microscope equipped with Gatan digital CCD camera (Netherlands) at 80 KV. The confirmation of insoluble precipitates as arsenic was done by SEM (Scanning electron microscope) equipped with EDAX.

Statistical Analysis

All graphs were created and statistical calculations were performed using Microsoft Excel 2007. The significance of the data obtained was checked by Student's *t*-test using the program SPSS Statistics 20.0 (IBM USA).

RESULTS

Endophytic Colonization under Arsenic Stress

In the colonization study, it was observed that pre-colonization of *P. indica* in paddy plant is a time dependent process and is unaffected by arsenic salt concentration that is otherwise toxic to plants. About 50–60% colonization was also observed at 2 days after inoculation (dai). Colonization increases up to 75% at 5 dai and is maintained during the study however colonization more than 75% was not observed (**Table 1**). No adverse effect of arsenic at given concentration on fungal colonization and sporulation was seen when *P. indica* was inoculated after arsenic treatment. Fungal colonization was confirmed by real-time PCR analysis and intracellular pear-shaped chlamydospores (**Figures 1A,B**). Real-time PCR analysis shows that four times higher fungal load in the As treated root than untreated roots. A similar pattern of colonization was also observed by confocal microscopy in the 25 day old roots (**Figures 1A,B**; Supplementary Figure 1).

Endophyte Recovers Plant Growth from Arsenic Toxicity

A distinct morphological and biomass changes observed in plant colonized with *P. indica* as compared to non-colonized plants (used as a control) (**Figures 1C,D**). Plants treated with arsenic shows shoot stunting and poor growth as compared to control plants (As; **Figure 1D**). We found that plants colonized with *P. indica* first and treated with arsenic at day 3 showed increased

TABLE 1 | Effect of arsenic on colonization of root.

S. No.	Dai (days after inoculation)	Percent colonization (control plant)	Percent colonization (as treated plant)
1	1	∼10	∼10
2	2	∼60	∼60
3	3	∼75	∼75
4	4	∼75	∼75
5	5	∼75	∼75

The chlamydospores distribution within the root was considered as an indicator for presence of fungus and this was applied to study colonization. Percent colonization was calculated for the inoculated plants according to the method described previously (Mcgonigle et al., 1990).

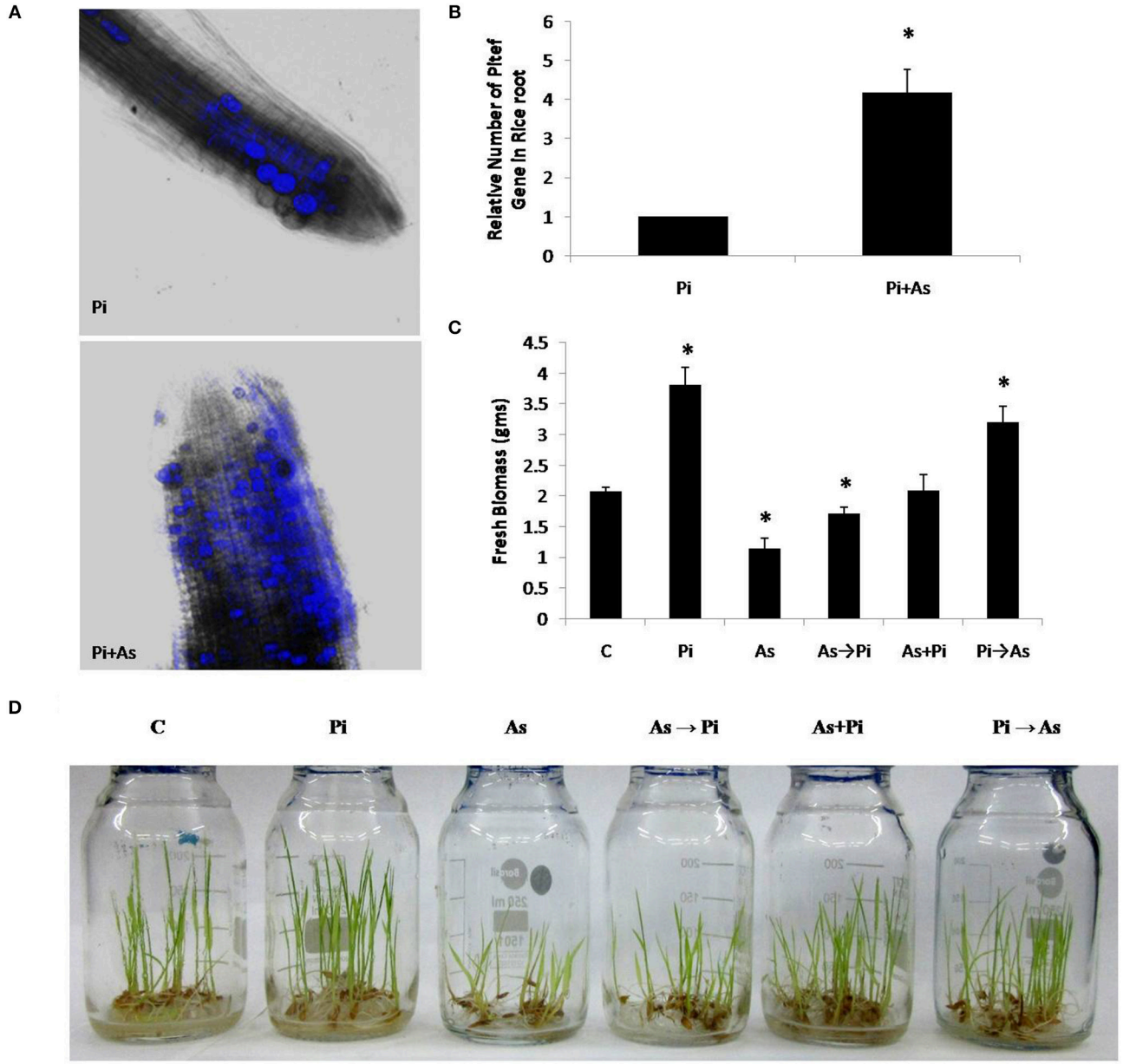

FIGURE 1 | Colonization of *P. indica* under arsenic stress. (A) Confocal microscope and **(B)** real time PCR analysis was done to characterize colonization of fungus *P. indica* under arsenic stress 100 μM sodium arsenate Pi+As after 25 dai (days after inoculation) in the rice root, the hyper-colonization was observed than the untreated control Pi. **(C,D)** Alternate and simultaneous inoculation of *P. indica* was done to show the pattern of recovery at the level of biomass of rice plant to the arsenic-treated plants. **(C)** Rice plants grown for 25 days without any fungus were used as a control; Pi, rice plants inoculated with *P. indica* alone at day 0 and grown for 25 days; As, rice plants treated with arsenic at day 0 and grown for 25 days; As→Pi, rice plants first treated with arsenic at day 0 and at day 3 inoculated with *P. indica* and grown for a total of 25 days; Pi+As, rice plants inoculated simultaneously with both fungi and arsenic at day 0 and grown for 25 days; Pi→As, rice plants first inoculated with *P. indica* at day 0 and at day 3 treated with arsenic and grown for a total of 25 days. Maximum recovery in the biomass is maximum when already colonized plant was treated with arsenic. Asterisks show values significantly different from those of the controls ($P < 0.05$).

overall growth as compared to arsenic treated non-colonized plant that was equivalent to control plants (Pi→As; **Figure 1D**). Similar morphological patterns were also observed in delayed fungal inoculated treated plants and it showed improved root and shoot growth (As→Pi; **Figure 1D**).

A significant increase was also observed in biomass i.e., 1.7 fold in 25 days old paddy plants colonized with *P. indica* as compared to non-colonized plants ($P < 0.05$) (Pi; **Figure 1C**). Plants treated with arsenic showed a 1.72 fold decrease in dry weight of 25 days old rice plants as compared with control plants

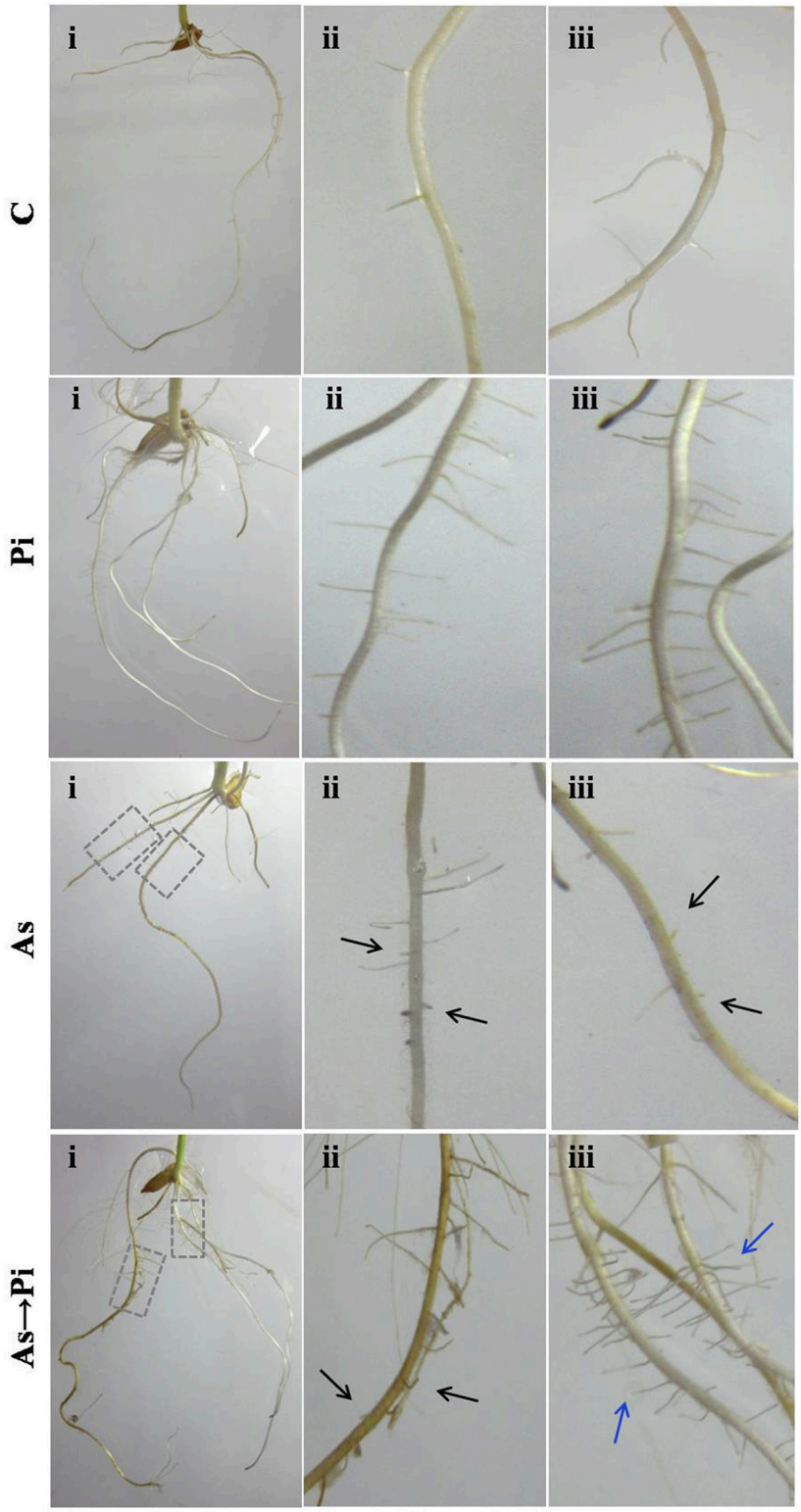

FIGURE 2 | *P. indica* recovers root damage by arsenic. Inoculation of *P. Indica* to the arsenic-treated plants induced new healthy root formation. Damaged root branches in arsenic-treated plants As are shown by black arrows, and recovery in root branches in *P. indica* colonized plants (As→Pi) are shown by blue arrows. C, rice plants grown for 25 days without any fungus were used as a control; Pi, rice plants inoculated with *P. indica* alone at day 0 and grown for 25 days; As, rice plants treated with arsenic at day 0 and grown for 25 days; As→Pi, rice plants first treated with arsenic at day 0 and at day 3 inoculated with *P. indica* and grown for a total of 25 days.

(non-colonized) ($P < 0.05$) (As; **Figure 1C**). Inoculation of *P. indica* at day 3 after arsenic treatment resulted in improved biomass yield i.e., 1.8 fold increase in dry weight as compared to plants treated with arsenic and is comparable to control plants ($P < 0.05$) (**Figure 1C**). In another case, where plants treated simultaneously with *P. indica* and arsenic at day 0 showed 1.16 fold increases in dry weight in comparison to control (plants without fungus and arsenic treatment) (**Figure 1C**). Our study shows that *P. indica* colonization promoted overall plant growth even in presence of toxic dosage of arsenic as compared to control plant (**Figures 1C,D**).

