Final Report
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NATIONAL INSTITUTE FOR FOREST PRODUCTS INNOVATION

Innovative nursery management solutions to sustainably manage root disease, improve nursery utilization, and enhance resilience and productivity of planted pines

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Gippsland Centre

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NATIONAL INSTITUTE FOR
FOREST PRODUCTS INNOVATION
GIPPSLAND

Innovative nursery management solutions to sustainably manage root disease, improve nursery utilization, and enhance resilience and productivity of planted pines

Prepared for

National Institute for Forest Products Innovation

Gippsland

by

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Australian Government
Department of Agriculture, Fisheries and Forestry

Executive Summary

Softwood plantation growers in Gippsland and throughout Australia depend on efficient nursery practices to reliably supply robust high-quality plants for their plantation establishment/re-establishment operations. Currently, these supply chains are threatened by annual losses of ~20% of nursery stock plants due to disease and ill health, and further losses post-planting due to a lack of resilience. These figures are predicted to worsen in the coming decade as we experience more frequent extreme climate events. This project tested and developed evidence-based management strategies to reduce these losses by optimising the balance of fertiliser, fungicide, and beneficial microbe treatments used in nursery. The project had five main objectives: (1) communicate and co-design experimentation throughout the project through virtual and on-site stakeholder workshops, (2) Develop recommendations on target nutrition levels to balance nursery plant health and maintenance of a diverse beneficial root microbiome, (3) screen and optimise the best practice for fungicide choice and application, (4) trial new avenues for the control of foliar pathogens, and (5) provide post-project support to aid with adoption and tailoring of project findings. While nursery focused, the project also generated post-planting seedling resilience measures in a series of field trials in both Gippsland and Eastern Australia. To achieve these aims, the project brought together an exceptional team of university and government researchers with extensive experience in forestry and microbiome management as well as four commercial grower nurseries from both Gippsland and other regional areas. These latter partners provided critical input into the design of the project through their invaluable practical know-how and ongoing support throughout the project by applying their experience in running nursery and field experiments to validate project recommendations.

Overall, the project has provided a series of outputs relevant to both Gippsland, as well as the forestry industry. Firstly, we have provided a set of recommendations with regards to reduction of fertiliser regimes and tailored choices of fungicides and fungicide levels. This will benefit the industry by reducing costs of fertiliser and fungicides used annually in the nursery while maintaining, or improving, the growth and resilience of their seedling and cuttings post-planting. Adoption of these recommendation will also benefit the industry by improved environmental sustainability; reductions in fertiliser reduce their reliance on a limited resource and improve the carbon footprint of nursery production. Improved uptake of fertilisers through microbiome management will also reduce runoff and negative impacts on the environments surrounding nurseries. This project has also highlighted how the use of hyperparasites, beneficial fungi that target and control pathogenic fungi, could be used as a means of sustainably controlling foliar pathogens. Further investigation of this means of biocontrol would also support sustainability of the industry and improve seedling retention and reduce annual losses of cuttings and seedlings. Additionally, this project has created, presented and shared training materials (PowerPoints, face to face workshops and an online meetings) providing nationally consistent messages about nursery management of plant health and disease through microbiome management.

The industry has already begun to adopt the recommendations from the project through reduced fungicide use and through alterations to fungicide choice and treatment regimes. To continue to gain benefit from this, industry is encouraged to integrate our findings on the factors that improve microbiome health and, thereby, boost plant vigour and disease resistance. Continued engagement with the project partners will also bring benefit across the industry nationally as we can advise on tailoring our findings in a manner that is feasible and tailored to their growing conditions.

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Introduction

Australia's forests, encompassing both native, planted, and urban zones account for one of the largest forest estates in the world (Montreal Process Implementation Group, 2013). Contributing significantly to the Australian economy and environmental stability (Montreal Process Implementation Group, 2013; Tovar et al, 2017), these assets are managed carefully across jurisdictions by >109 stakeholder groups. These latter groups include state and local government, the forest wood product sector, the building industry, and the general community (Mohammed et al, 2011). In Gippsland, plantation forestry accounts for the region's single largest land use with 90,000 hectares of plantations (23% of Victoria's total plantations). With log production in this region fully committed, the supply chain of young plants for the re-establishment of plantations and for any new plantations is key to the ongoing economic success of this region's forestry industry. However, this supply chain is threatened by an ever-increasing loss of nursery plants to disease and spread from disease in the nursery to plantation. Therefore, current disease management practices need to be revised and novel plant health techniques need to be prioritised.

The success of nursery operations relies on the judgment of nursery managers to balance issues of nutritional, chemical and biological inputs against the variable factors of climate and disease expression in order to produce quality plants. Evidence-based examples of management options are required to assist nursery managers to closely follow each crop to meet customer specifications, field performance and nursery performance targets. In containerised production, seedlings/cuttings start in sterilised potting mix and containers. While this is an excellent start, it is also open ground for establishment of unwanted microbes. *Fusarium* species, a number of which are pathogenic or parasitic, are ubiquitous in environmental samples and are known to frequently establish within containerised potting mix. Once there, they have the potential to cause a range of undesirable phenotypes, including needle loss, root rot/stunting and reduced root collar diameter. Pathogenic species from the Nectriaceae family have also been found in several nurseries, including those in Gippsland, and are suspected causal agents of tip death and mortality in pine cuttings. Compounding these two soil-borne diseases are foliar pathogens that take advantage of weakened plant health. *Botrytis cinerea* is a key necrotrophic pathogen common in nurseries that parasitise weak plants causing browning or decay of needles and rot.

Fungicides remain the first line of defence against fungal pathogens in most nurseries, applied either preventatively or in response to symptoms of disease. While these treatments can be effective, they come with a cost, both financial and environmental, and their continued use over time can lead to a build-up of fungicide resistance (Lucas et al., 2015; French et al., 2021). Compounding these challenges, fungicides can also negatively impact the beneficial microbial communities. Paradoxically, because these beneficial microbes occupy resource niches, their reduction through applications of fungicides may actually increase pathogen prevalence by leaving nutrients and soil niches open to colonization to this latter group of fungi (Naidoo et al., 2019; French et al., 2021). As a result, new sustainable strategies to combat fungal pathogens are needed.

One of the most promising non-chemical ways to reduce disease is through the use of beneficial microbes (Reglinski & Dick, 2005). These microbes assist in disease resistance through priming plant immunity, increasing overall plant health and resilience and competing directly with pathogens for resources. Plantation trees have the additional ability to associate with ectomycorrhizal (ECM) fungi, which colonise plant roots, protecting the plant from pathogens and providing it with nutrients in exchange for sugars. Pines, in particular, are highly dependent on these ECM symbionts to establish in forest soils (Dickie et al., 2010). While the importance

of beneficial microbes for overall plant health and in preventing disease has been demonstrated, there is no clear consensus on the combination of beneficial microbes best suited to individual crops and environments, nor how these microbes interact with other treatments, such as fertilisers or fungicides (Stuart & Plett, 2020). Therefore, additional research is needed in this area for it to be leveraged appropriately within the Australian forestry industry.

Beneficial microbes, particularly ECM fungi, are sensitive to nutrient levels within the soil. High levels of fertilisation reduce root colonisation by these fungi and favour nitrophilic species (Stuart & Plett, 2020). These nitrophilic species may not be able to efficiently support tree health once the plant leaves the nutrient rich nursery environment and is put into a plantation as they are less adept at mining essential nutrients from soil organic matter. Much of the research in this area pertaining to pine plantations has been conducted in the Northern hemisphere, thus there is a general lack of knowledge in how fertilisation interacts with beneficial microbes in an Australian context.

In addition to beneficial microbes, other novel methods and technologies are being developed to combat diseases. These include biocontrol agents, such as hyperparasites, which parasitise and feed off fungal pathogens (Wilson et al., 2020), hormone applications, which alter the natural immune system of the plant to enhance defence responses (Reglinski & Dick, 2005, Zhu & Tian, 2012; Plett et al, 2014), or dsiRNA (environmental RNAi), which specifically targets and degrades the mRNA of key pathogen genes halting the ability of the pathogen to colonise the host plant (Wong-Bajracharya et al., 2021). All of these methods are advantageous in that they produce little-to-no off-target effects or environmental residues. Their application, however, has typically been limited to a number of controlled experiments and they require additional research in Australia for their use both within a pine-based system and at a larger scale.

