PMI Performance Metric for FY18: Using genomics-based techniques, develop an approach to explore the functioning of plant-microbe interactions.

Q2 Metric: Describe progress towards understanding fungal relationships with plants.

Introduction

The Plant-Microbe Interfaces (PMI) project is a Scientific Focus Area directed towards understanding the dynamic interface that exists between plants, microbes and their environment. Project efforts are focused on characterizing and interpreting systems comprising the poplar tree (*Populus*) and its microbial community, in the context of favorable plant microbe interactions. We seek to define the relationships among these organisms in natural settings, dissect the molecular signals and gene-level responses of the organisms using natural and model systems, and rebuild the complexity of these systems using sequence characterized plants and microbes. *Populus* is an ideal host system for examining interfaces between plants and microbes and a leading candidate for bioenergy production. It is a dominant perennial component of many North American temperate forests and among only a few plant species that host both endo- and ectomycorrhizal fungal associates. Numerous other types of microorganisms can be found within, or closely associated with, various *Populus* tissues, and these organisms may range from highly beneficial to pathogenic with respect to effect on host fitness. Ultimately, an improved fundamental understanding of plant-microbe interfaces will enable the use of indigenous or engineered systems to address challenges as diverse as bioenergy production, environmental remediation, and carbon cycling and sequestration.

The investigation of *Populus*-fungal relationships is one of the PMI project's primary goals. A particular focus is on understanding the key molecular factors, mechanisms, and gene networks involved in symbiosis formation and function using the *Populus-Laccaria bicolor* model system. The knowledge acquired from the *Populus-L. bicolor* interaction is being extended to other *Populus* associated fungi which are explored as emerging and complementary models to better understand such beneficial associations and its connections to ecological processes in forest ecosystems.

Overview of *Populus*-fungal interactions

Populus is an economically important genus of woodland and riparian trees that forms associations with highly diverse rhizospheric fungi from across the fungal kingdom (Ascomycota, Basidiomycota, Glomeromycota and Zygomycota) (Karlinski *et al.,* 2013; Gottel *et al.,* 2011). Mutualistic mycorrhizal symbionts are of major importance as drivers of ecological and evolutionary processes in forest ecosystems. *Populus* associates with ecto- and endo- (arbuscular) mycorrhizal fungi (Karlinski *et al.,* 2010). These fungi enhance (i) absorption of mineral nutrients, such as nitrogen and phosphorus, thereby stimulating plant growth, (ii) provide protection against certain diseases, (iii) augment the ability of plants to withstand drought and (iv) increase plant establishment and survival. In addition to these intimate biotrophic interactions, *Populus* trees also interact with a wide diversity of endophytic fungi which also play key roles in the rhizosphere and soil community (Shakya *et al.,* 2013). Among the many pathogens that infect poplar trees, *Melampsora* and *Septoria spp.* fungi, which cause rust and leaf spot diseases in plants, are responsible for considerable damage in poplar plantations.

Because of the availability of host genome sequence (Tuskan *et al.,* 2006), experimental glasshouse and agroecosystems, and the diversity of fungal interactions, *Populus* is increasingly recognized as an excellent model tree for the study of host-fungal interactions in relation to tree growth and underlying physiology and genetics.

Fungal community survey, analysis and model systems

The fungal community associated with *Populus* has been extensively characterized using both traditional mycological approaches as well as molecular-based surveys. The PMI team has developed a collection of over 2000 fungal strains representing multiple functional groups (guilds) which interact with *Populus*: these include arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (EMF), endophytic fungi (ENF), as well as pathogens. AMF and EMF both form mycorrhizal structures that provide the plant host with nutrients and protection from pathogens in exchange for photosynthetically fixed carbon. The function of ENF is less well understood; they are more diverse than AMF, and EMF are increasingly being recognized as important members of the plant microbiome community which also confer numerous growth benefits to plants. One major objective of the PMI SFA is to identify how these diverse symbiotic fungi associate and communicate with their *Populus* hosts.