Root morphology is an important health recovery parameter to analyze arsenic toxicity. Arsenic treated plants are badly

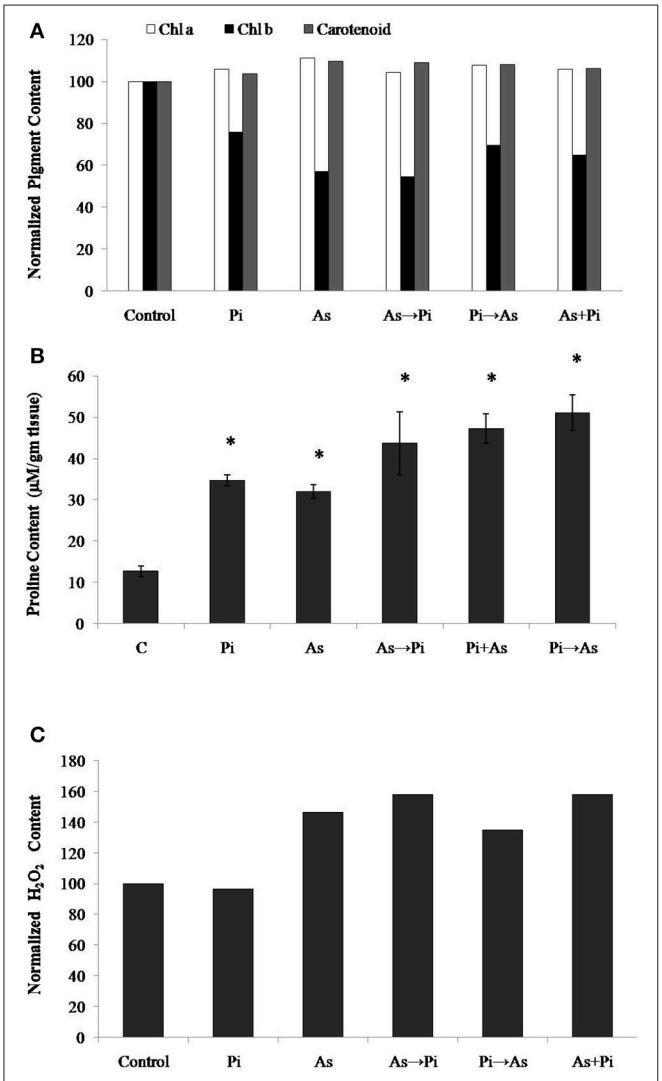

FIGURE 3 | Biochemical changes induced by *P. indica* may protect plant from arsenic-induced toxicity. (A) Normalized chlorophyll a, b, and carotenoids content; **(B)** Proline content; and **(C)** H_2O_2 content in the plants alternate and simultaneously treated with *P. indica* and arsenic. All experimental conditions were the same as described for **Figure 1**. Asterisks show values significantly different from those of the controls ($P < 0.05$).

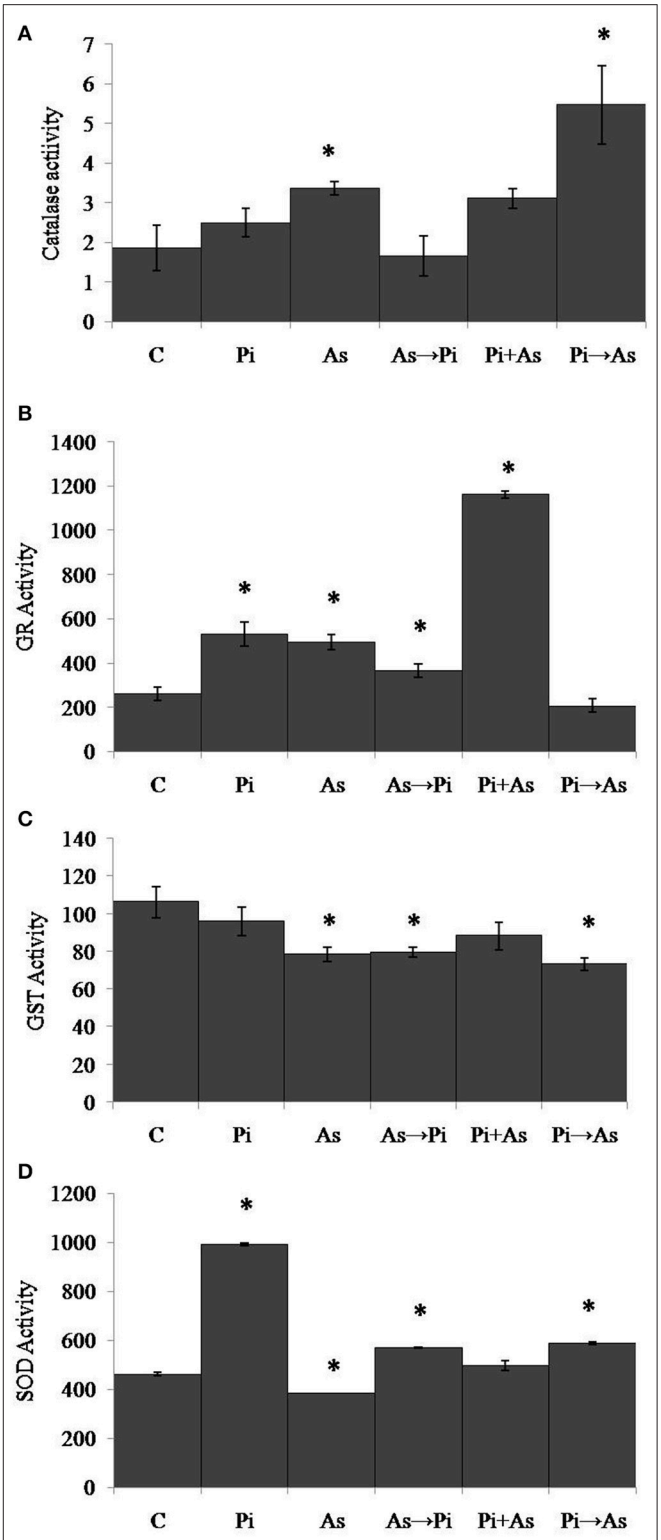

FIGURE 4 | Modulation of redox enzyme in rice root after treatment of *P. indica* and arsenic works as a defense against oxidative stress. (A) CAT, **(B)** GR, **(C)** GST, and **(D)** SOD specific activities compared with those of control plants **(C)**; asterisks show values significantly different from those of the controls ($P < 0.05$). All experimental conditions were the same as described for **Figure 1**.

damaged at the root level and show a poor growth of primary and almost nil secondary roots as compared to control plant (As; **Figure 2**). Colonization of *P. indica* shows an increase in growth of primary root and number of secondary root as compared to control plant (Pi; **Figure 2**). *P. indica* inoculation in arsenic-treated plants improved root growth and number of secondary root. Further new roots emerged bearing healthy secondary roots (As→Pi; **Figure 2**).

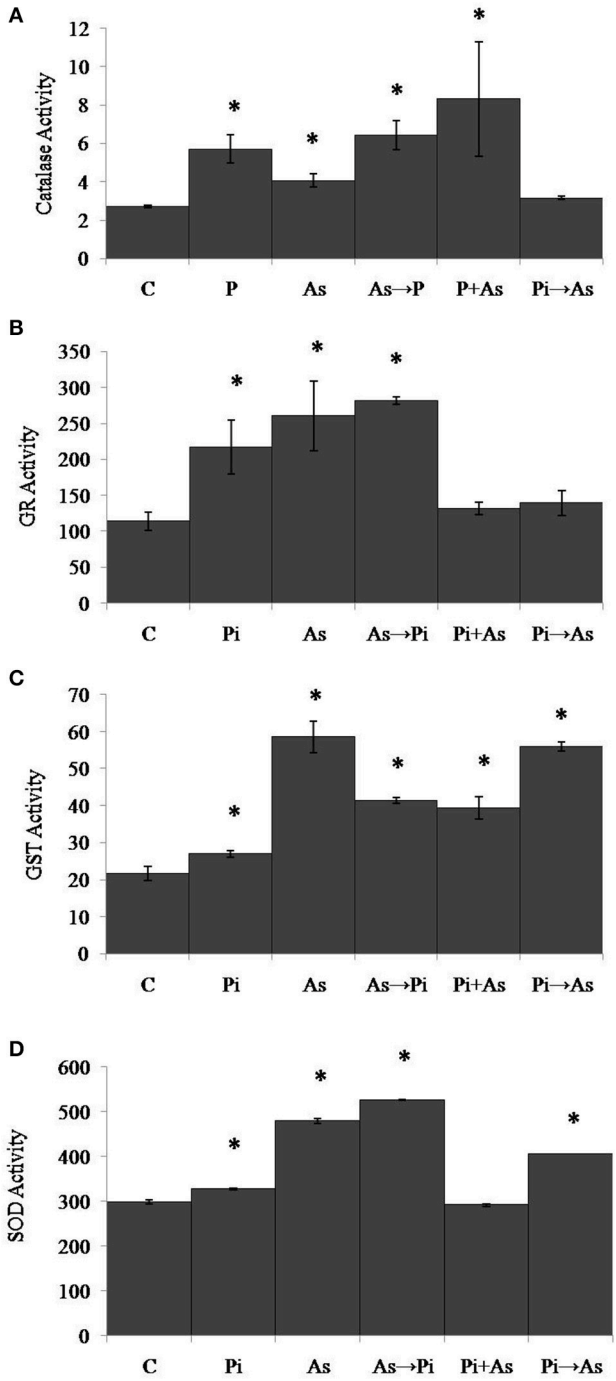

FIGURE 5 | Modulation of redox enzyme in rice shoot after treatment of *P. indica* and arsenic works as a defense against oxidative stress. (A) CAT, **(B)** GR, **(C)** GST, and **(D)** SOD specific activities compared with those of control plants **(C)**; asterisks show values significantly different from those of the controls ($P < 0.05$). All experimental conditions were the same as described for **Figure 1**.

P. indica Protects Rice Plant from Arsenic Toxicity

Arsenic treatment decreased total chlorophyll content by 10% and chlorophyll-b content by 43% while 11 and 9% increase was observed for chlorophyll-a and carotenoids respectively as compared to control (**Figure 3A**). However, *P. indica* increases the pigment contents upon colonization both in presence and absence of arsenic as compared to arsenic-treated plants (**Figure 3A**). Arsenic treatments increased the proline contents in rice seedlings indicating the influence of enhanced oxidative stress. The proline contents were increased by about 1.5–3 times higher on arsenic treatment while colonization of endophyte increases the proline content about two to five times higher than the control throughout the study. Similarly, the delayed and alternate treatment of arsenic and endophyte increases proline contents up to five times and was equivalent to untreated colonized plant (**Figure 3B**). Long-term induction of proline in colonized plant is a characteristic phenomenon of *P. indica* colonization (Jogawat et al., 2013).

The H_2O_2 level is a measure of oxidative environment in the rice plant upon exposure of biotic and abiotic stresses. H_2O_2 contents in arsenic-treated plant was 50% higher as compared to control, however, it was 4% lower in fungus colonized plant. In case of arsenic treatment before colonization (As→Pi) and simultaneous treatment of arsenic and endophyte (As+Pi), H_2O_2 level was measured 10% higher as compared to arsenic-treated plants in both cases (**Figure 3C**) while in case of arsenic treatment after colonization (Pi→As), H_2O_2 level was measured 8% lower as compared to arsenic-treated plant.

P. indica Regulates Anti-oxidative Enzyme in Rice Plant

To analyze the effect of arsenic and fungus on the redox immunity of plant, antioxidative enzyme activity was measured. Exposure to arsenic increased catalase (CAT) activity by 1.8

TABLE 2 | Fungal colonization and arsenic translocation.

S.No.	Sample	Arsenic content in root (mg/gm root biomass)	Arsenic content in shoot (mg/gm shoot biomass)
1	Control	0.13 ± 0.055	0.03 ± 0.004
2	Pi	0.02 ± 0.003	0.08 ± 0.044
3	As	0.65 ± 0.011	2.16 ± 0.44
4	As→Pi	6.97 ± 1.246	0.21 ± 0.006
5	As+Pi	0.49 ± 0.009	1.34 ± 0.062
6	Pi→As	26.22 ± 0.81	0.039 ± 0.003

Treated and control plants were analyzed for arsenic uptake and effect of fungal colonization on arsenic translocation to shoot. The amount of arsenic present in the plant root and shoot is expressed in mg per gm biomass.

fold as compared to non-colonized plant root ($P < 0.05$) (**Figure 4A**). Under similar conditions the activity of glutathione reductase (GR) was increased significantly (1.9 fold) (**Figure 4B**). Glutathione-S-Transferase (GST) and Super Oxide Dismutase (SOD) activity decreased significantly as compared to control plants ($P < 0.05$) (**Figures 4C,D**). In the case of plants colonized with *P. indica,* a maximum of 1.3 fold increased activity was observed for CAT as compared to non-colonized plants. Under similar conditions, GR and SOD activities were found to be increased by 2 and 2.1 fold. No significant change in activity was observed for GST (**Figures 4B–D**). Inoculation of *P. indica* in plants treated with arsenic (As→Pi) resulted decrease in enzyme activity for CAT and GST while increase in activity up to 1.4 and 1.2 fold for GR and SOD respectively than control plants and were found significant ($P < 0.05$) (**Figures 4A–D**). In the case of simultaneous treatment of As and fungus, CAT and GR activities were observed 1.7 and 4.4 fold higher than control plants while an insignificant decrease was observed in case of GST and no change in activity was observed for SOD. Similarly, in case of plants previously colonized with *P. indica* and later treated with arsenic (Pi→As), CAT and SOD activities were significantly higher than control and As treated plants (**Figures 4A,D**) while significantly decrease in activity was recorded for GR and GST as compared to control (**Figures 4B,C**). Interestingly, decrease was observed in GST activity than control plants in all cases of alternate and simultaneous treatment of As and fungus (**Figure 4C**).