Collaborative pilot work between HVP in Gippsland and WSU prior to this project sought to determine if currently available fungicides can reduce pathogen load within their nurseries. While they had achieved some degree of control over the pathogen, based on cutting survival and on pathogen quantification by sequencing, treatments used at the time were highly variable in how the pathogens of interest are suppressed. Therefore, we needed to define better measures of fungicide selection and application procedures to reduce this inconsistency across the nursery sector. In that context, this project was begun to devise more tailored procedures that would promote consistent pathogen repression while further promoting plant resilience to support Australian forestry nurseries and growers.

Methodology

This project explored options to manage several common or topical disease issues through optimisation of fungicide, fertilisation, and microbiome management. This was achieved through a series of screening experiments under controlled conditions to identify the optimal range of fertiliser x fungicide x mycorrhizal inoculum. These results were then tested in nurseries across the collaborating jurisdictions to test their efficacy under normal operating conditions. These nursery trials were then planted as per normal forestry practices and their establishment and resilience will be tested in the coming years as part of the post-project support of growers and as part of the FWPA funded project VNC578-2122. Further, we trialled novel pathogen control options under controlled conditions that could be pursued in future for use in industry.

1. Testing of Fertilisation x Fungicide Treatment to Find Optimal Balance for Fusarium control (WSU Controlled Condition Experiment)

While *Fusarium* is moderately controlled with standard fungicide applications, its persistence in soils makes it an excellent model to create a baseline for the contribution of fertilisation x fungicide x mycorrhiza treatments to the control of soil borne pathogens that is broadly applicable across the industry. At Western Sydney University, we set up two seedling-based trials (one with radiata pine and one with a Southern pine hybrid) to challenge plants with *Fusarium* sp. as a model for common, undesirable soil-based pathogens that would have broad applicability for most softwood plantation nursery managers. In this experimental module ('Experiment 1a and 1b'), we sowed surface sterilised seeds into non-sterile potting mix obtained from HVP Plantations (for radiata pine, experiment 1a) and potting mix obtained from HQPlantations (for Southern pine, experiment 1b). In both experiments, a randomised block design was used, with 6 replicated blocks per treatment and 20 individual trees per block. Half of the plants in each block received a microbial slurry including mycorrhizal fungal hyphal fragments, endophytic fungi, and saprotrophic fungi (+inoculum) while the other half received sterile water (-innoculum).

For experiment 1a, the level of slow-release fertiliser in the potting soil was maintained as per current nursery practices, but four different fertigation procedures were used: (i) standard fertigation as per HVP Plantations, (ii) 0.5x standard fertigation as per HVP, (iii) matched nutrient profiles as per standard fertigation but with mineral nitrogen sources replaced with a commercially available amino acid mixture, and (iv) matched nutrient profiles as per 0.5x standard fertigation but with mineral nitrogen sources replaced with a commercially available amino acid mixture. For each of these four treatments, each was then further subdivided into different fungicide treatments: 1x, 0.75x, and 0.50x recommended doses of industrially relevant fungicides. These fungicides were chosen following an *in vitro* screen for efficacity against the target pathogen (*Fusarium commune*) and minimal or low repression of beneficial fungal growth rate. Seedlings were germinated and grown for four months after which half of each block received an inoculation with 10^8 spores of *Fusarium oxysporum.* Total plants used was 2,100 for this experiment. Following pathogen application, plants were grown for a further two months after which they were destructively harvested and plant height, root collar diameter, biomass, and mycorrhizal colonisation of the roots were scored. Samples of the needles were also used to quantify photosynthesis rate, chlorophyll content, phytohormone levels while samples of roots colonised by mycorrhizal fungi were taken for DNA extraction and identification via sequencing of the ITS2 barcode region.

For Experiment 1b, in consultation with our industry stakeholders, we varied both potting mix levels of slow-release fertiliser as well as fertigation regime. In the potting mix we used standard levels of slow release fertiliser and 50% levels of slow release fertiliser. The latter treatment, with 50% slow release, was divided into 3 fertigation groups: no fertigation, 25% of the nitrogen typically applied as slow-release returned as mineral nitrogen, and 25% of the nitrogen typically applied as slow-release returned as organic nitrogen in the form of amino acids. As with radiata pine, half of the plants received pathogen inoculation after four months of growth, and all treatments were further sub-divided into either 100% recommended dose for fungicide treatment based on manufacturer labels, or 50%. A total of 600 plants were grown for this trail. At the end of the trial, plants were destructively harvested and plant height, root collar diameter, biomass, and mycorrhizal colonisation of the roots were scored. Samples of roots colonised by mycorrhizal fungi were taken for DNA extraction and identification via sequencing of the ITS2 barcode region.

2. Testing of Fertilisation x Fungicide x Microbial Treatments for pathogen control (HVP and HQP Nursery Experiments)

Concurrent with the controlled environment studies outlined in Experiment 1 we collaborated with the HVPlantations nursery and HQP Plantations nursery who were conducting a series of nursery experiments also testing the impact of fertilisation, fungicide, and microbial treatment on plant health in the nursery. In the nursery trials at HVP (with *P. radiata* cuttings) we evaluedted how optimal tip preparation and potting mix fungicide options impacted the microbiome of radiata cuttings when using containerised production. At HQP (with Southern Pine hybrid seedlings), we undertook root microbial community profiling of plants that had been treated with different timed release profiles of slow-release fertilisers. In both experiments, the nursery managers were testing either naturally sourced inoculum (HVP; *Rhizopogon* fruiting bodies) or commercially bought inoculum (HQP). To introduce a pathogen stress, plants from the nursery trials were sent to WSU near the end of their growth cycle for inoculation with *F. oxysporum* and tested for disease severity based on nursery pre-treatment and the ability of *F. oxysporum* to establish in the containers of the different treatments.

3. Applying optimised Fertilisation x Fungicide Treatments to ascertain applicability to the control of Nectriaceae root pathogens (WSU Controlled Condition Experiment)

This experiment used controlled environment studies to determine if the results from Experiments 1 and 2 could be directly applied to the control of a second significant group of root rot pathogens frequently found in forestry nurseries, including those in Gippsland - the Nectriaceae. Seedlings were used for this experiment as opposed to the originally proposed cuttings due to experimental timing. All seedlings, both radiata pine and a Southern pine hybrid, were treated with the project standard fungal inoculation as used in Experiment 1a and 1b. Using the best conditions for pathogen control as determined by Experiment #1 and #2, two fertilisation levels (industry standard as a control followed by the best treatments based on the results of experiments 1 and 2) were used as were two fungicide treatments (industry standard and the best treatment as per experiment 1). Half of the plants were inoculated with either a sterile control while the other half with a Nectriaceae inoculum (3 pathogen isolates recovered by industry collaborators). A slightly shorter growth window was used for this trial with pathogen added after three months of growth, and plants harvested two months after that following six treatments with a rotation of two fungicides. At harvest, all plants were assessed for a range of attributes associated with general seedling health and immunity: growth parameters (collar diameter, height, above/below ground biomass etc), root rot symptoms and needle drop/tip damage. A pooled sample from each block was taken for analysis of Nectriaceae

load and a profiling of the root microbiome will be characterised using Next Generation sequencing.

4. Test a range of new products to control prevalent foliar-based pathogens (HVP Nursery)

An increasing number of products are becoming available to treat foliar pathogens of pine (e.g. Botrytis cinerea), but few have been rigorously tested for their ability to control disease in the Australian context. We ran two trial experiments at HVP across two seasons to compare and contrast the ability of new chemical or biological fungicides both individually and in combination in preventing *Botrytis* infection in recently-set cuttings in the Glasshouse. In the experiments, symptom development after inoculation with *B. cinerea* (isolated and grown by WSU for our nursery partners) in both potting mix conditions was be monitored along with the benefit of each fungicide/alternative control treatments.