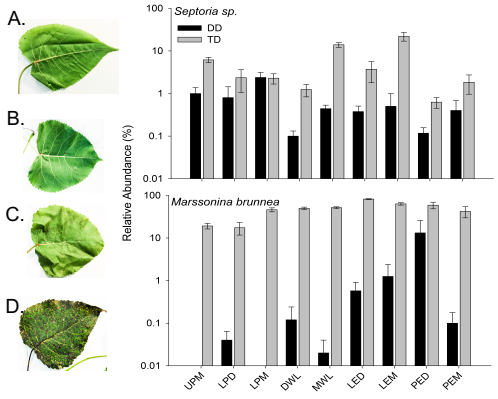

Figure 1: **Log relative abundance of OTUs classified as the *Populus* leaf pathogens *Septoria sp.* and *Marssonina brunnea* across leaf niches within *P. deltoides* (DD) and *P. trichocarpa x deltoides* hybrids (TD).** Bars represent mean values ±S.E. Any bars missing indicate that OTU is absent from all samples within that habitat category. *Septoria sp.* and *Marssonina brunnea* relative abundance differed across habitats and genotypes ($p \leq 0.05$). At left, representative of developing vs. mature leaves for *Populus deltoides* (A vs B) and TD hybrids (C vs D) respectively.

Our environmental metagenomic surveys of *Populus* species and genotypes have shown that fungal communities vary considerably within different rhizosphere compartments (Gottel *et al.*, 2011; Shakya *et al.,* 2013), across different plant habitats within the tree (Cregger *et al.,* 2018) and by tree species/genotype (Bonito *et al.*, 2013; Cregger *et al.,* 2018). Not surprisingly, across *Populus* species, susceptibility to fungal pathogen infection plays a large role in structuring aboveground fungal leaf communities (Cregger *et al.,* 2018; Figure 1). Further, native leaf fungal endophytes have also been shown to modify plant disease severity (Busby *et al.,* 2015).

Laccaria bicolor as a model symbiont

The basidiomycetes *Laccaria bicolor* is a common ectomycorrhizal fungus (ECM) that is a mutualist with many northern temperate forest trees including *Populus*. Such fungi are unique in having a simultaneous dual lifestyle, living both within the plant roots as symbionts and, at the same time, in the soil as facultative, transitory saprotrophs. *Laccaria* species have been a major experimental model for decades (Molina, 1982; Mueller & Gardes, 1991; Martin *et al.,* 1999; Labbé *et al.,* 2008) and *L. bicolor* was the first ECM fungus to have its genome sequenced (Martin *et al.,* 2008). The considerable investment in the generation of genomic and genetic tools

for *L. bicolor* (Kemppainen *et al.,* 2008) provided a useful system for studying the evolution of host and ecological specificity (Kropp & Mueller, 1999).

Laccaria bicolor has been used extensively in both basic and applied research. The physiological ecology of *L. bicolor* is well studied among ectomycorrhizal taxa, because it grows rapidly in culture and its mycorrhizas are easily established with tree roots under laboratory conditions (Kim *et al.,* 1998). *L. bicolor* is commonly used in microcosms and *in vitro* experiments in dual culture with *Populus* or conifer seedlings (Tagu *et al.,* 2001) and has become a model organism to investigate ectomycorrhizal symbiosis.

Effectors in *Laccaria bicolor*: regulation of the *Populus* host

The complex interplay between plant protein receptors and symbiotic effectors enables the formation of an ectomycorrhizal and mutualistic relationship. Several studies in host roots that have been colonized by ectomycorrhizal fungi have suggested that the colonizing symbionts have acquired the ability to actively suppress plant immunity (Martin *et al.,* 2007; Luo *et al.,* 2009; Tschaplinski *et al.*, 2014; Veneault-Fourrey, C. & Martin, 2011). One hypothesis was that this is achieved through the secretion of fungal protein effectors. We have shown that *L. bicolor* is indeed producing numerous Mycorrhiza-induced Small Secreted Proteins (MiSSPs). Within the PMI project, we have demonstrated that *L. bicolor* MiSSP7 and MiSSP8 are required for symbiosis establishment and development (Plett *et al.,* 2011).