In the case of shoot, plants treated with arsenic show significant increase in CAT, GR, GST, and SOD activities and were found 1.5, 2.3, 2.5, and 1.6 folds respectively as compared to control plants (**Figures 5A–D**). However, GR activity was reduced up to 40% in the case of arsenic-treated plant shoot (**Figure 5B**). An increased CAT activity (2 fold) was observed

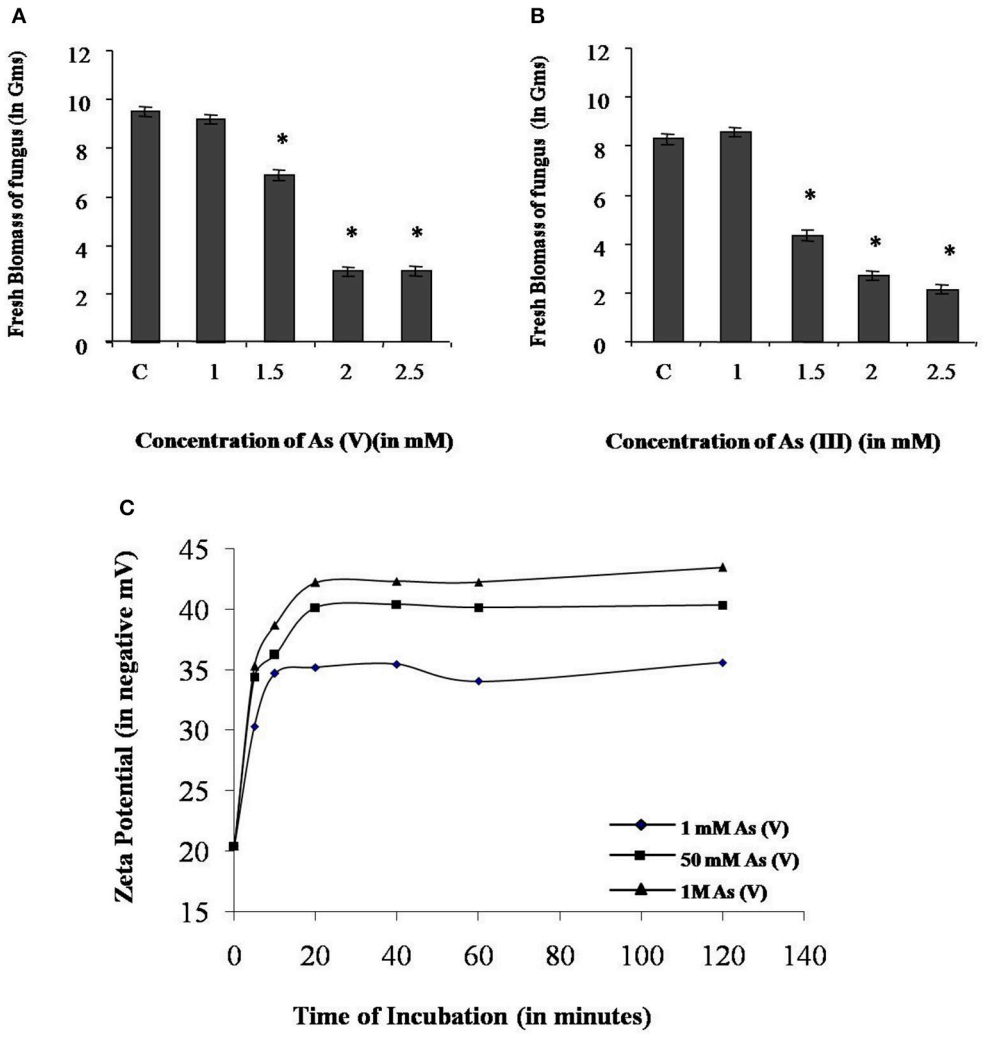

FIGURE 6 | Characterization of arsenic resistance by fungus *P. indica*. (A) Growth analysis of fungus at different concentration of sodium arsenate (As V). **(B)** Growth analysis of fungus at different concentration of sodium arsenite (As III). **(C)** Analysis of change in zeta potential on the cell wall due to adsorption of arsenic at 1 mM, 50 mM, and 1 M sodium arsenate (As V). Asterisks show values significantly different from those of the controls ($P < 0.05$).

in plants colonized with *P. indica* as compared to non-colonized plants (**Figure 5A**). Similarly, GR, GST, and SOD activities were found 1.9, 1.2, and 1.1 folds increased respectively (**Figures 5B–D**). In all three conditions of alternate and simultaneous treatment of arsenic and fungus, it was observed that CAT, GR, GST, and SOD were increased significantly as compared to control plants ($P < 0.05$) except SOD activity in case of simultaneous treatment of arsenic and endophyte (**Figures 5A–D**).

P. indica Reduces the Arsenic Load in Rice Plant

We compare the absorption of arsenic through the plant root in colonized and non-colonized state of the hydroponic culture. It was observed that pre-colonized plant root accumulated arsenic up to 26.22 ± 0.81 mg/g dry weight (40 fold increase) while non-colonized arsenic-treated plant root accumulates arsenic up to 0.65 ± 0.01 mg/g dry weight. The accumulation of arsenic in the shoot of pre-colonized plants was 0.039 ± 0.003 mg/g dry weight (55 fold decrease) and arsenic content in the shoot of non-colonized arsenic treated plants was 2.16 ± 0.44 mg/g dry weight. Interestingly, colonization alters the distribution of arsenic in the plant system and was mainly restricted to the root system of colonized plants (26.22 ± 0.81 mg/g dry weight) and a fraction of it translocated to shoot (0.039 ± 0.003 mg/g dry weight, **Table 2**). Results suggest a significant reduction in arsenic translocation from root to shoot in case of pre-colonized plant. The translocation factor is decreased to 1.48×10^{-3} in this treatment as compared to arsenic treated non-colonized plant where it was 3.32. A similar pattern was also observed in the case of simultaneous and alternate treatment of arsenic and endophyte in long-term experimental up to 25 dai (**Table 2**). Dithiozone assay was also done to visualize arsenic accumulation in the shoot. We observed the endophyte colonization restrict the arsenic distribution into the root, and shoot receives comparatively less arsenic than arsenic-treated plant and therefore shoot stained lighter (Supplementary Figure 2).

P. indica Can Tolerate Arsenic Axenically

In the axenic culture, *P. indica* is able to tolerate both sodium arsenate (As V) and sodium arsenite (As III) up to 1 mM. However, the growth of fungus was reduced 30 and 50% in presence of 1.5 mM As V and As III respectively. At 2 and 2.5 mM concentration of arsenic the growth of fungus was reduced up to 70% (**Figures 6A,B**).

P. indica Adsorbs Arsenic on Cell Wall and Accumulates in Vacuoles

Adsorption study was done to show the ability of the cell wall to capture dissolved toxic arsenic from the media. It was observed that cell wall was able to adsorb arsenic in a non-linear fashion and suggesting increasing the concentration satiate the As adsorption (**Figure 6C**). Adsorption of arsenic was 28.8 and 20% at 0.1 and 2 mM arsenic respectively. Arsenic adsorption saturated at 4 mg per gram cell wall (**Table 3**). To confirm this TEM analysis of the fungal cell was done to localize arsenic in the cell. We noticed an electron dense layer on the cell wall and vacuoles in cells treated with arsenic when treated with 100 μM arsenic salts (**Figures 7B,C**). Further, we also detected insoluble precipitates of arsenic in both TEM and SEM analysis (**Figure 8**). EDAX analysis also reveals the vacuolar content and precipitation of arsenic around cell wall, contains up to 0.53 and 3% arsenic respectively (Supplementary Figures 3, 4 and Supplementary Tables 1, 2). Some marked modification also observed in the arsenic-treated cell such as vacuolization, cell expansion, and changes in cell wall (**Figures 7**, **8**).

TABLE 3 | Adsorption studies of Arsenic on the cell wall.

S.No.	Sample Treatment (for 60 min.)	Amount of Arsenic adsorbed (per gram fresh weight of cell wall)
1	0.1 mM As (V)	0.216 ± 0.06 mg
2	1 mM As (V)	1.694 ± 0.43 mg
3	2 mM As (V)	2.913 ± 0.23 mg
4	50 mM As (V)	3.873 ± 0.16 mg
5	1 M As (V)	4.221 ± 0.13 mg

One gram of cell wall was incubated in 100 ml of arsenic solution for 60 min. and cell wall material was washed with 1X PBS three times. Adsorption analysis was done in triplicates independently.

DISCUSSION

In the natural habitat, almost all terrestrial plants make an association with arbuscular mycorrhizal fungi (AMF) except a limited number of plant (such as members of Amaranthaceae, Chenopodiaceae, Cyperaceae, Juncaceae, Proteaceae or with lupines, and Brassicaceae), resulting in a range of beneficial consequences to their hosts. AMF improves nutritional status, impart resistance to soil borne pathogens and tolerance to salt, drought, and heavy metals (Kumar et al., 2009, 2011; Orlowska et al., 2012; Jogawat et al., 2013; Spagnoletti and Lavado, 2015). Previous works have demonstrated the benefits of P. *indica* over AMF and its application (Varma et al., 1999; Kumar et al., 2009; Yadav et al., 2010; Jogawat et al., 2013). In the present study, we have established the role of *P. indica* in the bioprotection against arsenic toxicity and restricted accumulation of arsenic in host plant. In this study, we found that treatment of arsenic to plant reduces overall biomass, root, and shoot growth in rice and hampers the overall plant growth. *P. indica* colonization in plants helps in recovery from the hampered growth arising due to arsenic toxicity. Growth recovery and increase in biomass indicating the detoxification capacity of the *P. indica*. We have shown that the recovery in plant growth from arsenic stress in presence of *P. indica* is due to reduced arsenic translocation to shoot, immobilization of arsenic into root, storage of arsenic in fungal cells, and reprogramming the host cell redox status.

Our result showed that arsenic does not affect the colonization in the rice root initially but induces hyper-colonization in the arsenic-treated plant root without hampering physical traits (**Figure 1** and **Table 1**). Colonization of *P. indica* in rice plants significantly lowers the susceptibility to arsenic. *P. indica* improved plant growth, biomass, and root integrity even in