5. Determine how promotion of below-ground beneficial microbes or novel control treatments can affect systemic plant immunity to improve Botrytis control (WSU Controlled Condition Experiment)

In this experiment we determined if the addition of beneficial microbes x fertilisation treatments could naturally enhance foliar resistance to *Botrytis cinerea* in the absence of foliar fungicide treatment. In the first part of the trial, cuttings were set by HVP using standard procedure and standard potting mix. Once these had begun rooting, they were shipped to WSU where half of the plants were inoculated with the root fungal inoculum as used in Experiment 1. Then, both plant groups that were inoculated and uninoculated with this beneficial microbiome slurry were fertilised in one of two fertilisation regimes: (i) standard fertigation as per HVP, (ii) matched nutrient profiles as per standard fertigation but with mineral nitrogen sources replaced with a commercially available amino acid mixture. Following establishment of the beneficial microbial inoculum on the root system, we inoculated all plants with 10^6 spores per mL of *B. cinerea* and maintained a high humidity in the growth chamber. Disease development was then analysed based on needle discoloration on a score of 0-5 where a score of 0 equalled no visible disease. All treatments were compared to standard fertigation without mycorrhizal inoculation.

Using another set of HVP cuttings that were set using their standard procedure and standard potting mix and fertiliser regime, we also tested a series of six (6) novel *B. cinerea* control methods. In this experiment, cuttings were sprayed to saturation with 10^6 spores per mL of *B. cinerea* and maintained a high humidity in the growth chamber. The following day, plants were then sprayed with one six novel control methods: methyl jasmonate (MeJA) solution, 10^6 spores per mL of a fungal hyperparasite, treatment with two fungicides determined by *in vitro* testing to be superior for *B. cinerea* control when compared to currently used fungicides, and two double-stranded interfering RNA (dsiRNA) designed by WSU to target and knock-down fungal pathogenicity factors. Disease development was then analysed based on needle discoloration on a score of 0-5 where a score of 0 equalled no visible disease. All treatments were compared to standard fungicide treatment as used in the HVP nursery.

6. Assess the efficacy of optimised fertilizer x fungicide x microbiome treatment under industrially relevant conditions (Nursery Partners and Plantation Experiment)

As a final experiment, we took what we had learned in both controlled-condition and nursery-based experimentation to determine the next steps in establishing new best practice with regards to nursery management of diseases. To do this, we tested a set of fertilisation x fungicide x

mycorrhization regimes tailored to each of the partner growers in four states. Each of the four partner nursery established these trials, three of which were carried through to completion (an error in trial establishment in one partner nursery meant that their experiment had to be abandoned). Of the three trials that were carried to completion, two encompassed radiata pine and one utilised a Southern Pine hybrid, therefore we were able to test our findings across the tree species of interest to this project in a robust manner. While details of each trial changed depending on the particular nursery, they all incorporated our findings from Experiments 1-4, and involved reductions in fertilisation/fertigation, alterations to microbial inoculum, and amendments to choices of fungicides used. All experiments ran for the full growth cycle of the nursery, plants were graded, and then were all sent to field trials for establishment across a gradient of site productivity. A selection of plants from each of the nursery trials were also shipped to WSU for our the standardised measurments developed for this project and our industry partners: plant height, root collar diameter, biomass, foliar nutrient analysis and mycorrhizal colonisation of the roots were scored. Samples of roots colonised by mycorrhizal fungi were taken for DNA extraction and identification via sequencing of the ITS2 barcode region. We also developed and applied new diagnostic pipelines to monitor the diversity and quantity of pathogens present in the seedlings at the end of the growth period. For those field trials established early in the second year of the project, health and survival (i.e. resilience) post planting were determined. For those field trials established at the end of this grant, our nursery partners have committed to continue monitoring their survival and growth for the first two years, and WSU will be following the evolution of the root and soil microbiome of these trials as part of the separately funded FWPA project VNC578-2122.

Results

Radiata pine shows equivalent growth at lower levels of fertilisation

Under controlled conditions we tested the growth and nutrition of radiata pine seedlings at different levels of fertigation based on recommendations from our nursery partners. We found that we could reduce fertigation by 50% without negative impact on germination, or overall growth (**Figure 1**). All of the plants under these conditions met grower specifications for height and root collar diameter. In plants receiving organic nitrogen, the plants had a significantly larger shoot weight when compared to plants receiving the equivalent level of inorganic nitrogen fertiliser. When foliar nutrition was analysed, it was found that plants with 50% less fertigation had no significant difference in either macro or micronutrients (**Figure 2**).

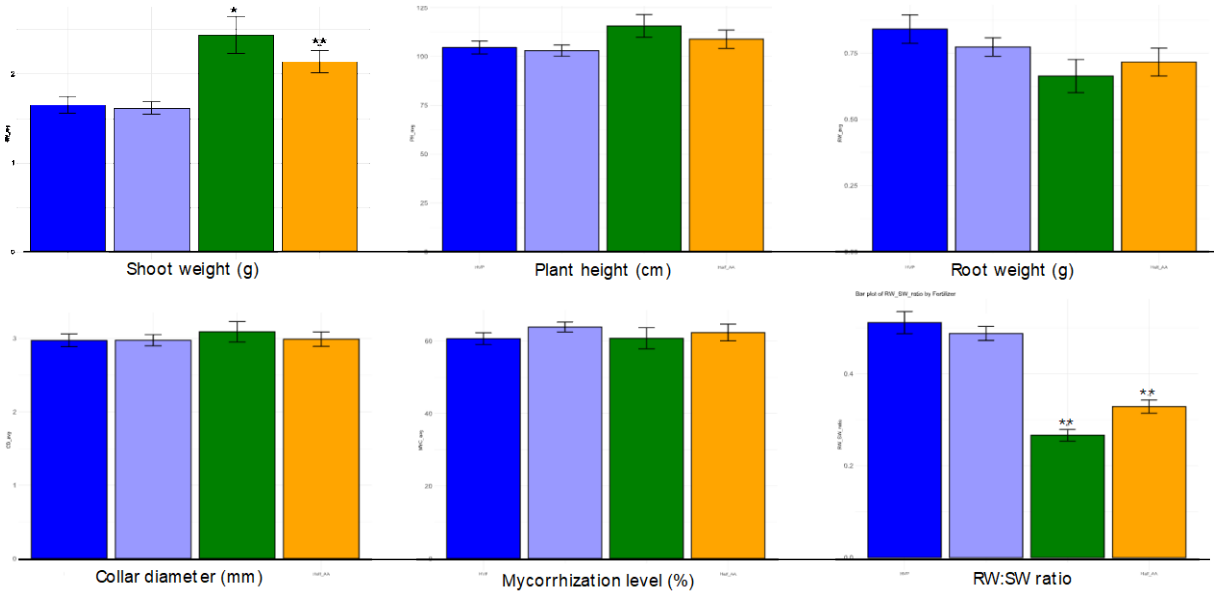

Figure 1: Physiological parameters of radiata pine at two levels of fertigation. Dark blue bars represent values of plants grown at industry standard fertigation, light blue bars represent values of plants grown at 50% industry standard fertigation. Green bars indicate equivalent nutrition to dark blue bars but with mineral nitrogen replaced with organic nitrogen. Similarly yellow bars are equivalent to light blue bars but with mineral nitrogen replaced with organic.

Based on these results, we tested a wider range of slow-release fertiliser levels in the potting mix (0-100% standard dose) and different levels of fertigation (100%, 75% standard application concentration) under nursery conditions. The initial seedling germination rate in potting mixes with reduced slow-release fertiliser was not significantly affected, while seedling vigour decreased in a near linear fashion with fertiliser reduction (**Figure 3**). Despite this, germination and vigour under a reduction in slow-release fertiliser of 25% was found to be acceptable by our industry partners.

Following six and 12 months of growth in nursery, we found a very interesting, non-linear relationship between growth and the amount of slow-release and fertigation the plants received. At six months, the largest plants (based on root mass) were found at both industry standard fertilisation and fertigation (i.e. 100RD +100RD) and at 75% industry dosing (i.e. 75RD + 75RD; **Figure 4**). However, in this same time period, plants receiving 75% industry

dose of slow release fertiliser, but 100% fertigation were significantly smaller than those receiving 75% fertigation. Similarly, plants receiving 50% slow-release fertiliser and 75% fertigation were significantly larger than those with the same slow-release profile and 100% fertigation levels.