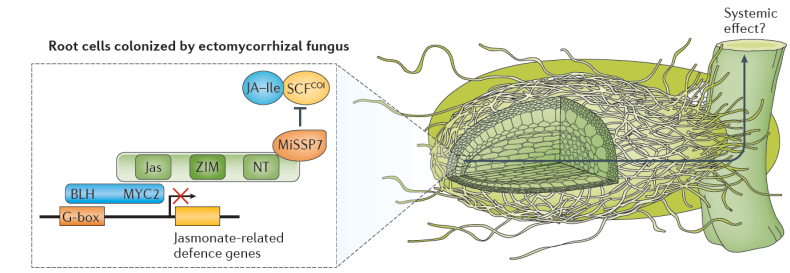

Figure 2. Establishment of ectomycorrhizal symbiosis requires the repression of plant defences that would otherwise prevent fungal growth inside the root; therefore, the ectomycorrhizal fungus *L. bicolor* uses the effector MiSSP7 to ensure the suppression of jasmonate-responsive genes. After wounding caused by the colonization of the apoplastic space by its hyphae, *L. bicolor* secretes MiSSP7 as an effector protein that suppresses jasmonate-related defence mechanisms by binding to JAZ6, which prevents its recognition by JA–Ile–COI1 and thus its proteasomal degradation, thereby maintaining the inhibition of MYC2 and the repression of jasmonate-responsive genes.

Our past efforts have focused on the molecular characterization of MiSSP7 involved in the establishment of the symbiosis between *L. bicolor* and poplar roots. MiSSP7 is a 7kDa protein that accumulates in the hyphae and is secreted into the extracellular environment after sensing of diffusible plant signals. Secreted MiSSP7 is imported into root cells, where it accumulates rapidly in root cell nuclei. In the host nuclei, MiSSP7 interacts with the transcriptional repressor, JASMONTA ZIM DOMAIN protein 6 (PtJAZ6), which is a master regulator of the jasmonate signaling pathway (Plett *et al.,* 2014). The interaction between MiSSP7 and PtJAZ6 prevents the proteasomal degradation of PtJAZ6 that would otherwise be activated by the accumulation of jasmonate triggered by fungal colonization. This stabilization of PtJAZ6 maintains repression of part of the jasmonate-signaling pathways, allowing fungal colonization of the root apoplastic space (Plett *et al.,* 2014) (Figure 2). Interestingly, among the jasmonate-induced genes that are repressed by the MiSSP7-PtJAZ6 complex, several are related to plant immunity and others function in plant cell wall modification (such as those encoding chitinase, extensin and pectin esterase). This suggests that, in addition to inhibiting jasmonate-induced defence mechanisms, MiSSP7 is able to modify the composition of the plant cell wall, probably ahead of the hyphal progression in the middle lamella.

Efforts in the PMI project have also shown that plant cell wall modifications within ectomycorrhizal roots arise from cell wall–modifying enzymes of fungal origin. These results were counter-intuitive as ECM fungal genomes contain a very low number of CAZymes active against the plant cell-wall (Veneault-Fourrey *et al.,* 2014; Kohler *et al.,* 2015). We have shown that *L. bicolor* LbGH5-CBM is an endocellulase active against cellulose extracted from aspen roots and is required for symbiosis development (Zhang *et al.,* 2018). We also found that MiSSP8 is required for symbiosis, likely through a dual role. One could be linked to fungal hyphae aggregation while the other is related to plasmodesmatal function (Pellegrin *et al.,* 2017).

In parallel with functional studies, we have performed genomic and transcriptomic studies of several ectomycorrhizal, orchid mycorrhizal and ericoid mycorrhizal interactions. We showed that 7 to 38% of the genes that are up-regulated during mycorrhization are taxon-specific genes that are restricted to a single mycorrhizal species (Kohler *et al.,* 2015). Among all symbiosis-upregulated genes, 8 to 28% encode candidate-secreted effector proteins named MiSSPs. We thus combined gene expression profiling, genomic studies and in planta sub-cellular localization to identify putative candidate effectors for further functional investigation in the emerging fungal model *Cenococcum geophilum* also associated with *Populus* (Pereira de Freitas *et al.,* 2018). We identified a set of 22 MiSSPs that showed a high presence-absence polymorphism among the studied *C. geophilum* strains suggesting an evolution through gene gain/gene loss. Finally, we showed that six CgMiSSPs target four distinct sub-cellular compartments such as endoplasmic reticulum, plasma membrane, cytosol and tonoplast (Pereira de Freitas *et al.,* 2018).