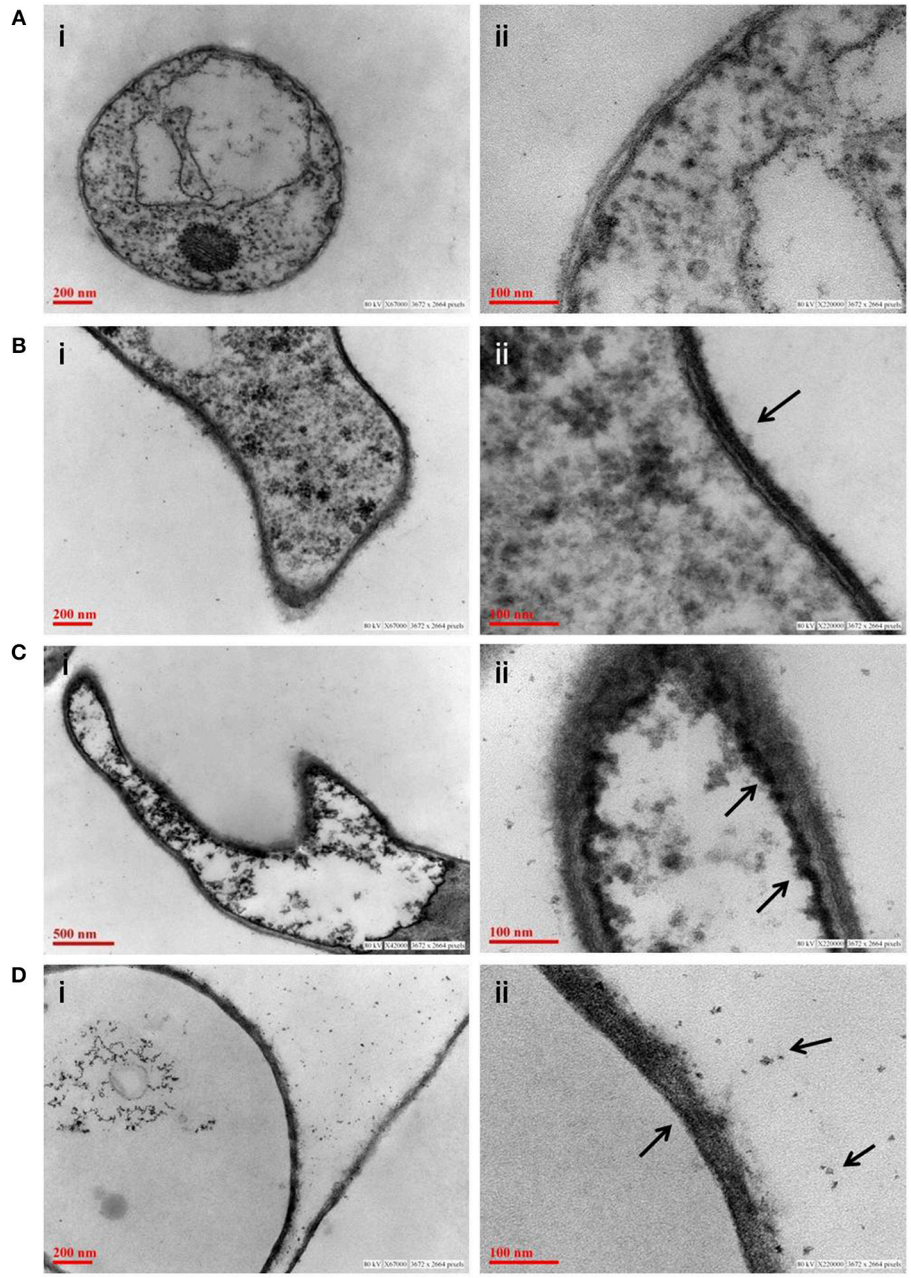

FIGURE 7 | Transmission electron micrograph of fungus *P. indica* showing arsenic adsorption, accumulation and precipitation. (A) Untreated fungus; **(B)** accumulation of arsenic on cell wall of fungus (marked with arrows) treated with sodium arsenate (As V); **(C)** accumulation of arsenic on in vacuole of fungus (marked with arrows) treated with sodium arsenate (As V); **(D)** synthesis of insoluble precipitates of arsenic (marked with arrows) on cell wall of fungus treated with sodium arsenite (As III).

presence of toxic dosage of arsenic. *P. indica* induces abiotic-tolerance and arsenic resistance in hosts (**Figures 1**, **2**). It was observed that the arsenic treatment reduces the chlorophyll b only but no change was observed for chlorophyll a and carotenoids. Endophyte colonization helps the plants in recovery from arsenic-induced chlorosis by enhancing the chlorophyll b contents in the arsenic-treated plants (**Figure 3A**). However, no improvement in anthocyanin content was observed under the influence of fungal colonization (Supplementary Figure S5). Further, we observed a significant higher proline content in the colonized plants (both treated and untreated) than the arsenic-treated and control plants (**Figure 3B**). It has been observed that endophyte induces proline accumulation in the plant (Jogawat et al., 2013) which help to overcome the adverse effects of ROS by pro-pro cycle and reduces the damage to plant (Liang et al., 2013; Signorelli et al., 2014). We observed 1.9 times

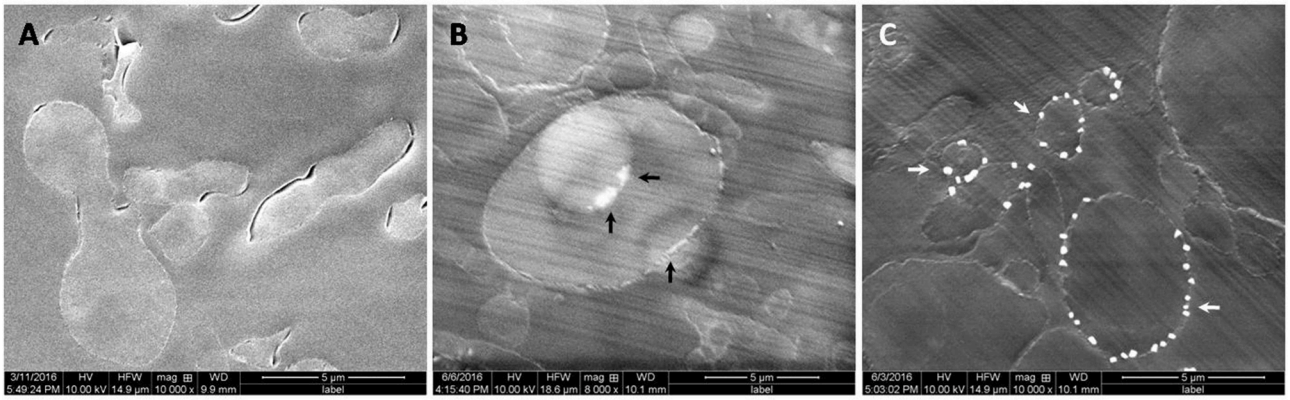

FIGURE 8 | Scanning electron micrograph of fungus *P. indica* showing arsenic adsorption, accumulation, and precipitation. (A) Untreated fungus; **(B)** accumulation of arsenic in vacuoles and on cell wall of fungus (marked with black arrows) treated with arsenic (100 μM As); **(C)** Synthesis of insoluble precipitates of arsenic (marked with white arrows) on cell wall of fungus treated with arsenic (500 μM As).

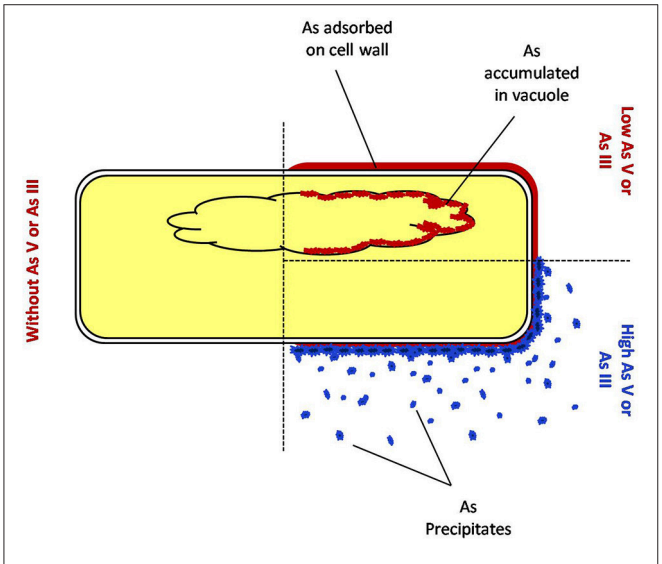

FIGURE 9 | Mechanism of detoxification of arsenic by fungus endophytic fungus *P. indica*.

higher accumulation of hydrogen peroxide (H_2O_2) in arsenic-treated rice plant than control (**Figure 3C**) as shown previously (Nath et al., 2014). At the same time arsenic treatment to rice plant significantly induces CAT (**Figures 4A**, **5A**) and GR (**Figures 4B**, **5B**) activity but a reduction in GST (**Figure 4C**) and SOD (**Figure 4D**) activity was observed, which may impair the antioxidative system of the plant and results in the accumulation of H_2O_2. *P. indica* colonization significantly reduces H_2O_2 content in arsenic treated plants but was still higher than the control plants. However, untreated colonized plants do not show any significant accumulation of H_2O_2. It is evident that an established mutualism between plant and fungus *P. indica* suppresses the accumulation of H_2O_2 in colonized plant (Schäfer et al., 2007) and this may be a possible explanation of reduction of H_2O_2 content in treated plants colonized by fungus. *P. indica* strongly induces CAT, GR, and restore the SOD activities in treated colonized plant which help plant to reduce reactive oxygen species and minimize damage caused by arsenic-mediated oxidative stress. It is suggesting that *P. indica* induces a systemic redox reprogramming leads to induction of resistance in plant and helping plant to recover its biomass and normal proliferated roots.

Interestingly, this was observed that a lower amount of arsenic was accumulated in shoot in all the cases of *P. indica* colonized treated plants as compared to arsenic-treated plants (**Table 2** and Supplementary Figure 1) and colonization of endophyte in the root reduces the translocation factor drastically in the plant (Marchiol et al., 2004). These results suggest that reduction in arsenic accumulation in shoot is either due to *P. indica* mediated induction of immobilization of arsenic in plant root or *P. indica* itself function as an arsenic-screen and restricting arsenic to be translocated to the shoot. We performed assays and observed that there is no inhibitory effect on fungal growth of arsenic at a concentration of 1 mM which revealed that endophyte has tolerance to arsenic toxicity (**Figures 6A,B**). Further, we observed that fungal cell wall has a great capacity to adsorb arsenic on the cell wall (**Figures 6C**, **7**) and which is up to 4 mg per gram fresh weight of cell wall (**Table 3**). This result suggesting that the induced arsenic-tolerance imparted by the fungus is due to selective clearance of arsenic by the extra-radical hyphae of fungus from the media and this is why root receives a large amount of arsenic while a small fraction of it can mobilize to shoot. In colonized state, extra-radical hyphae may apply alternate strategy to remove a large amount of arsenic which is not possible by adsorption only, as it requires a large amount of fungal cell to adsorb arsenic. This hypothesis was supported by one of our observation in which arsenic exposure induces a vacuolization (formation of multiple or larger vacuoles) in *P. indica* and these vacuoles store a significant amount of arsenic (**Figures 7C**, **8**). Therefore, it is suggested that the process of compartmentalization of arsenic may be a unique

mechanism which may also play role in detoxification of the arsenic during colonization and that has to be studied. In a previous study a metal tolerant fungus, *Exophiala pisciphila*, (H93) was shown to accumulated over 5% Cadmium (Cd) of its dry weight intracellularly (Zhang et al., 2008) and enhances colonized maize plant's tolerance to Lead, Zinc, and Cd (Li et al., 2011). Similarly, our study has also revealed that the endophyte imparts tolerance to arsenic using not only vacuolar entrapment and cell wall adsorption but it also precipitates arsenic out side of the fungal cell (**Figure 8**). We observed arsenic precipitation around cell wall containing arsenic up to 3% and this may also play important role in the immobilization of arsenic. The process of immobilization of arsenic into the root is mainly due to adsorption, compartmentalization into the vacuole and transforming arsenic into precipitates (**Figure 9**). These fungal mediated processes reduce the amount of soluble arsenic around colonized root and therefore minimize the arsenic transportation and accumulation in the shoot. The bioprotective and arsenic screening nature of *P. indica* can be utilized in the agricultural field contaminated with arsenic.

The fungal mediated tolerance against arsenic in plant is led by decrease in free or available arsenic in the surroundings with an additive effect on the anti-oxidative metabolite and anti-oxidative enzyme system of the plant. The plants possess a potent cellular environment to deal with arsenic mediated toxicity such as reactive oxygen species (ROS) in which both the anti-oxidative metabolites and enzyme systems play a crucial role. However, the susceptible varieties of plant were unable to induce both of these systems (Rai et al., 2011; Shankar et al., 2016). It was interesting to note that in the present study, both parameters enhanced under arsenic stress in the colonized plants (sensitive variety; IR 64) which does not negatively affect biomass yield of rice. Further, the capacity of immobilization of arsenic by *P. indica* reduces the arsenic load in rice shoot makes crop safer to consume. Exploitation of axenically culturable endophytic fungus *P. indica* may not only add value to modern crop-growing strategies in arsenic contaminated area but may also serve as a model system to study molecular traits of metal resistance in plant-microbe interaction and bioremediation.

AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration between all authors. Fungal colonization analysis, Antioxidant enzyme activities in rice plants and Estimation of Chlorophyll and Proline estimation were done by SM and MK. Dithizone test and Estimation of H_2O_2 production was done by JShukla and SM. Arsenic adsorption analysis was done by MK. Transmission Electron Microscope, Scanning Electron Microscope and EDAX analysis were done by JShukla, ASK, MK, JShankar, NA, and PNS. Confocal imaging was done by SM and RN. Arsenic accumulation studies in plants were done by SM. Arsenic estimation in plants was done by KM and SKR. MK defined the research theme. MK coordinated the project and wrote the manuscript. All authors have contributed to, seen and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00754/full#supplementary-material

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Non-edible Oil Cakes as a Novel Substrate for DPA Production and Augmenting Biocontrol Activity of *Paecilomyces variotii*

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The present study investigated the use of waste non-edible oil cakes (Jatropha, Karanja, Neem, and Mahua) as a substrate for the growth of *Paecilomyces variotii* and dipicolinic acid (DPA) production. Previous researches proved the efficacy of DPA in suppressing certain pathogens that are deleterious to the plants in the rhizosphere. DPA production was statistical optimized by amending non-edible oil cakes in growing media as nitrogen and sugars (Dextrose, Glucose, and Lactose) as carbon source. Plackett-Burman design (PBD), indicated that Jatropha cake, Karanja cake, and Dextrose were the most significant components ($p < 0.05$) of the media and were further optimized using response surface methodology (RSM). Jatropha cake, Karanja cake, and Dextrose at the concentration of 12.5, 4.5, and 10 g/l, respectively, yielded 250 mg/l of DPA, which was 2.5 fold more than that obtained from basal medium. HPLC analysis of the optimized medium (peak at retention time of 30 min) confirmed the enhanced DPA production by *P. variotii*. The scanning electron microscopy (SEM) images showed that optimized medium impose a stress like condition (due to less C:N ratio) for the fungus and generated more spores as compared to the basal medium in which carbon source is easily available for the mycelial growth. The antimicrobial activity of the fungal extract was tested and found to be effective even at 10^{-2} dilution after 72 h against two plant pathogens, *Fusarium oxysporum* and *Verticillium dahlia*. Statistical experimental design of this study and the use of non-edible oil cakes as a substrate offer an efficient and viable approach for DPA production by *P. variotii*.