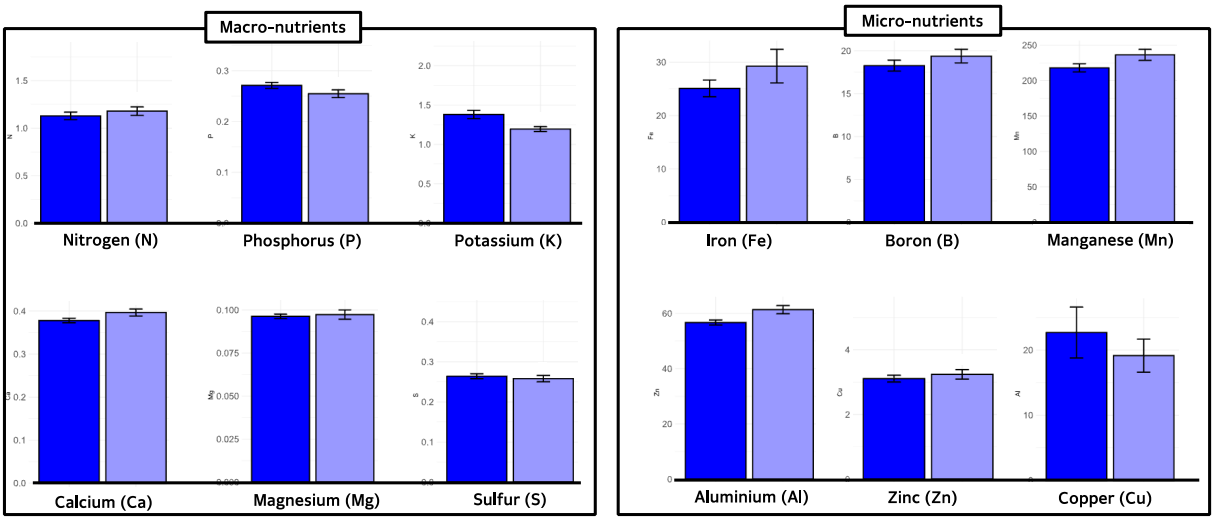

Figure 2: Foliar nutrient parameters of radiata pine at two levels of fertigation. Dark blue bars represent values of plants grown at industry standard fertigation, light blue bars represent values of plants grown at 50% industry standard fertigation.

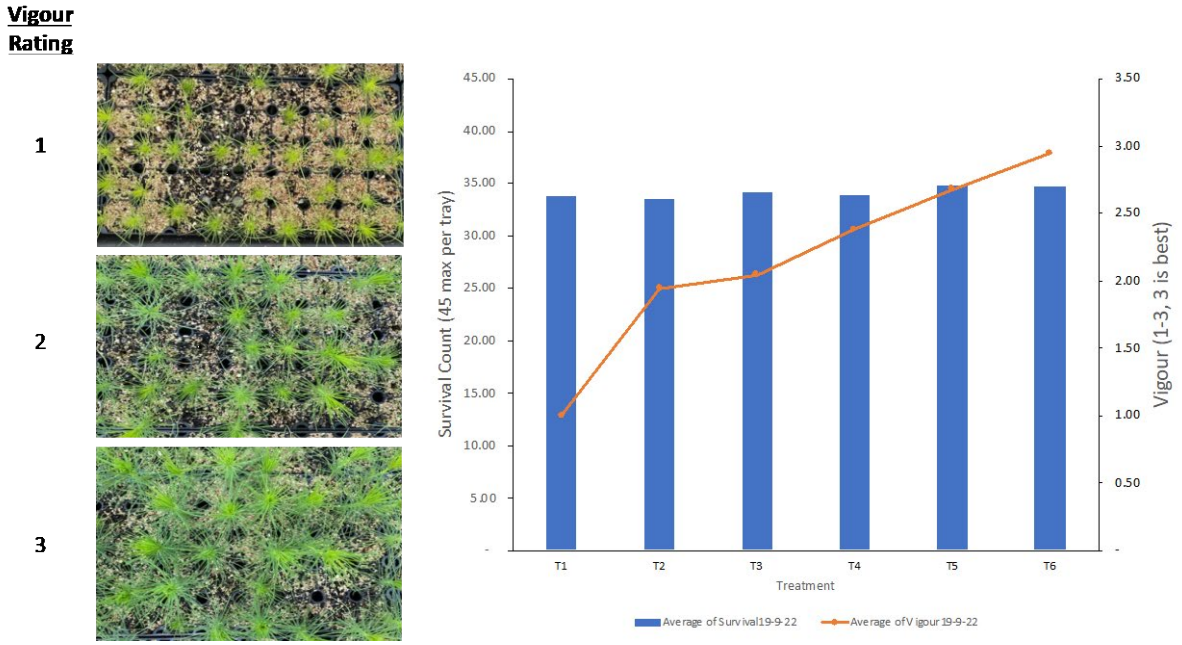

Figure 3: Germination and vigour of young radiata pine seedlings under six slow-release fertiliser treatments in nursery. T1 represents 0% slow release fertiliser, T2 -75% slow release fertiliser, T3 -50% slow release fertiliser, T4 -25% slow release fertiliser, T5 standard slow release fertiliser, T6 standard slow release fertiliser plus granular etridiazole fungicide

Even more marked was the fact that there was no significant difference in between root biomass at dispatch (12 months growth) between 100% fertilisation/fertigation and 50% fertilisation + 75% fertigation. Therefore, fertilisers can be significantly reduced in nursery conditions. However, there is a limit as the plants with no addition of slow release fertiliser grew very poorly. Therefore, slow release fertiliser must be included in potting mix to achieve maximal plant growth.

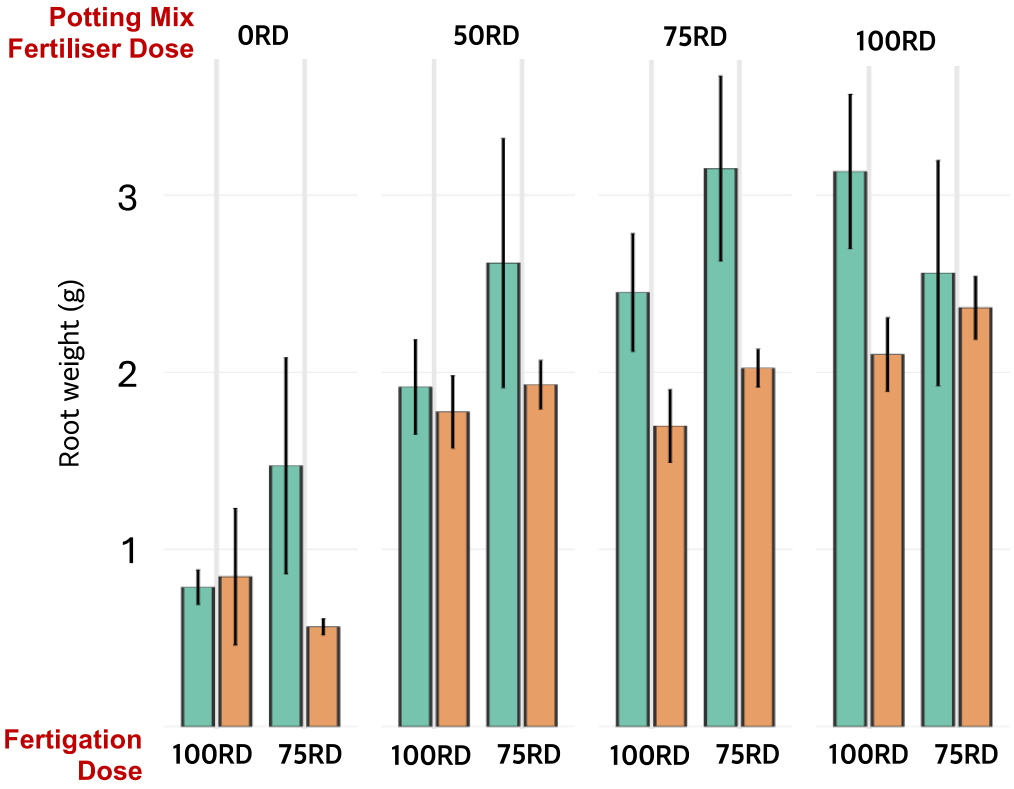

Figure 4: Root mass of radiata pine with four slow-release fertiliser and two fertigation levels. Fertiliser dosing is recorded here as "recommended dose" (RD). Green bars are root masses after six months of growth in the HVP nursery, orange bars from the same experiment after 12 months (ie pre-dispatch)

Reduced or altered fertiliser application increases microbial diversity

Using samples from these previously described experiments, we analysed the microbial diversity in the root systems of the pine seedlings. We found that as fertiliser level decreased, there was a commensurate and linear increase in overall fungal microbial diversity (**Figure 5A**). When we investigated how the top 20 genera of fungi changed in relative abundance across this fertilisation gradient, we found that the occupancy of *Rhizopogon,* a mycorrhizal fungus, decreased substantially to be replaced by *Thelephora, Wilcoxina,* and *Serendipita,* all mutualistic fungi of pine (**Figure 5B**). Tailoring the ferrtiliser application away from mineral-based nitrogen to organic nitrogen, aimed at feeding beneficial microbes at the expense of disease causing fungi, we found that fungal diversity in the roots of pine increased significantly over traditional fertilisation regimes (**Figure 6A**). Functionally, this increase in diversity was correlated to an actual increase in nitrogen concentrationsin the needles of the pine (**Figure 6B**).