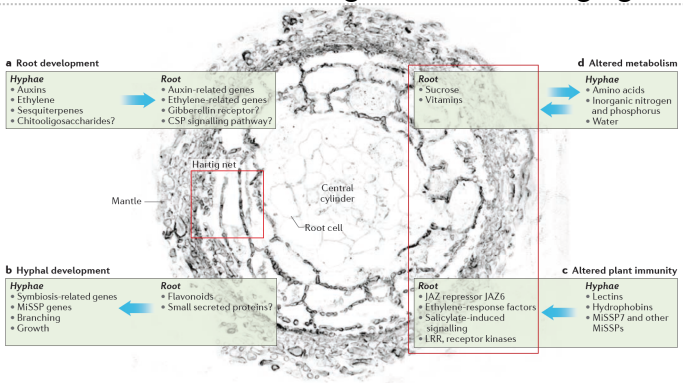

Figure 3. A model for the establishment of ectomycorrhizal symbioses. Insights gleaned from genomic and molecular studies enable the formulation of a conceptual summary of the signalling pathways, and key genes and proteins, that are involved in the development and function of ectomycorrhizal symbioses, at least for the model organisms Populus-Laccaria that have been studied. The model can be formulated as four steps that must be achieved by the fungus: modulation of host root development; hyphal development; suppression of plant defences; and modulation of the metabolism of host root cells.

Our findings, coupled with other studies on symbiotic effectors, have drastically changed the way we view mutualistic fungi. We now know that mutualistic fungi use mechanisms similar to plant pathogenic fungi to manipulate and control root immunity and development. Our work within the PMI project highlights that the study of ECM symbiosis is at the intersection of studies on root development, root immunity and plant metabolism (Martin *et al.,* 2016). Figure 3 presents our current model for the molecular mechanisms that entail the development of ectomycorrhizal symbioses. In the first step, the host plant has a restricted set of genes that are induced during the pre-infection phase and during the colonization of the apoplastic space (Duplessis *et al.,* 2005; Larsen *et al.,* 2016). Second, ectomycorrhizal fungi can alter root metabolism so that the intruding hyphae are accommodated, similar to what has been observed for plant pathogens and arbuscular mycorrhizal symbionts (Luo *et al.,* 2009; Tschaplinski *et al.,* 2014). Prior to physically contacting the plant root, colonizing ectomycorrhizal hyphae alter endogenous auxin metabolism, signalling and responses in root cells, through the use of mechanisms that may include a range of diffusible chemical signals (such as fungal and plant auxins, and fungal sesquiterpenes), such that an increase in short roots are produced, thereby providing a larger surface area to colonize (Felten *et al.,* 2009; Vayssières *et al.,* 2015; Krause *et al.,* 2015; Ditengou *et al.,* 2015). Third, attenuated expression

of genes that function in chemical-based and hormonal defence pathways occurs in the host plant during the initial steps of the fungal invasion of plant tissue, during which MiSSP effectors are secreted (Plett *et al.,* 2014a and b; Doré *et al.,* 2015; Kohler *et al.,* 2015). This mechanism of weakening plant defences is probably crucial for enabling hyphal penetration into the root apoplastic space. However, the host plant may respond to the developing ectomycorrhizal interaction by secreting its own effector-like proteins and chemical signals, which might, in turn, control the secretion of fungal effectors (Plett *et al.,* 2017). Fourth, fungal effectors, such as symbiosis-upregulated plant cell wall degrading enzymes that are upregulated during symbiosis, modify cell-to-cell attachments and plant cell wall rigidity to enable further hyphal penetration into the root tissues (Kohler *et al.,* 2015; Veneault-Fourrey *et al.,* 2015; Zhang *et al.,* 2018).

Further studies of the ability of mutualistic symbionts to interfere with host plant signalling will provide novel insights into the mechanistic basis for fungal regulation of the development and immunity of host plants. Obtaining a global view on the hormonal regulation of poplar roots during ECM development becomes thus an important following focus.