Keywords: jatropha cake, karanja cake, dipicolinic Acid (DPA), *Paecilomyces variotii*, medium optimization, biocontrol

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INTRODUCTION

Previous researches proved the efficacy and adaptability of *Paecilomyces* in effectively controlling different pathogens under various environmental conditions (Dunlap et al., 2007; Anastasiadis et al., 2008; Sharma et al., 2012; Barakat and Saleh, 2016). *Paecilomyces* is morphologically characterized by having flask-shaped phialides, which is differentiated into swollen base and a distinct neck and generate conidia that are arranged end to end in a chain. *Paecilomyces* species

is known to utilize a variety of substrates due to its ability to consume high ammonia and nitrogen rich substrates (Liu et al., 2016). Furthermore, produces bioactive substances such as dipicolinic acid (DPA), which has been reported to suppress certain pathogens that are deleterious to the plants in the rhizosphere (Asaff et al., 2005, 2006). Use of microbial inoculants as biocontrol agents for controlling disease and pests of crop plants is continuously gaining attention as an alternative tool against the use of chemical pesticides in developing countries (Dale et al., 2007). Very few experimental studies have been reported on the DPA production from *Paecilomyces* strain and its use as an agent for control of plant pathogens.

Reported by several workers that media constituents simply affect the virulence of biocontrol agents against plant pathogens (Cliquet and Jackson, 2005; Nisha and Kalaiselvi, 2016) and its composition plays a significant role in growing fungal mycelia with increased sporulation (Sun and Liu, 2006; Gao et al., 2007). Liu and Chen (2002) reported that fungi can use wide range of substrates as carbon and nitrogen sources. However, low cost and simple media will be a better choice for their mass-production and also for field application. For successful use of a mycopesticide in biocontrol, it is essential to produce high yields of propagules which are pathogenic toward target pathogen. The quality as well as quantity of propagules is affected by medium composition and culture conditions (Gupta et al., 2016). Carbon-Nitrogen ratio as well as type of sugar in media plays an important role in determining the pathogenicity of the fungus (Vidal et al., 1998; Gao et al., 2007; Zhao et al., 2013; Nisha and Kalaiselvi, 2016). Therefore, nitrogen and carbon sources were screened for the production of bioactive molecule because they may influence the activity of phytopathogens, and enhances antibiotics production in biocontrol strains and contribute to the variability of biocontrol in different soils and also on host crops (Milner et al., 1995; Thomashow and Weller, 1996; Cliquet and Jackson, 2005; Moorthi et al., 2015).

Previously, media optimization studies reported the use of one factor at a time for various biomolecules production which is laborious and time consuming process, it also often misleads the understanding of the system behavior, generates confusion, and variation in prediction (Singh and Chhatpar, 2010; Saharan et al., 2011; Nisha and Kalaiselvi, 2016). Plackett and Burman's design (PBD), when applied to evaluate medium components, will screen the insignificant factors out of a large number of possible factors at fairly early stages of the experiment. Box Behnken Design (BBD) is useful in determining the effect of key factors, by minimum number of experiments for further optimization (Singh and Chhatpar, 2010; Zhao et al., 2013). No published reports to the best of our knowledge are available for increased production of the bioactive DPA from *P. variotii* using response surface methodology (RSM). The findings of this study strongly support the hypothesis that better yields can be achieved by optimization of C and N sources using RSM technique.

MATERIALS AND METHODS

Microorganism and Culture Condition

Paecilomyces variotii strain employed in this study was procured from the culture collection of International Centre for Agriculturally Important Microbes (ICAIM), PUSA, New Delhi, India. The potato dextrose agar (PDA) was used to grow the fungus on plates at 28°C for 7 days and it stimulated the production of conidia. These conidia were sub-cultured under submerged fermentation (SmF) in Erlenmeyer flask (500 ml) containing 100 ml of the medium containing (per liter): Glucose (30 g), yeast extract (3 g), KH_2PO_4 (0.39 g), $Na_2HPO_4.12H_2O$ (1.42 g), $MgSO_4.7H_2O$ (0.60 g), NH_4NO_3 (0.70 g), and KCl (1.00 g) (Fargues et al., 1992). The medium pH was adjusted to 5.6 before sterilization and inoculated with 4 ml of 10^7 conidia/ml from conidial suspension. The flasks were then incubated at 27°C for 7 days in an incubator (Scigenics Biotech, India) and the inoculum thus prepared was used for harvesting conidial spores. The spores were collected in sterile solution (0.05% Tween 80) and 50% glycerol stocks were maintained from Tween 80 conidial suspension culture and stored at −20°C. This spore suspension was utilized as inoculums in all further experiments. For using oil cakes as media, the non-edible oil cakes i.e., Jatropha (*Jatropha curcas*), Karanja (*Pongamia pinnata*), Neem (*Azadirachta indica*), and Mahua (*Madhuca indica*) were oven dried at 55°C for 24 h, grinded, and sieved to obtain 0.8- and 2.0-mm particle size. C:N ratio in the cakes was analyzed. Oil cake was soaked in water overnight, then heated for 2 h and final volume was adjusted by adding water to prepare cake broth.

Analytical Methods

Biomass Estimation

Gravimetric analysis was used to determine biomass in submerged fermentation as described by Soundrapandian and Chandra (2007). The liquid from flask was filtered through pre-weighed Whatman filter paper Grade 1 (pore size, 11 μm) and dried at 70°C under vacuum (0.6 atm) until reaching the constant weight, by determining the weight difference of the filter paper. Spore count was done with a Neubeaur's hemocytometer.

Chemical Analysis of the Samples

The culture filtrate was centrifuged at 10,000 rpm for 10 min and supernatant was filtered through nylon filter (pore size 0.45 μm) and divided into two parts for analysis. One part was kept for spectrophotometric analysis of DPA and second part (50-ml aliquots) was freeze-dried and then extracted using sonication with 50 ml methanol acetate at 25°C for 10 min.

Qualitative Analysis

The extracts with methanol and ethyl acetate were used for Thin Layer Chromatography (TLC). Silica gel GF_{254} plate (E. Merck, Dramstadt, Germany) was used with 1-butanol/water/acetic acid (12:5:3, by vol.) solvents. The plates were exposed to UV light (254 nm) to visualize the bands and confirmation was done by spraying a 0.051 M cerium ammonium sulfate solution and heating on a hot plate for color development. A DPA standard (Sigma) (Rf 0.90) was used for determining the presence of DPA (Asaff et al., 2005).

High-Performance Liquid Chromatography (HPLC) analysis of the collected samples were performed using a Thermo-separation (Constametric 4100) chromatograph using following conditions: UV detector and a Phenomenex OOH-0138-KO column, temp 30°C, mobile phase 5 mM H_2SO_4, flow rate of

0.6 ml/min and 210 nm wavelength for compound detection (Asaff et al., 2005).

DPA Estimation

DPA content was quantified using a UV-visible spectrophotometer (Asaff et al., 2006). Sample (0.8 ml) to be quantified was mixed with 0.2 mL of ferrous ammonium sulfate (1 g) and ascorbic acid (1 g) and dissolved in 100 ml of 0.5 M acetate buffer and with a final pH of 5.5. Absorbance was measured at 440 nm wavelength in UV-visible spectrophotometer (PerkinElmer model Lambda 25 UV/VIS, Massachusetts, USA).

Experiment Design

Culture medium was optimized using non-statistical as well as statistical experimental designs. Medium components were selected by using one-factor-at-a-time approach. The preliminary experiments revealed the optimal range of carbon (Glucose, Dextrose, and Lactose) and nitrogen sources (Jatropha oil cake, Karanja oil cake, Neem oil cake, and Mahua oil cake) and its effect on sporulation and DPA production by the fungus (**Table 1**). This data was utilized for designing further experiments using PBD.

All experiments were performed in triplicate in 500 ml Erlenmeyer flasks with 100 ml working volume. The average crude DPA concentration and spore count were taken as the dependent variables or response (Y). A correlation was also determined between production of DPA and spore of the fungus.

PBD for Identification of the Significant Variables

To increase DPA production, carbon and nitrogen sources were optimized using PBD. PBD screen "n" variables in just a minimum of "n + 1" number of experiments (Plackett and Burman, 1946). It was used to identify the relatively important medium constituents i.e., C and N sources. PBD design evaluates the impact of main factors, as well as their interaction in production of DPA and produce optimal or near optimal responses. A total of seven factors were studied as described in **Table 2** including their low (−1) and high levels (+1). The PBD was based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

Where Y is the response (DPA production), β_0 is the model intercept, and β_i is the variable estimates. The factor level with confidence level above 95% is considered the most significant factor that affects the DPA production. A 25 PBD leading to 30 sets of experiments were performed in triplicate to verify the most significant factors affecting the DPA production, as shown in **Table 3**.

Optimization of the Selected Trace Concentration by Response Surface Methodology (RSM)

RSM is an efficient method to answer multivariate problems and optimizing several responses by minimizing the number of experiments (Kumar et al., 2016). Box Benkhen design (BBD) and

TABLE 1 | Growth and bioactive substance (DPA) production by *P. variotii* on non-edible oil cakes used as solid medium and broths (after 10 days).

Non-edible oil cake	Treatment	Solid medium		Broth		
		Growth/day (cm/day)	Spore count (spores/g of substrate)	Dry mycelial weight (g/100 ml)	Spore count (spores/ml)	DPA production (mg/l)
C1	Alone	0.42 ± 0.01^{c}	6.7×10^{9}	0.29 ± 0.04^{d}	2.7×10^{9}	102 ± 3.5
	+ PDA (1:1)	0.35^{c}	9.0×10^{9}	0.15 ± 0.016^{a}	7.9×10^{8}	110 ± 1.2
	+ dextrose	0.36^{d}	5.1×10^{10}	0.31 ± 0.02^{d}	3.4×10^{9}	135 ± 2.3
C2	Alone	0.44^{d}	5.1×10^{9}	0.271 ± 0.02^{d}	2.0×10^{9}	70 ± 1.2
	+ PDA (1:1)	0.32 ± 0.02^{d}	6.2×10^{9}	0.135 ± 0.005^{a}	3.1×10^{8}	78 ± 3.5
	+ dextrose	0.23 ± 0.01^{c}	2.9×10^{10}	0.28 ± 0.04^{d}	1.9×10^{9}	83 ± 1.3
C3	Alone	0.25 ± 0.02^{d}	4.5×10^{9}	0.275 ± 0.02^{d}	2.1×10^{9}	108 ± 2.1
	+ PDA (1:1)	0.31 ± 0.02^{d}	1.0×10^{9}	0.13 ± 0.01^{a}	3.0×10^{8}	116 ± 1.1
	+ dextrose)	0.35 ± 0.01^{d}	2.1×10^{10}	0.28 ± 0.04^{d}	1.8×10^{9}	136 ± 2.5
C4	Alone	0.18 ± 0.015^{b}	7.1×10^{8}	0.20 ± 0.03^{b}	10×10^{7}	60 ± 3.4
	+ PDA (1:1)	0.18 ± 0.01^{c}	8.3×10^{7}	0.12 ± 0.04^{a}	8.7×10^{7}	52 ± 1.8
	+ dextrose)	0.20^{c}	7.7×10^{7}	0.18 ± 0.01^{b}	6.5×10^{7}	45 ± 3.4
Control	PDA (Potato Dextrose Agar)	0.26 ± 0.01^{c}	2.5×10^{9}	0.249 ± 0.01^{c}	1.5×10^{9}	91 ± 1.0

C1, Jatropha oil cake; C2, Neem oil cake; C3, Karanja oil cake; C4, Mahua oil cake. Same letter used in each column indicates insignificant difference among the treatments ($p < 0.05$) according to DMRT. Value are means of $n = 4$.

TABLE 2 | Levels of various Carbon and Nitrogen sources tested in Plackett-Burman Design (PBD).

Variables code	Independent Variables	Levels (g/l)	
		Low level −1	High level +1
x_1	Glucose	15	60
x_2	Dextrose	15	60
x_3	Lactose	15	60
x_4	Karanja	10	40
x_5	Jatropha	12	48
x_6	Neem	12	48
x_7	Mahua	17	56

PBD are part of RSM. In this experiment PBD was used initially to select the components affecting DPA production and BBD was used to optimize the major variables designated as x_1, x_2, and x_3. Seventeen experimental runs were generated by the software comprising of different combinations of three factors. A second-order polynomial function was fitted to correlate the relationship between independent variables and response for predicting the optimal point. For three factors this equation is

$$Y_{pred} = \beta_o + \sum \beta_i x_i + \sum \beta_{ij} x_j^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

Where, Y_{pred} is the predicted response, xi and x_j are the input variables which influence the response Y. β_i is the i^{th} linear coefficient, β_{ij} is the ij^{th} interaction coefficient and β_o is the constant. A quadratic polynomial equation was used to fit the data obtained.