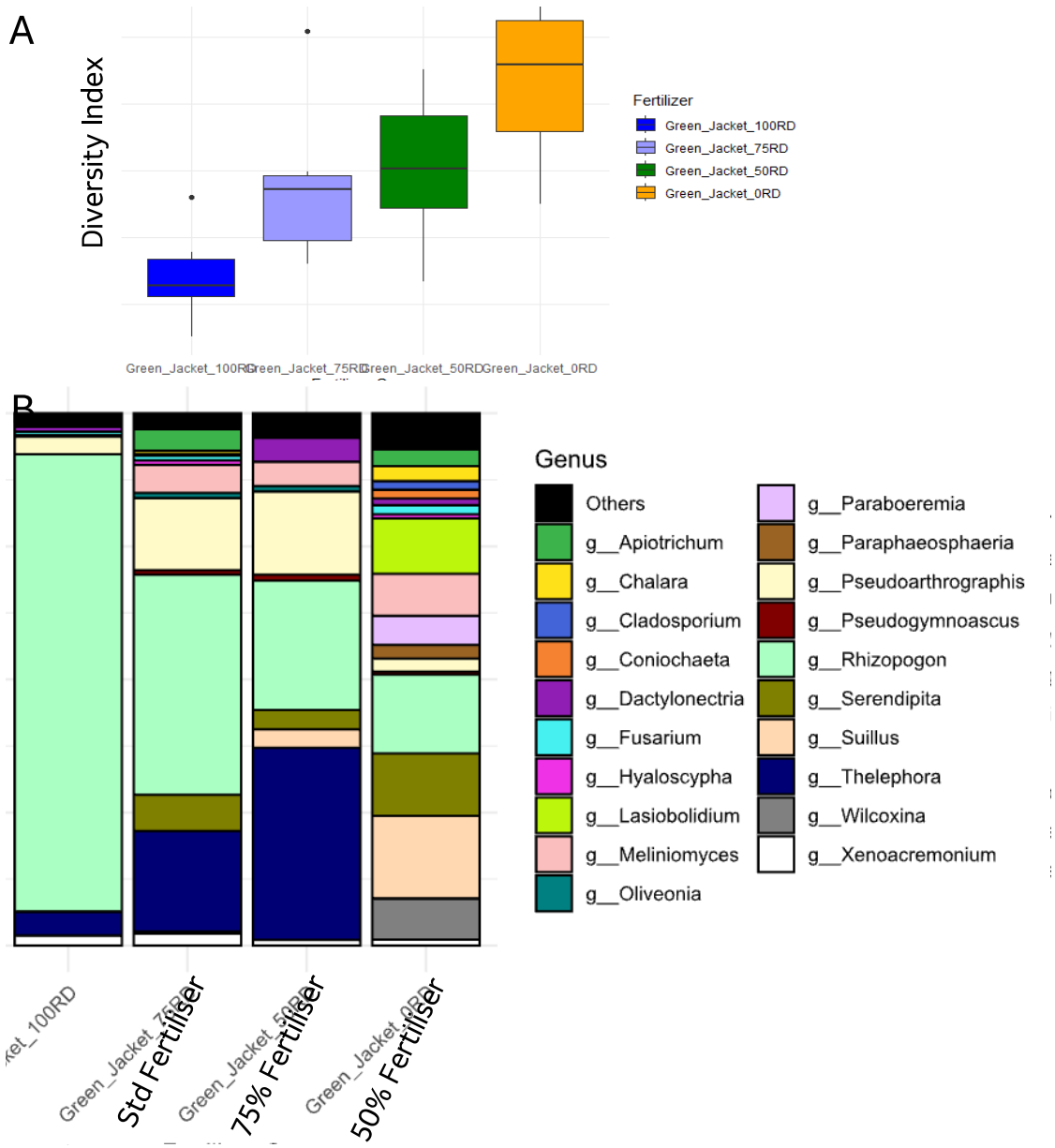

Figure 5: Microbial diversity under three different fertiliser regimes. (A) Shannon diversity of total mycobiome for standard fertiliser (dark blue), 75% standard fertiliser (medium blue), and 50% standard fertiliser application (green). (B) Relative abundance graph of the top 20 fungal genera recovered from radiata pine roots experiencing three different fertilisation regimes.

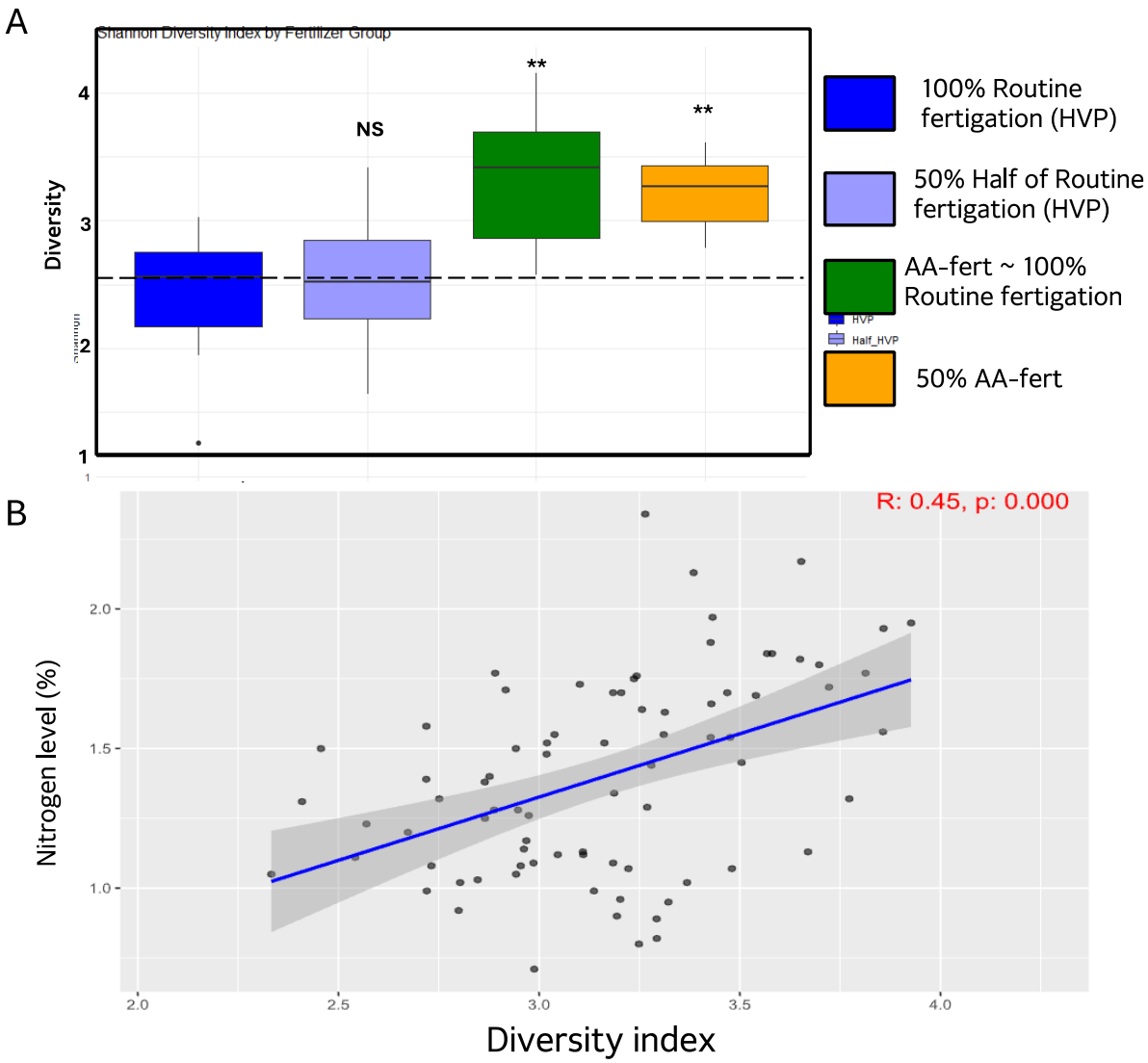

Figure 6: Relationship of fertilisation to microbial diversity and diversity to plant nutrition. (A) Fungal diversity compared between plants receiving mineral nitrogen versus organic nitrogen. (B) Relationship between total fungal diversity and foliar nitrogen concentration of radiata pine.