Effectors in *Populus:* regulation of the symbiont *L. bicolor*

A large body of evidence now demonstrates how symbiotic microbes evade host defenses by deploying signaling molecules such as effectors. In contrast, little is known about how the host-plant contributes to the recruitment of symbionts. Within the PMI project, we have made significant advances in demonstrating that host-plants do possess genetic features that target specific microbes to promote interaction. To this end, we describe below two specific examples of host features that drive recruitment of the fungal symbiont *L. bicolor* by *Populus spp*.

a) *Populus* Small Secreted Proteins (SSPs)

It is widely accepted that fungi can use small secreted proteins (SSPs), usually <250 amino acids in length, to influence their hosts in order to support their metabolic requirements during symbiosis. However, the potential role of plant SSPs in plant-fungi symbiosis has not been defined. We hypothesized that plants can also use SSPs as messengers to communicate with their fungal partners for building mutual beneficial relationship. To test this hypothesis, we performed RNA sequencing of *Populus trichocarpa* roots in mutualistic symbiosis with the ectomycorrhizal fungus *Laccaria bicolor*. Through computational analysis of the RNA-seq data, we identified 417 plant-encoded putative SSPs that were significantly regulated during this interaction. The predicted secretion of poplar SSPs was tested using a yeast complementation assay in which the survival of a *Saccharomyces cerevisiae suc2* mutant depends on the secretion of a truncated SUC2 protein that lacked its native secretion signal and was fused to the poplar SSP candidates. We found that on average 15 of the 40 (38%) poplar SSPs that were tested complemented the *suc2* mutation. Furthermore, we demonstrated that four of the five poplar SSPs tested in an *in-vitro*

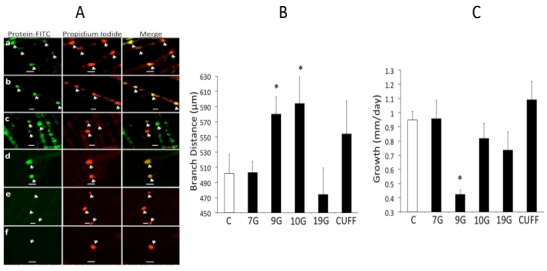

Figure 4. ***In-vitro*** **feeding of synthetic PtSSPs on** ***Laccaria bicolor***. (**A**) Uptake and sub-cellular localization of Populus trichocarpa small secreted proteins (SSPs) into fungal hyphae. *L. bicolor* colonies were incubated with 1 µM of either FITC-labelled SSP or FITC alone (control) in PBS (pH 7) for 4 hours after which they were rinsed briefly in PBS, fixed in 4% paraformaldehyde and then stained with 0.1% propidium iodide. Green signal indicates SSP-FITC (mature protein lacking secretion signal) florescent localization, red indicates propidium iodide fluorescence as a nucleus marker. Nuclei are marked by an arrow. (a) CUFF.29946 (represented by "CUFF"); (b) Potri.009G063200 (represented by "9G"); (c) Potri.010G251000 (represented by "10G"); (d) Potri.007G006800 (represented by "7G"); (e) Potri.019G121200 (represented by "19G"); (f) FITC control (represented by "C"). (**B**) Distance between hyphal branches in colonies of *L. bicolor* treated 1 µM of PtSSPs or FITC. (**C**) Growth rate of *L. bicolor* hyphae treated with 1 µM of PtSSPs or FITC. From Plett *et al*., 2017.

feeding experiment could enter *L. bicolor* hyphae and accumulate in the nucleus (Figure 4A). Further, two of these poplar SSPs significantly affected hyphal growth and morphology (Figure 4B-C; Plett *et al.*, 2017). These results indicate that plants encode proteins that appear to function as effectors that may regulate symbiotic associations.

b) Receptor-like Kinase: a key player in host-symbiont specific recognition

A key hypothesis driving the development and deployment of unprecedented genomic resources in *Populus* was that the plant host plays a critical role in selecting its associated microbial symbionts. As described above using microbial community profiling, we have observed that there were significant differences in microbial community profiles across different host genotypes grown in the same environment. This difference was especially strong across *Populus* species, *P. trichocarpa* and *P. deltoides*. Labbé et al. (2011) had also demonstrated that *L. bicolor* exhibited preferential colonization of *P. trichocarpa* (T) when compared to *P. deltoides* (D). With these observations in mind, we sought to establish robust genomic resources for interspecific TxD QTL mapping pedigrees 52-124 and 54B to facilitate segregation analysis of colonization efficiency. To that end, whole-genome resequencing to a minimum 30× depth for parental genotypes 93-968 (*P. trichocarpa*), ILL-101 (*P. deltoides*) and D124 (*P. deltoides*) was performed by JGI. Pairwise comparisons among these genotypes and stringent curation yielded 5,390 single nucleotide polymorphisms (SNPs) with known genomic positions. These were subsequently used to design the *Populus* Illumina 5K bead array (Muchero *et al.,* 2015).