Software for Experimental Design

The "Design-Expert" software version 7.0 (Stat-Ease, Inc., Minneapolis, USA) was used for the construction and evaluation of the statistical experimental design. The RSM contour plot analysis provides the optimal C and N values for DPA production.

Preparation of *P. variotii* Samples for Morphological Study

The *P. variotii* cells were harvested by centrifugation from the run showing a maximum yield of DPA and control after the completion of BBD. The harvested cells were prefixed with 2.5% glutaraldehyde solution overnight at 4°C. The cells were centrifuged and washed thrice with 0.1 M sodium phosphate buffer (pH 7.2) and serial dehydration of the cells was done using different gradient of ethanol. Later, the cells were dried at "critical point" (Tyagi and Malik, 2010).

For SEM standard protocol was used to study the morphology of the *P. variotii* cells under SEM (ZEISS EVO 50, Germany). The SEM images were clicked using following parameters: EHT = 20.00 kV, WD = 12.0 mm, Signal A = SE1.

Biocontrol Activity *In vitro*

The antagonistic activity of the *P. variotii* against two phytopathogenic fungi *Fusarium oxysporium* and *Verticillium dahilae* was studied by dual culture test by following the method of Rahman et al. (2009). *P. variotii* was inoculated on both the sides of Potato dextrose Agar (PDA) plate which contained 6 mm disc of pre-grown phytopathogenic fungi at the center. The biocontrol activity of *P. variotii* against the fungal pathogens was observed in terms of measuring the diameter of zone of inhibition. Phase Contrast Microscope (Leica Model, DME, Germany) at 10 and 40X magnification after 2–5 days of incubation at 28°C was used for identification of fungal mycelium along the edges of the inhibited colonies facing *P. variotii*.

RESULTS AND DISCUSSION

Carbon and nitrogen are known to be most essential nutrients required by fungi for their growth. C:N ratio in the medium is important since it significantly affects the spore yield of the bioherbicidal fungi (Engelkes et al., 1997; Gao et al., 2007). Media having 15:1 C:N ratio promote more conidial production along with biocontrol efficacy. Results showed that C:N ratio is more important for the sporulation of *P. variotii*. The cakes of Jatropha, Karanja, Neem, and Mahua were found to have C:N ratios of 2.76, 2.29, 3.28, and 6.3 respectively.

PBD for the Screening of Important Factors for DPA Production

Seven medium elements which have the possibility to influence the production of active substance, namely Glucose, Dextrose, Lactose (carbon sources), and Jatropha oil cake, Karanja oil cake, Neem oil cake, and Mahua oil cake were selected for a 2-level PBD. The predicted regression equation for the DPA (Y) production as a function of the coded values of critical variables is given in the following equation:

$$\begin{aligned} Y = {} & 98.68 - 6.09x_1 + 5.09x_2 - 3.97x_3 + 6.75x_4 + 4.53x_5 \\ & - 0.97x_6 - 1.47x_7 - 9.70x_1x_2 - 5.39x_1x_3 - 5.6139x_1x_4 \\ & - 3.53x_1x_6 - 0.84x_1x_7 + 3.94x_2x_4 + 4.92x_2x_5 + 1.27x_2x_7 \\ & + 4.30x_3x_7 + 5.67x_4x_5 - 3.32x_4x_6 + 1.57x_5x_6 \end{aligned} \quad (3)$$

From Equation (3) it can be observed that Karanja oil cake (x_4), due to its higher coefficient effect was the most significant factor. Although, the results have also given the picture that the Jatropha oil cake and dextrose are also necessary in certain amounts to switch on the maximum production of DPA. These factors contain some important elements that may influence the activity of some enzymes (Deqing, 2002; Saharan et al., 2011) and some enzymes included in non-ribosomal peptide synthetase (NRPSs). The biochemical mechanism related to the yield of DPA by non-edible oil cakes needs further research. The results of *F*-test showed significant difference ($p < 0.01$) for variance between the average of observation of two-level experiment and center point. The result revealed that the optimum point was in accordance with the design used in the experiment.

TABLE 3 | PBD and results of the fractional factorial design.

Run	x_1	x_2	x_3	x_4	x_5	x_6	x_7	DPA (mg/l)	
								Predicted	Observed
1	−1	1	1	−1	−1	−1	−1	89.4	88.2
2	−1	1	−1	−1	1	−1	−1	90.1	94.3
3	1	1	−1	−1	−1	1	−1	79.7	78.2
4	1	1	−1	−1	−1	−1	1	89.6	90.2
5	−1	−1	−1	1	1	1	−1	90.9	91.2
6	−1	−1	−1	1	−1	−1	1	88.6	88.6
7	−1	−1	−1	1	1	1	1	96.2	94.6
8	1	−1	1	1	1	−1	1	93.1	94.2
9	1	−1	−1	1	1	−1	−1	140.1	138
10	−1	−1	1	−1	−1	1	−1	96.2	98.4
11	−1	1	−1	−1	−1	1	1	99.4	100.6
12	−1	−1	1	1	1	−1	−1	104	105.4
13	−1	−1	1	−1	−1	−1	1	96.8	96.8
14	−1	1	−1	1	1	1	−1	152	155
15	1	1	1	1	1	−1	−1	94	91.2
16	1	−1	1	1	−1	−1	−1	95.2	94.2
17	1	−1	−1	−1	−1	1	1	97.3	98.8
18	−1	1	1	1	−1	−1	1	130.2	132
19	−1	1	1	−1	1	−1	1	93.3	94.4
20	1	−1	1	−1	1	−1	−1	91	89.8
21	−1	1	1	−1	1	1	−1	98.2	97.8
22	1	1	−1	1	−1	−1	−1	99.7	100.4
23	1	1	−1	−1	1	1	1	93	92.2
24	−1	−1	−1	−1	1	1	−1	93	92.3
25	−1	−1	1	1	−1	1	1	100	99.6
26	1	1	1	1	1	1	1	68.7	70.3
27	1	1	−1	1	−1	1	1	88.3	88.6
28	1	1	1	−1	−1	1	1	92.4	93.2
29	1	−1	−1	−1	1	−1	1	92	91.2
30	1	−1	1	1	1	1	−1	92.4	93.6

TABLE 4 | Coded and real values for Box Behnken design (concentration g/l).

Variables	Level of variables		
	−1	0	1
Dextrose	5	10	15
Jatropha	1	2	3
Karanja	2	4	8

Optimization of Selected C & N Sources by RSM Using BBD

Screening of the factors helped in the detection of gross curvature in the design space. The BBD was applied to estimate quadratic effects, pure process variability and reassess gross curvature, with active substances produced as response (Wang and Liu, 2008). Based on the variable identified by the PBD, a 3-level BBD was developed for variables significantly affecting DPA production (**Table 4**). The BBD of the three screened variables in coded format, along with DPA concentration as responses is depicted in **Table 5**. The maximum yield of DPA production, experimentally observed, was 250 mg/l in run 12. In order to get

TABLE 5 | Response from Box Behnken design experiment (conc. g/l).

Run	x_1	x_2	x_3	DPA (mg/l)
1	−1.000	0.000	−1.000	172.5
2	0.000	0.000	0.000	251.2
3	0.000	0.000	0.000	249.8
4	1.000	0.000	1.000	107
5	0.000	0.000	0.000	250.4
6	−1.000	0.000	1.000	138
7	−1.000	1.000	0.000	155
8	0.000	−1.000	1.000	130
9	1.000	0.000	−1.000	99.2
10	0.000	−1.000	−1.000	140
11	1.000	1.000	0.000	115
12	0.000	0.000	0.000	248.2
13	0.000	0.000	0.000	253
14	−1.000	−1.000	0.000	140
15	0.000	1.000	1.000	128
16	0.000	1.000	−1.000	175
17	1.000	−1.000	0.000	115

TABLE 6 | Significance of quadratic model coefficients for DPA production.

Factor	Coefficient values	*F*-values	*p*-value
Intercept	341.2	115.35	<0.0001
x_1	47.0	69.50	<0.0001
x_2	119.6	5.59	0.0501
x_3	225.5	16.99	0.0045
x_1^2	−2.66	1.09	0.3309
x_2^2	−13.1	8.68	0.0215
x_3^2	−97.1	6.64	0.0367
x_1x_2	0.37	363.08	<0.0001
x_1x_3	2.83	225.96	<0.0001
x_2x_3	−6.16	244.14	<0.0001

TABLE 7 | ANOVA for response surface quadratic model obtained for DPA production.

Source	Sum of Squares	Mean Square	DF	*F*-value	*P* > *F*
Model	53514.61	5946.07	9	115.35	<0.0001
Residual	360.85	51.55	10		
Lack of Fit	295.32	49.22	6	3.004	0.22
Pure Error	*65.53*	16.382	4		
Total	53875.46		19		

CV % = 4.26; R^2 = 0.9833; Adj R^2 = 0.9847; Predicted R^2 = 0.8962.
Value of "p >F" less than 0.05 indicate model terms are significant.
The CV value >4 indicates adequate precision in the model.

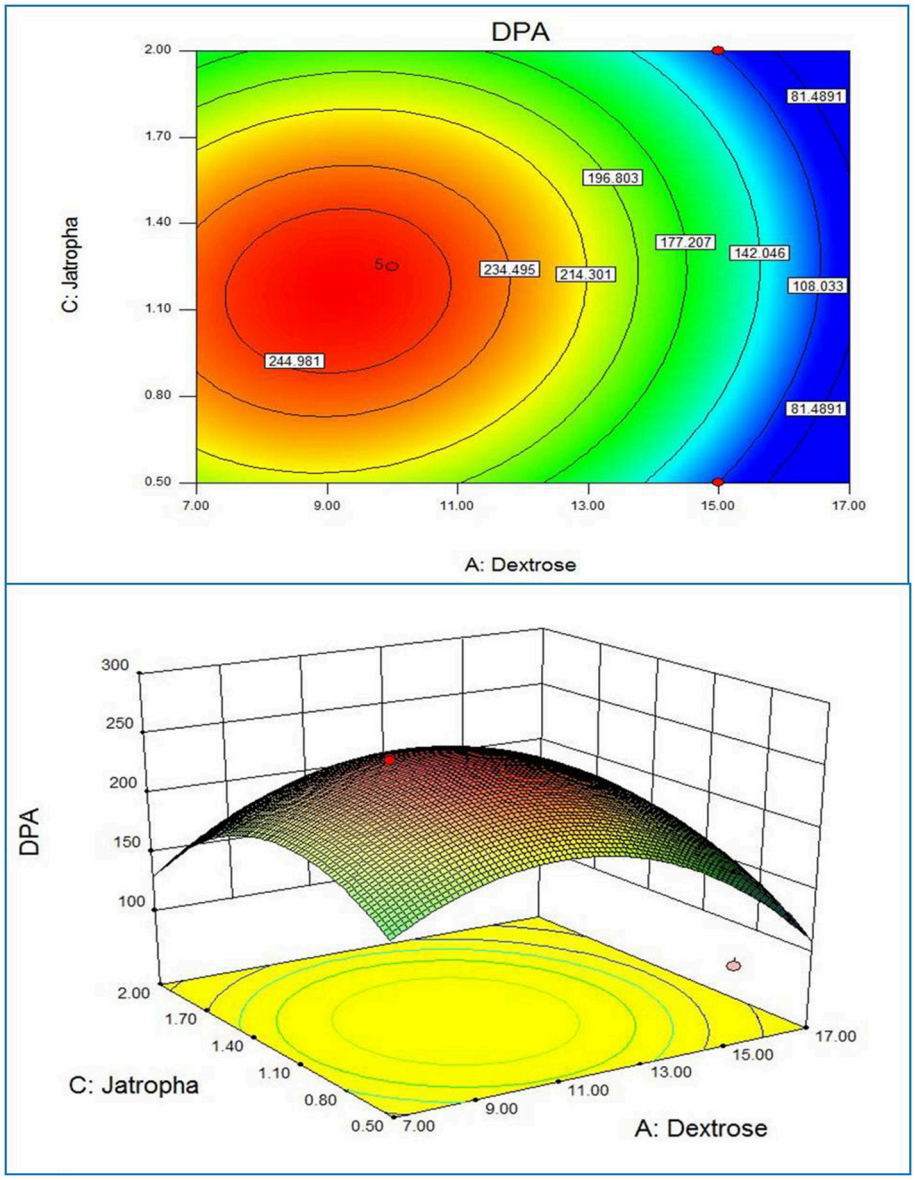

FIGURE 1 | 2D contour plot and 3D response surface curve of Jatropha oil cake and Dextrose predicted by the full quadratic model.

the optimum values of the variables which correspond toward maximum DPA production, a second-degree polynomial model was proposed. By applying a second-degree polynomial equation (Equation 4) in multiple regression analysis, explains the function of each variable and their interactions in the production of active substance:

$$Y_{pred} = 341.2 + 47.0x_1 + 119.6x_2 + 225.5x_3 - 2.66x_1^2 - 13.1x_2^2 - 97.1x_3^2 + 0.37x_1x_2 + 2.83x_1x_3 - 6.16x_2x_3 \quad (4)$$

Where Y is the predicted active substance (DPA) yield, x_1 is Dextrose, x_2 is Karanja oil cake, and x_3 is Jatropha oil cake.