In vitro screening of fungicide efficacy: Dactylonectria and Illyonectria

We conducted *in vitro* assessments of fungicide effectiveness against four pathogens from the Nectriaceae family, specifically: *Dactylonectria pinicola*, *Ilyonectria capensis*, *Ilyonectria palmarum*, and *Dactylonectria estremocensis.* All isolates were sourced from the pine root system of Australian-grown pine. Our goal was to identify three fungicides that not only demonstrate high efficacy against the pathogens but also exert minimal effects on beneficial microbes. Despite all pathogens being from the Nectriaceae family, certain fungicides showed specificity towards particular pathogenic species (**Figure 7**). Fungicides exhibiting substantial effectiveness against all targeted fungal pathogens included active constituents cyprodinil + fludioxonil, tebuconazole, carbendazium, and N-trichloromethylthio-4-cyclohexane-1,2-dicarboximide as well as a biological control (*Bacillum subtilis*). These were also tested against beneficial microbes. In the testing of seven beneficials (*Thelephora terrestris, Umbelopsis isabelline, Mortierella sp., Serendipita sp., Rhizopogon pseudoroseolus, Rhizopogon subaustralis,* and *Resinicium bicolor*), we found that there was also a fungal species-specific

response. For example, *Rhizopogon subaustralis* showed very little inhibition by all fungicides tested while *Thelephora terrestris* was almost universally inhibited by the fungicides tested. For the ongoing trials under control conditions, N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide, carbendazim, tebuconazol, and etridiazole were chosen for fungicide treatment and used in rotation.

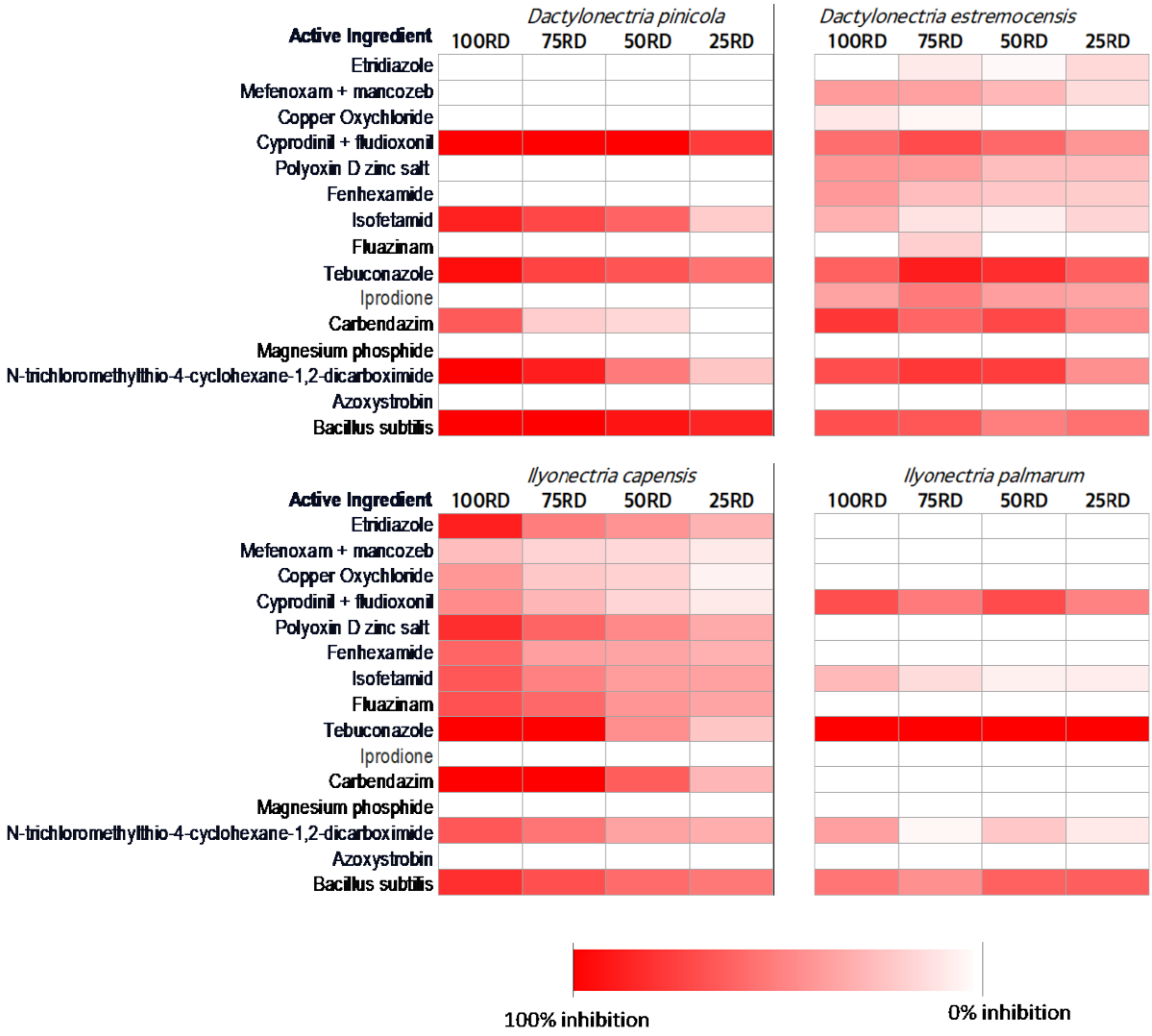

Figure 7: *In vitro* screening of fifteen fungicides against Nectriaceae pathogen species commonly occur in pine seedling root system. Fungicides tested are recorded as their active ingredient(s). The scale bar illustrates the percentage of inhibition, with red signifying 100% inhibition and white indicating 0% inhibition. Each fungicide was tested at four concentration level where 100RD = 100% of label recommended dose, 75RD = 75% of label recommended dose, 50RD = 50% of label recommended dose, and 25RD = 25% label recommended dose.

In vitro screening of fungicide efficacy: Fusarium oxysporum

We also conducted *in vitro* assessments of fungicide effectiveness against *Fusarium oxysporum* sourced from syptomatic pine root system. As above, our goal was to identify three fungicides that demonstrate high efficacy against the pathogens with minimal effects on beneficial microbes. As growers use a different set of fungicides to control this root rot pathogen, we tested 15 fungicides, many that overlapped with *Dactylonectria* and *Illyonectria* control, as well as some that were specific to *F. oxysporum* contorl. Very few of fungicides tested showed the

ability to control *F. oxysporum* growth (**Figure 8**). Fungicides exhibiting substantial effectiveness included active constituents pyraclostrobin + boscalid, cyprodinil + fludioxonil, prochloraz, fluazinam, azoxystrobin, and tebuconazole. For the ongoing trials under control conditions, N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide, carbendazim, tebuconazol, and etridiazole were chosen for fungicide treatment and used in rotation.