We used the bead array to genotype 712 individuals from the 52-124 pedigree and 299 individuals from the 54B pedigree and this exercise yielded a high density genetic map for 52-124 consisting of 3,568 markers and an improved 54B map with an additional 677 SNPs. Both genetic maps allowed us to finely map and identify candidate genes underlying a major QTL associated with *L. bicolor* colonization previously identified in the 54B pedigree by Labbe *et al.* (2011). This analysis identified a whole-gene deletion in *P. deltoides* involving a G-type lectin receptor-like kinase (PtLecRLK1) which segregated consistently with *L. bicolor* colonization efficiency across the pedigree. The presence of the functional *P. trichocarpa* copy conferred approximately 2X the colonization efficiency when compared to *P. deltoides* deletion variants. The role of the G-type lectin receptor-like kinase in mediating colonization by *L. bicolor* was validated using heterologous expression in *Arabidopsis* which converted a non-host into a host for the fungal symbiont (Figure 5). We have also demonstrated that this PtLecLRK1 functions by suppressing the host-defense pathways to allow compatible interactions.

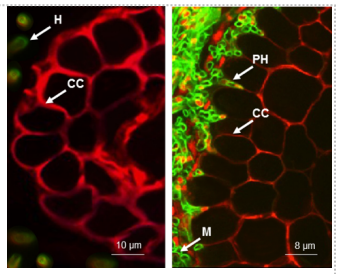

Figure 5: Microscopic confocal images of a transversal section of wild type plant Col-0 (left) and *35S:PtLecRLK1* transgenic line (right) co-cultivated with *L. bicolor*. Propidium iodide (red) was used to stain root cell walls and UVitex 2B (green) was used to stain fungal cell walls. H, hyphae. CC, cortical root cell. PH, penetrating hyphae. M, mycelium.

Emerging and complementary fungal model systems

The *Populus* root microbiome harbors a diverse community of endophytic and ectomycorrhizal (EMF) fungi that promote nutrient acquisition and plant health. *Populus* genotypes also vary in their ability to form symbioses with different root-associated fungal taxa. Over the past few years, we have gained an extensive collection representative of fungal endophytic associates that we recently extended with a focus on ectomycorrhizal communities associated with *Populus trichocarpa* across its range in the Pacific Northwest (from five core watersheds in Oregon and Washington). In order to further explore other emergent models, the development of co-culture

systems using spore prints or pure isolated cultures of different EMF collected during the field campaigns were initiated.

Indeed, this effort resulted in over 300 collections of EMF including pure cultures, spore prints and bulk soil that we further used in bioassay studies with different *Populus* genotypes (Figure 6). Taxonomic identification of fungal specimens is aided by multilocus DNA barcodes as well as morphological features. Many of the EMF fungi are uniquely host-specific with *Populus*. A diverse collection of root associated fungi have been identified and tested to evaluate their compatibility with different *Populus* genotypes representing different geographical ecotypes of *P. trichocarpa*. Selected species of *Populus*-associated EMF including *Lactarius, Russula, Hebeloma, Cenococcum, Laccaria* and *Paxillus* were inoculated with different *Populus* species and *P. trichocarpa* genotypes to define new experimental systems for greenhouse and sterile *in vitro* studies, to address plant-fungal compatibility and function, and to study ectomycorrhizal formation under controlled laboratory conditions.