In first order, main effect of Jatropha oil cake ($p = 0.0501$) and Karanja oil cake ($p = 0.0045$) are less significant than their quadratic main effect ($p < 0.001$) indicating the effect of Jatropha and Karanja oil cake in DPA production (**Table 6**). Similarly, the interaction of Dextrose—Jatropha ($p < 0.0001$) and Dextrose—Karanja ($p < 0.0001$) and the quadratic are also quite significant among all interactions. Results of second order response surface model using ANOVA (analysis of variance) are shown in **Table 7**. The low probability value obtained from Fisher's *F*-test [(P model > F) < 0.0001] showed the high significance of the model. The coefficient ($R^2 = 0.9833$) described the fitness of the model, which ascertained that > 98% sample variation was ascribed to the variables and only <2% of the total variance could not be explained by the model (**Table 7**). The model significance was also justified by the adjusted determination coefficient (Adj $R^2 = 0.9847$) and predicted determination

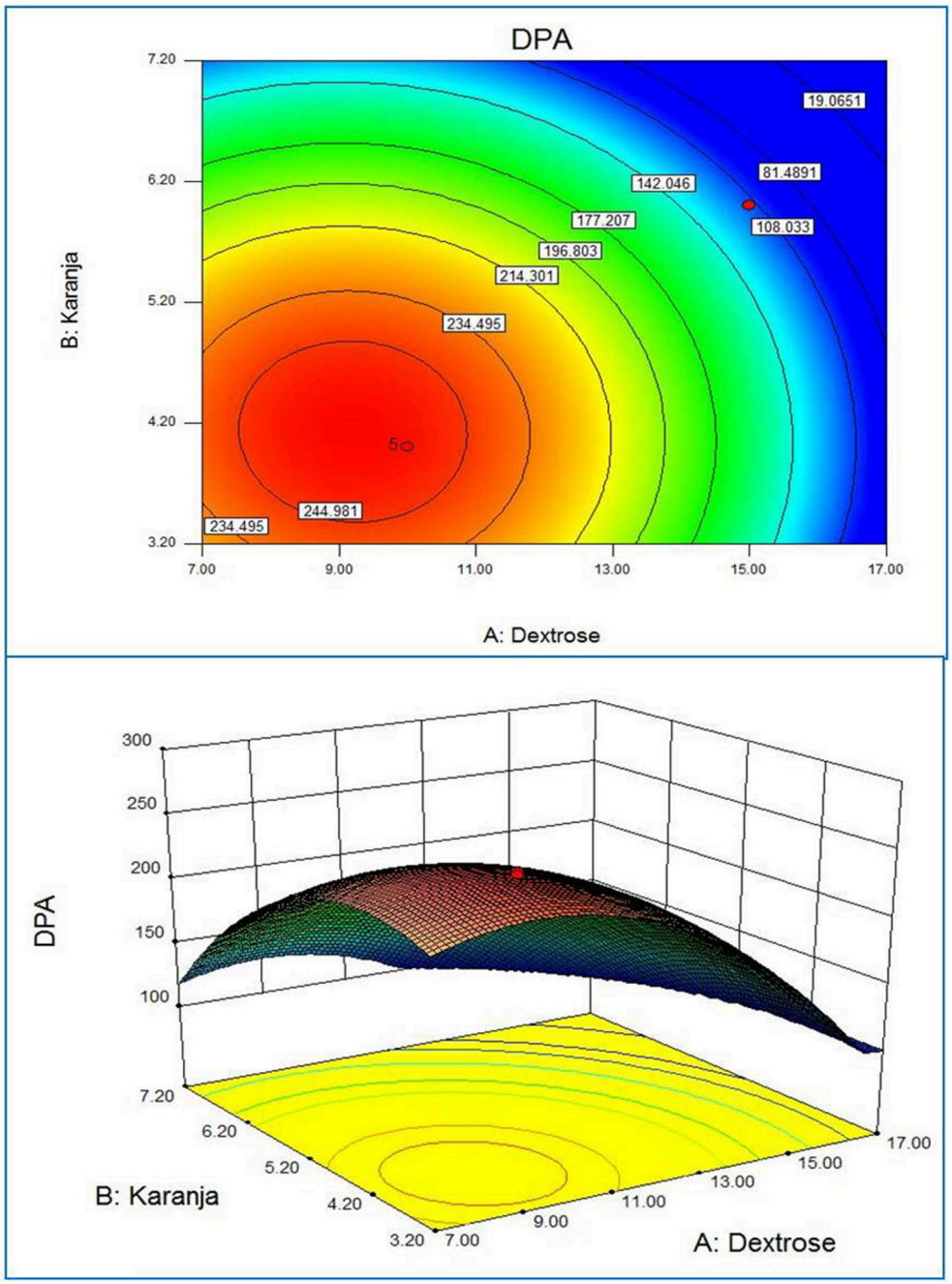

FIGURE 2 | 2D contour plot and 3D response surface curve of Karanja and Dextrose predicted by the full quadratic model.

coefficient (0.8962). The statistically insignificant lack of fit was also shown by model [(P model > F) = 0.22], making the model adequate for prediction within the range of variables employed. A coefficient variation having low value (CV = 4.26%) proposed the preciseness and reliability of experiments. The linear pattern in the normal plot of residuals, demonstrated normality in the error term, i.e., there were no indications of any problem in the data (Draper and Smith, 1981). The correlation coefficient of 0.94 reveals the strong positive correlation between the both spore formation and DPA production. Increase in the number of spore's leads to more production of DPA by the fungus.

Table 7 inferred the significance of each coefficient which was determined by F-test and P-value. The greater the magnitude of F-value the lesser the p-value, the more significant is the corresponding coefficient. The generated response surfaces of three variables tested expressed a linear effect on the response

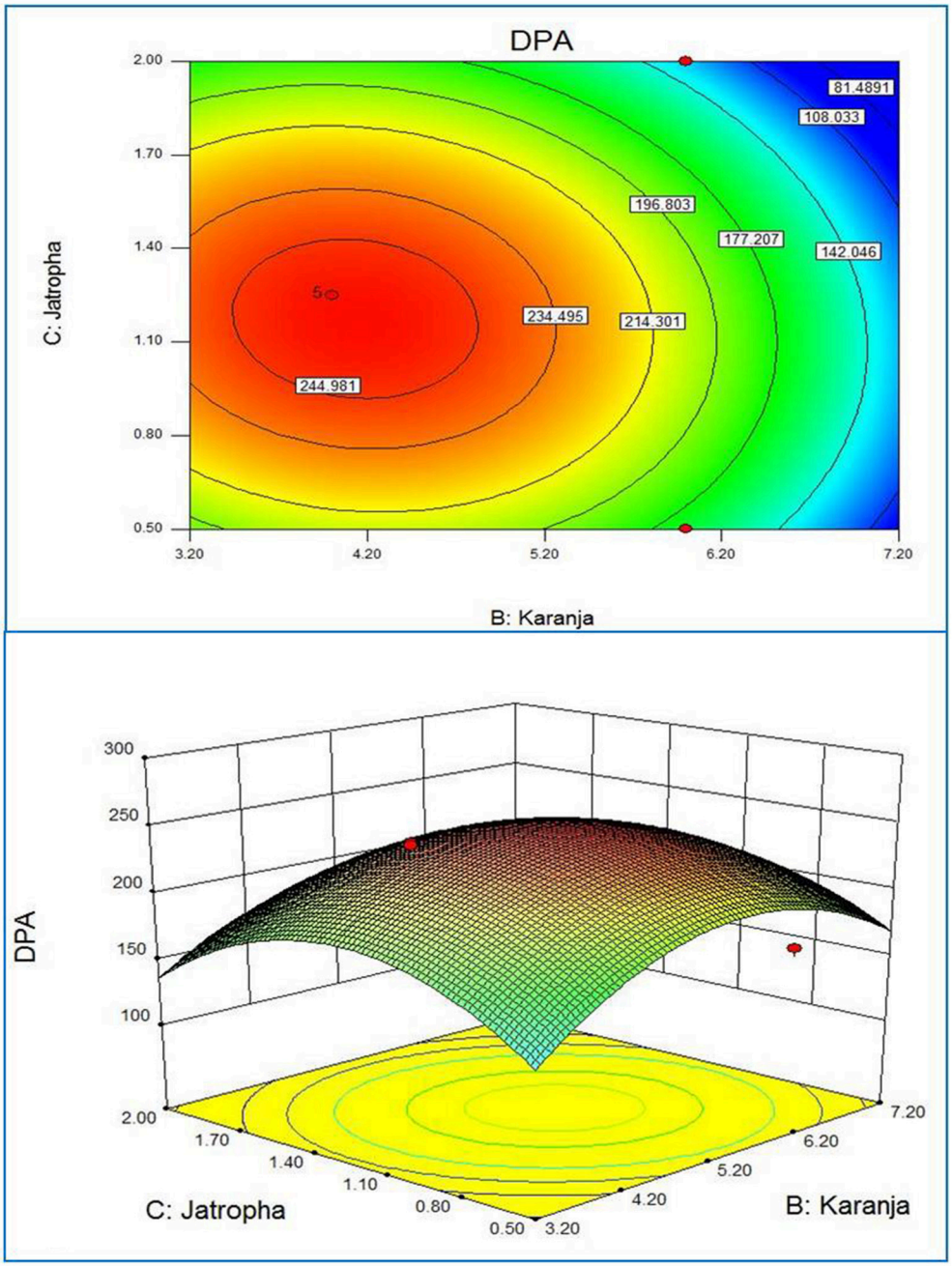

FIGURE 3 | 2D contour plot and 3D response surface curve of Karanja and Jatropha predicted by the full quadratic model.

($p < 0.001$). Dextrose, Karanja, and Jatropha showed a positive effect on the production of bioactive substance.

Response surface plots of the model generated by the software gave the visualization of the role of the independent variables on the dependent one (Xu et al., 2008). The RSM plot presents a system to foresee the yield of active substances for various values at different test variables and the contours of the plot generated are useful in recognition of the kind of interaction lies in test variables. The contour plots generated by using the fitted quadratic polynomial equation obtained from regression analysis are shown in **Figures 1–3**. Each figure shows the effect of two independent variables on the active substances production, while the other variable was held at zero level. The circular contours of the plot surfaces implicate the negligible interaction between the corresponding variables whereas an elliptical or saddle contours indicates the significant interactions between the related variables (Murthy et al., 2000). The effect of *Jatropha* oil cake and dextrose on the DPA production at the fixed Karanja oil cake level is

shown in **Figure 1**. Similarly, the **Figures 2**, **3** correspond to the effect of two variables on the production of DPA, holding the 3rd variable at zero level. All three cases, revealed a clear optimal convergence. The optimal levels provided three independent variables on DPA production. A significant interaction was noted between the carbon source dextrose and the nitrogen source Karanja and Jatropha oil cakes. The C:N ratio directly influenced the growth of the fungus and accumulation of the metabolites (Ramachandran et al., 2007; Abd EL-Aziz et al., 2015). Although the actual situation may be more complex than explained, an attempt for the medium optimization has been made by RSM.

Validation of the Predicted Concentration in the Optimal Medium

Based on medium optimization, the maximum production of DPA was 250 mg/l predicted by quadratic model when the dextrose, Karanja oil cake, and Jatropha were at 10, 40, and 12.5 g/l, respectively. Validation experiment was performed in a 5 l fermentor and compared with the predicted data from the model to verify the predicted results. Fermentor experiment was done only for validation of the results obtained from the batch fermentation and was carried out in exactly same conditions. The volume was increased from 100 ml to 5 l. Scale up experiment with 5l fermentor was done at 27°C and 150 rpm (Scigenics Biotech, India) for 7 days. In the 5 l fermentor, the average concentration of DPA in the broth was 373.5 mg/l, showing 50% increase in DPA yield, suggesting that the accuracy of the model is over 95%. Where as, the predicted value for DPA by the model was 251 mg/l which translates into a deviation of only 4.64%. The level of DPA production obtained using optimized carbon and nitrogen sources amendment was 2.5 folds higher than the non-optimized basal medium for fungus. This is the first report on DPA production optimization using RSM designs in synthetic medium.

Morphological Alteration of *P. variotii*

SEM images show the morphological changes in *P. variotii* spores grown in basal medium and in the oil cake (**Figure 4**). Spores in the best-selected concentration of oil cakes (Jatropha, Karanja) underwent morphological alterations considerably in comparison to the control when observed under SEM (**Figure 4**). Control spores are spotted round and spherical and less in number, also there is more mycelial content (**Figure 4A**) while the oil cakes treated spores are numerous and oval shaped having ornamented rough surface (**Figure 4B**).