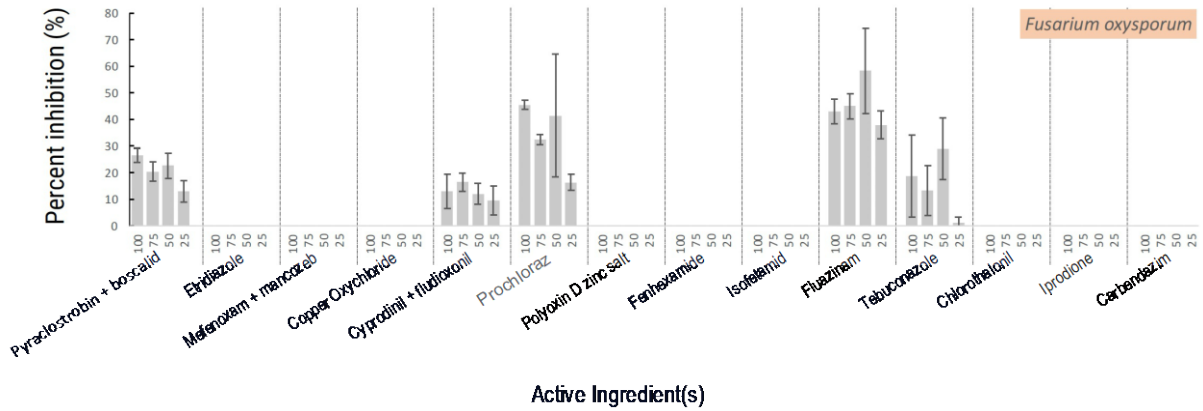

Figure 8: *In vitro* screening of fifteen fungicides against *F. oxysporum* isolated from symptomatic roots of pine seedling. Fungicides tested are recorded as their active ingredient(s). The scale bar illustrates the percentage of inhibition of growth across four concentrations 100 = 100% of label recommended dose, 75 = 75% of label recommended dose, 50 = 50% of label recommended dose, and 25 = 25% label recommended dose.

Beneficial microbiome addition reduces pathogen establishment

In an experiment run by our nursery partners HQPlantations, addition of three microbial inoculants were tested for their ability to control pathogen establishment and/or increase in abundance in the root systems of a Southern pine hybrid. When compared to no microbial inoculation (i.e. Nil; **Figure 9A**), all three inoculants reduced the presence of *Fusarium* sp. to nearly undetectable levels and reduced *Dactylonecriacea* abundance by 30-50% (**Figure 9A**). In a complementary controlled conditions experiment with radiata pine, we tested the ability of a WSU formulated mycorrhizal inoculum to control the establishment of *Fusarium* and other pathogens. In this instance, three isolates of *F. oxypsporum* were intentionally inoculated onto the pine root system and their establishment and growth tested after three months. Only in the instance where the WSU beneficial microbial inoculant was pre-applied to the roots prior to pathogen introduction were these three isolates of the pathogen either controlled (i.e. no pathogen increase in population over that added) or were repressed (**Figure 9B**, inset box). Therefore, addition of microbial inoculants containing beneficial fungi (e.g. mycorrhizal fungi) can actively reduce or neutralise the establishment of disease-causing pathogens. We also tested the ability of increasing microbiome diversity to inhibit the establishment of the second focal pathogen class, *Dactylonectriacea,* and found that there was a significant negative correlation between root fungal diversity and pathogen presence (**Figure 10**).

In planta testing of virulence of the B. cinerea:

We successfully inoculated and obtained disease in our controlled conidition trials (**Figure 11)**. We collected visual disease score data, providing us with an overview of the treatment effects on *Botrytis* control (**Figure 12A**). Our analysis revealed that routine fertilizer-treated plants that were not inoculated with mycorrhiza showed higher level of susceptibility compared to those inoculated with mycorrhiza. We also observed that the amino acid fertilizer treated plants were

significantly less susceptible to *B. cinerea* compared to HVP-fertilizer treated plants regardless of mycorrhizal inoculation. Furthermore, when we assessed the disease score data of plants treated with various controlling agents, we found that only the hyperparasite-treated plants showed a significantly lower susceptibility compared to the control plants (**Figure 12B**). Intriguingly, plants treated with Methyl-Jasmonate hormone displayed a high level of susceptibility, even surpassing that of the control plants. The altered fungicide regimes 1 and 2, based on selection of fungicides most active against *B. cinerea* in our *in vitro* testing, did not significantly repress *B. cinerea* disease symptoms compared to control. However, there was a distinct trend for reduced disease symptoms in these plants. Similarly, dsiRNA treatment did not significantly repress diease symptoms, but dsiRNA #2 showed an overall decrease in disease. Further testing of difference dsiRNA or fungicide concentrations may yield stronger inhibition.

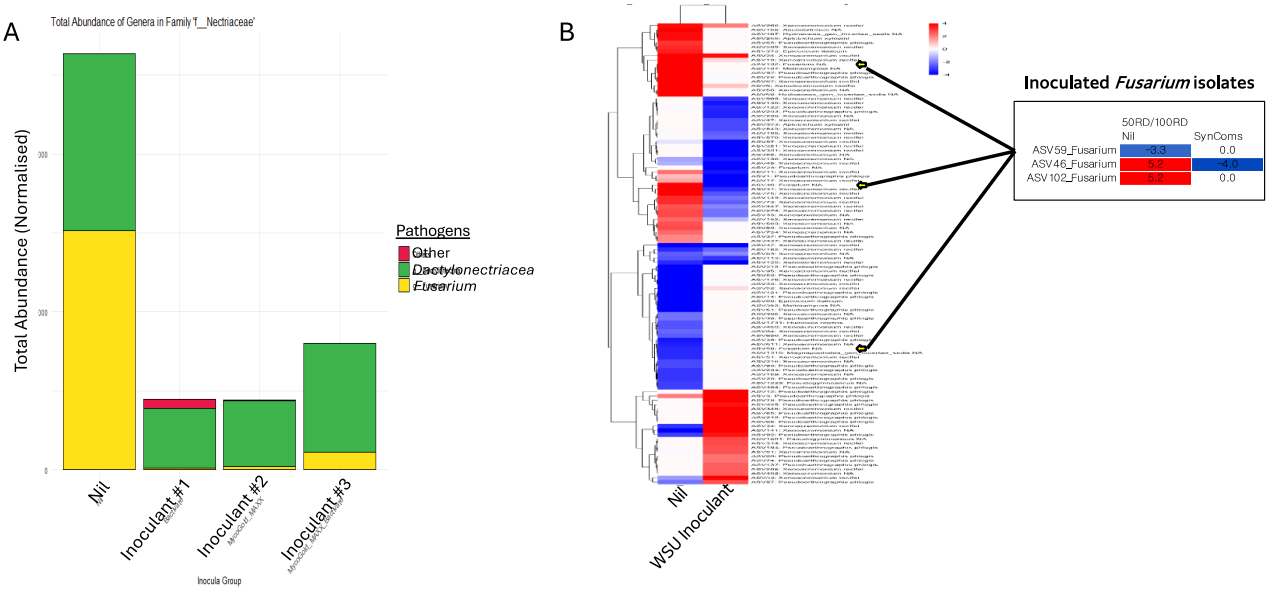

Figure 9: Impact of beneficial fungal pre-inoculation of pine roots controls establishment of disease causing *F. oxysporum*. (A) Control of *F. oxysporum* by three beneficial microbial inoculants in a Southern Pine hybrid. (B) Control of three inoculated isolates of pathogenic *F. oxysporum* by the WSU beneficial microbial inoculant.

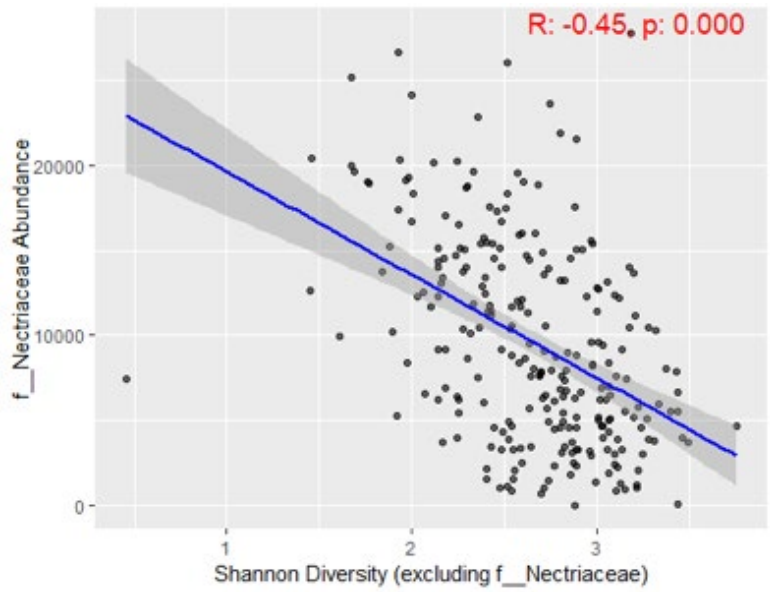

Figure 10: Correlation between nectriaceae abundance and total fungal biodiversity in pine roots.