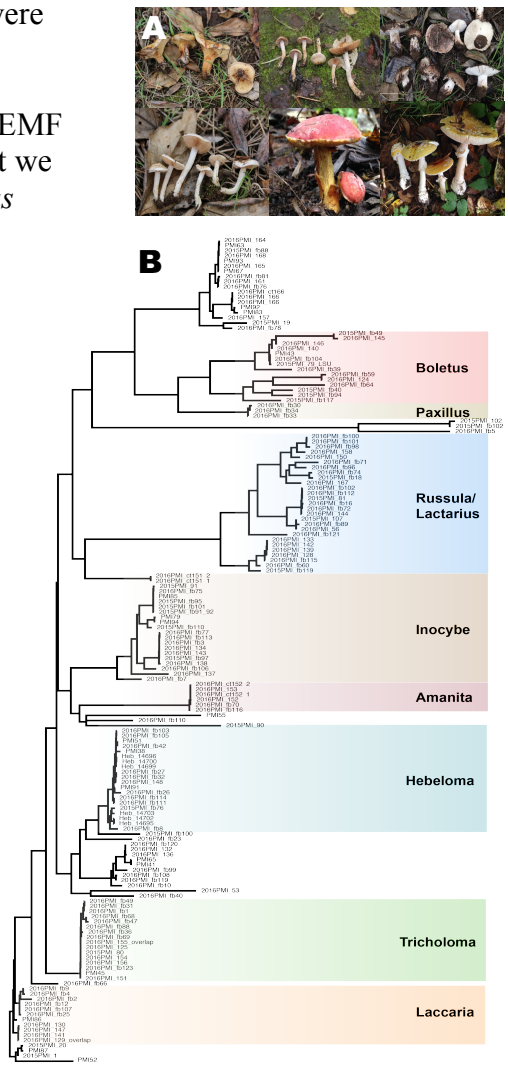

Figure 6. Key groups of ectomycorrhizal fungi collected during surveys in 2015 and 2016 in the *P. trichocarpa* range. (A) Images of some specimens in their native ecosystem; (B) RAxML phylogenetic tree of different fungal species observed based on ITS within some common fungi associated with *Populus*.

Complementing this unique PMI collection of ~2,000 isolates, 64 *Populus*-fungal genomes and transcriptomes (endophyte and EMF) have now been sequenced by DOE JGI. These include *Mortierella*, *Attractiella*, *Ilyonectria* and 13 members representative of the entire family of the Russulaceae (Looney et al. 2018) plus 6 more sequenced members of the Russulales order. Our unique collection of Russulaceae cultures include those that were previously known to be challenging or "unculturable". This Russulaceae collection allowed us to experimentally characterize and define the physiology and growth (i.e., pH, temperature, specific nutrient need) patterns of these understudied organisms. Also, we initiated the development of plant-fungi co-culture systems for which we have so far obtained, in greenhouse, mycorrhized *P. trichocarpa* with *L. deliciosus*, *L. populinus*, *M. Ochricompacta*, *R. cerolens* and *R. amoenolens*. Among the other EMFs, spore prints from 21 different *Hebeloma sp*., 12 *Laccaria sp.*, 4 *Boletus sp.*, 2 *Paxillus sp.*, 4 *Tricholoma sp*. and 3 additional *Lactarius sp*. have been obtained. In addition, EMF and endophyte cultures available from field collection campaigns are being used to establish split-root systems and *in vitro* formation of mycorrhiza for further characterization by transcriptomics and metabolomics (Figure 7).

This newly characterized fungal collection provides an opportunity to compare findings on colonization and signaling with those defined in our initial model system, *Populus-L. bicolor*. Evaluating the similarities and differences between these emerging fungal models will lead to a deeper understanding of fungal colonization and symbiosis.

Figure 7. Development of co-culture systems in greenhouse. (A) Ectomycorrhizal fungi (EMF) spores print inoculation of single species on eight different genotypes of *P. trichocarpa*; (B) and (C) *P. tricocharpa* in vitro and split-root system for establishment of new EMF models on *Populus*.

Summary

The PMI SFA project at ORNL has pioneered the investigation of *Populus*-fungal relationships. Field-based surveys of the native community and fungal isolations have defined the variety of fungal species interacting with *Populus,* while focused molecular studies, coupled with -omics based measurements, have helped to decipher the molecular mechanisms involved in recognition and colonization. Key molecular factors have been identified, gene interaction networks have been defined, and, critically, knowledge on how to manipulate these complex systems has been gained. The detailed understandings derived from the parent *Populus-Laccaria* model system are now being extended to a vast array of fungal species. These new model systems represent the diverse types of fungi that interact with *Populus* and will serve to deepen our understanding of *Populus*-fungal relationships and the functioning of *Populus's* microbiome in effecting the host plant's performance. Further details on the PMI project's efforts to understand plant-fungal interactions, and other aspects of plant-microbe interfaces, can be found in research publications derived from the project. A complete listing project publication can be found at: http://pmiweb.ornl.gov/index.php/publication-list/.

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