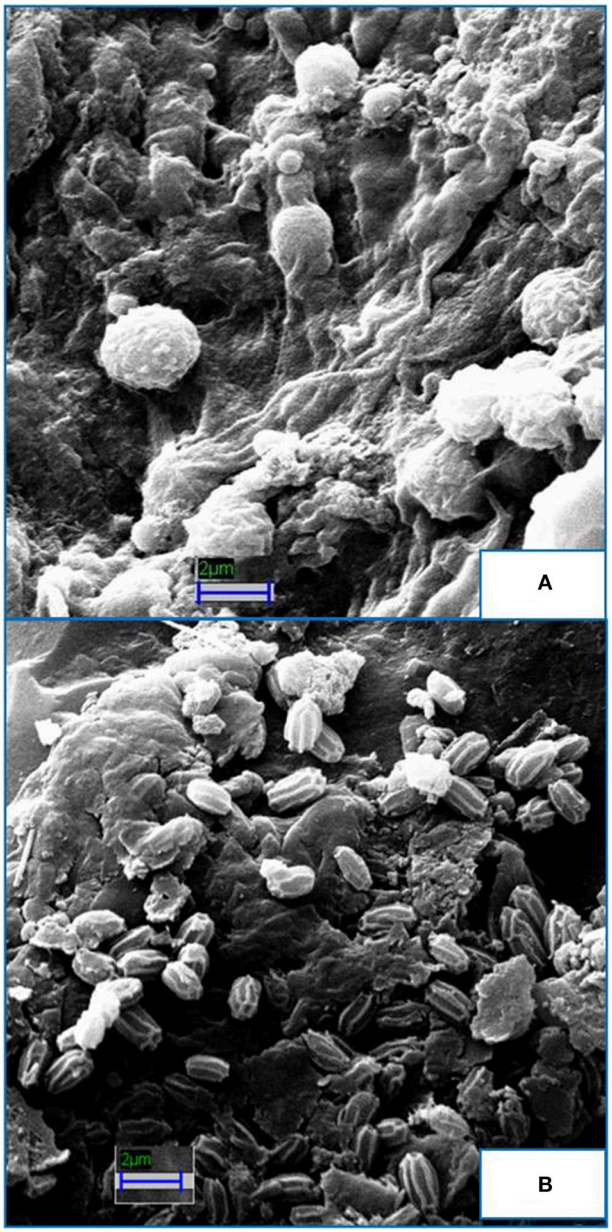

FIGURE 4 | Scanning Electron Micrographs of *P.variotii* spores grown in basal medium and optimized non-edible oil cake medium. (A) Spores in normal PDB are less in number, spherical, and with smooth surface. **(B)** Spores in optimized non-edible oil cake medium are numerous, oval shaped, and ornamented surface.

Biocontrol Activity

Production of bioactive substance establishes that *P. variotii* confirmed strong inhibition in dual culture after 2–5 days of incubation against *F. oxysporium* and *V. dahilae*. On further incubation, the pathogenic fungal mycelia (*F. oxysporium* and *V. dahilae*) growing toward the interacting zone inhibited and continuously lost vigor due to degradation. *P. variotii* mycelium was grew faster and covered the most part of the petriplate. It showed strong inhibition activities *in vitro* against above mentioned phytopathogenic fungi tested.

The fungistatic and fungicidal concentrations of *P. variotii* extract contains DPA as observed by spectrophotometric assay are presented in **Figure 5**. Fungal filtrate exhibited the strong antimicrobial effect even at very low concentrations. The Minimum Inhibitory Concentrations (MICs) against both the pathogens (*F. oxysporum* and *V. dahilae*) was identified at the concentration C3 i.e., 1E-2 dilution of the filtrate (**Figures 5A,B**).

This study is novel in terms of optimization of media for DPA production by *P. variotii,* a low cost raw material

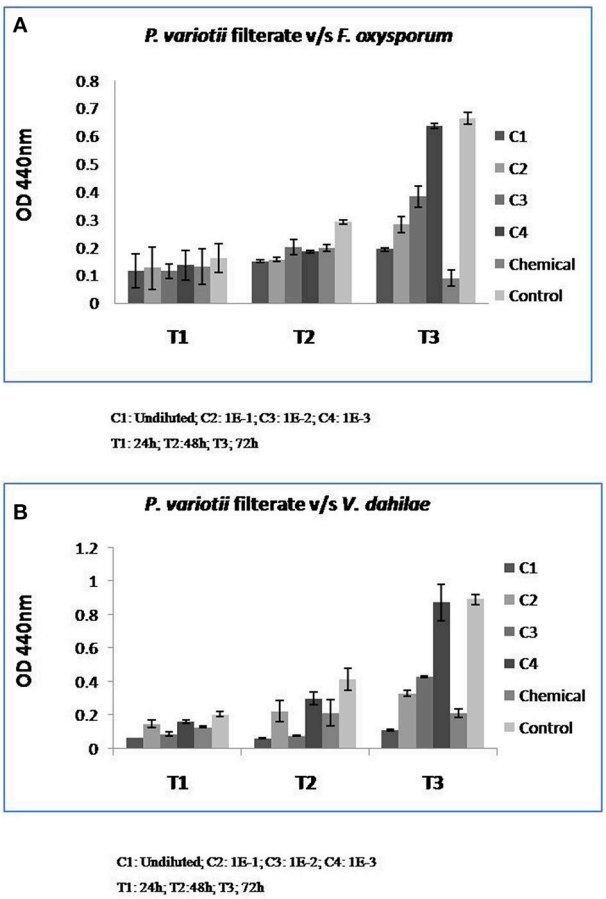

FIGURE 5 | Minimum Inhibitory Concentration (MIC) analysis of *P. variotii* filtrate against *F. oxysporum* (A) and *V. dahilae* **(B)**.

such as non-edible oil cakes and in studying biocontrol of plant pathogens by the fungal extract. Studies and field trials proved that *P. variotii* is potential in controlling *Fusarium* and *Verticillium* wilt of tomato was aided by production of various bioactive compounds such as DPA, oxalic acid, protease, etc. The plants that were applied with DPA and fungal extract of *P. variotii* were tolerant to the severe attack of *Fusarium* and *Verticillium* wilt pathogens. Biopesticidal applications involve high amount of bioactive components which can be attained by medium components optimization for exploiting a low cost material such as non-edible oil cakes (NEOCs) (Abd EL-Aziz et al., 2015). Use of NEOCs in biodiesel sector for the production of DPA could be a solution for oil cakes disposal, since these cakes are toxic and cannot be used as animal feed like other edible oil cakes. Several factors predominantly medium constituents are known to influence production of DPA. Particularly C and N are significant for the production of these bioactive compounds. Ping (2000) and Ramachandran et al. (2007) have reported, various industrial applications of oil cakes in biotechnological and fermentation processes and their value addition by implementation in feed and energy source as well. Sharma et al. (2012) in their study used the non-edible oil cakes as a potential substrate for *Paecilomyces lilacinus* and its application as biopesticidal agent against termites. PBD was useful in recognizing *Jatropha* oil cake, Karanja oil cake, and Dextrose as vital factors affecting DPA production by *P. variotii*. No earlier studies are available on the effect of NEOCs on DPA production by *P. variotii*. Furthermore, the interaction effect among *Jatropha* oil cake, Karanja oil cake, and Dextrose was highly significant in enhancing the DPA production by *P. variotii*. The scaling up in a 5 l fermentor in an optimized medium promote enhanced DPA production by 50%. Like other metabolites maximum DPA production was also attained in the stationary phase. Our results are in agreement with Azeredo et al. (2004) and Singh and Chhatpar (2010), who observed that maximum enzyme production, obtained at the stationary phase of growth.

The HPLC analysis of the optimized medium also confirmed the enhanced DPA production by *P. variotii* (**Figure 6**). The SEM analysis showed that oil cakes create a stress like condition (due to less C:N ratio) for the fungus and it generated more spores as compared to basal medium in which carbon source is easily available for the mycelial growth. The dramatic change in the morphology of the fungal cells has been observed. These results are supported by our previous observations of the DPA production during sporulation (unpublished data). During the biocontrol experiment at laboratory level the fungal extract (containing DPA) was found to be effective against *F. oxysporum* and *V. dahilae* even at low concentrations. This might be due to the Pyridine and amide moieties present in DPA. The antimicrobial activity of DPA might be due to the presence of the amide linkage groups and nitrogen heterocyclic rings, which enhances their activity (Al-Salahi et al., 2010). Moreover, the plate studies revealed the absence of any direct contact between *P. variotii* and the pathogenic fungi. This suggested that the inhibition of phytopathogenic fungi was due to exocellular, bioactive substance that diffuse through the agar medium, which substantiates the production of DPA and other bioactive substances that might inhibit the pathogen growth (Mohammadi et al., 2016). Visual observation under a phase contrast microscope, showed that the pathogenic fungal mycelia growth toward the *P. variotii* were thin and that the cytoplasmic contents of the cell became collate. However, on the control petriplate, pathogenic fungal mycelia showed regular radial growth. The results of the current work correlates with the studies conducted by Wang and Liu (2008), which optimized the medium for antifungal substance from *Paenibacillus* sp.

This study indicated that statistical experimental design recommends an efficient and realistic methodology for optimizing the requirements for DPA production by *P. variotii*. The maximum DPA production (251 mg/l), was 2.5 folds more when compared to the basal medium, achieved by optimizing carbon and nitrogen sources. This study will also contribute toward improving the DPA production by other microorganisms and the usage of trace elements. Integrated into a broader study of carbon and nitrogen sources on the production of DPA as bioactive substance, this work should help to build more rational

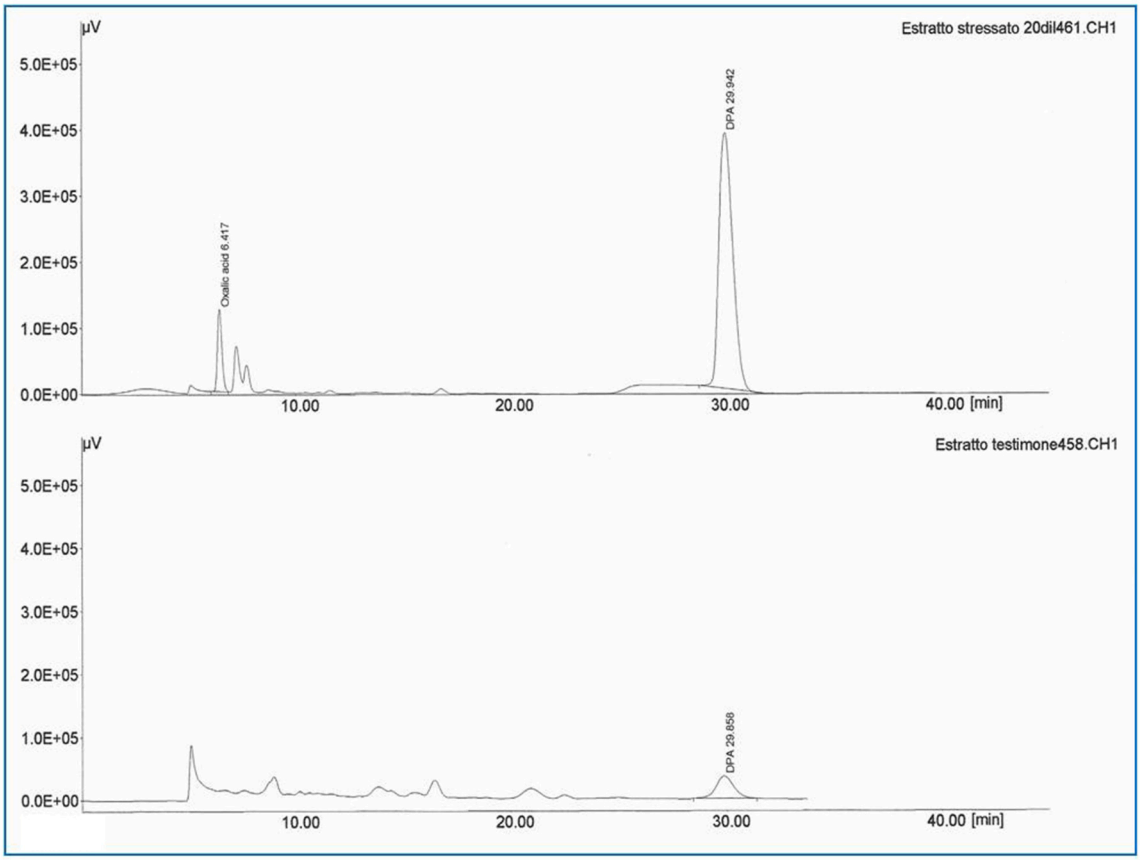

FIGURE 6 | High-Performance Liquid Chromatography (HPLC) for DPA production of control and best NEOCs (Karanja oil cake + Jatropha oil cake + Dextrose) treatment (at Retention Time 30 min).

control strategy, possibly involving scale-up production of DPA.

AUTHOR CONTRIBUTIONS

KA performed the experiments, applied RSM, and prepared the manuscript. SS designed the experiments and reviewed the manuscript. AK, SK, and JA edited and revised the manuscript.

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