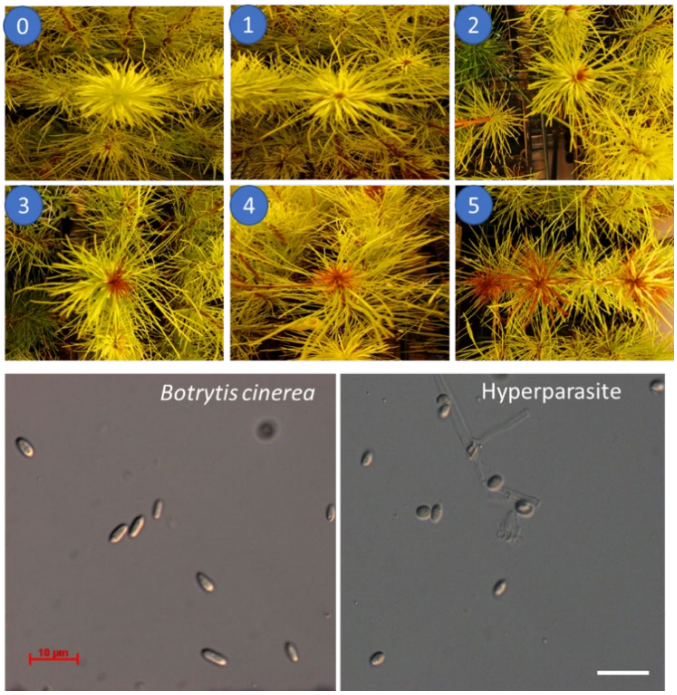

Figure 11: Visual disease scoring of *Pinus radiata* cuttings inoculated with Botrytis cinerea spore. Disease scores were assessed on a scale ranging from 0 to 5, with 0 indicating the least diseased plant, characterized by no needle death on the crown, and 5 representing the most severely diseased plant, featuring complete crown death. The lower panel displays Botrytis cinerea spores and hyperparasites, both applied at a concentration of 10^6/mL and 1 mL per plant.

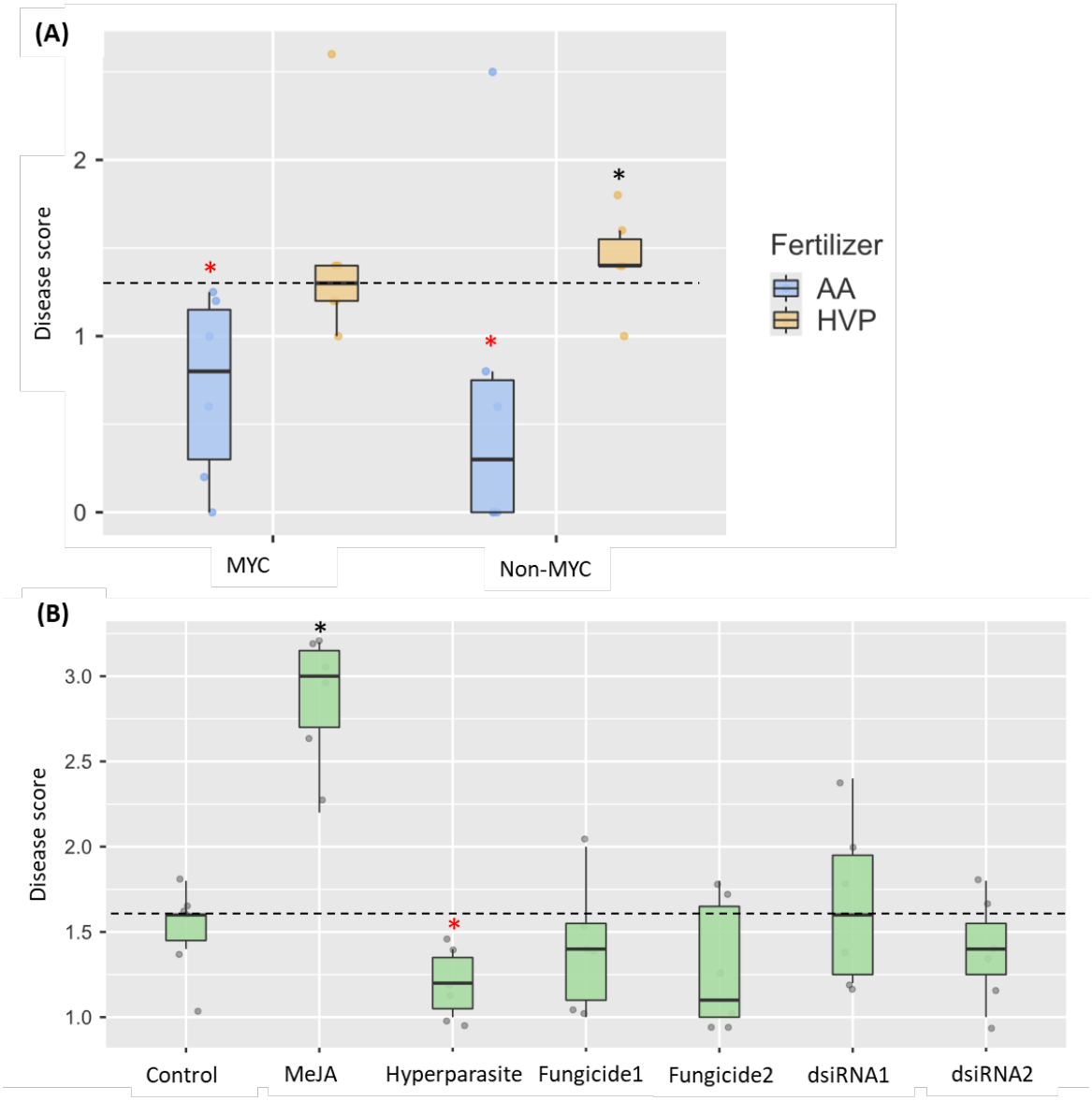

Figure 12: Effects of treatments on visual disease scores of *Pinus radiata* cuttings inoculated with *Botrytis cinerea*. (A) Impact of mycorrhizal inoculation and fertilizer types on disease scoring. (B) Impact of various controlling agents on disease scoring. AA= Organic Amino Acid fertilizer, HVP = HVP Plantation routine fertilizer, MYC= Mycorrhizal inoculation, and Non-MYC= Mycorrhizal not inoculated. Red and black asterisks denote treatments with significantly lower and higher impacts compared to the control (p value <0.05), respectively. The disease scores were derived from six replications, with each replication representing the average scores of 4 to 5 plants.

Discussion

This project advanced our understanding of how containerised nursery management of pine seedlings good be varied in order to more holistically manage plant vigour and disease. We were able to provide concrete advice to our forestry partners through three main innovations:

1. Optimisation of fertiliser x fungicide treatment by using knowledge of root microbiome ecology:

Beneficial fungi and bacteria found living on/in tree roots are known to promote disease resistance in plants. However, typical nursery treatment of plants through over-fertilisation and use of broad-spectrum fungicides can repress or kill these beneficial partners, thereby weakening plant health. Our first set of experimentation provided key information on the optimal conditions in radiata pine to control a soil-borne pathogen and improve the plant's natural immunity to increase survival while minimising fungicide requirements. We also ran similar experiments with Southern Pine to ascertain the broader application of the results. In an interesting finding, fertilisation reductions were far more effective in radiata pine than in Southern pine hybrids. A comparison of the total nutrition typically delivered to both crops found that Southern pine was already being given far less nutrition than radiata pine. Therefore, it is possible that Southern pine nurseries are already operating at the threshold limit of nutrition, a level below which is detrimental to both the plant and the mycorrhizal community.

We had a number of industry relevant findings when we tested the efficacity of fungicides sold to control the focus pathogens of this project. First, the majority of those tested had no direct impact on fungal growth. It is still possible that some of these compounds act to repress reproduction of the fungus, but growers would prefer to stop the growth of the pathogen before it reaches this point. Second, we found a number of unrelated active compounds were still useful, but that their recommended dose was far higher than needed. This could be because under nursery conditions, a great deal of water is used over the course of a day/week to combat transpired water, and this water would act to dilute fungicides applied. However, our growers found that pathogens were adequately controlled at 75% of the recommended dose of fungicides selected. A more worrisome finding was that the 'specificity' of the fungicides was minimal, with most of the strongest fungicides targeting both pathogen and mutualistic fungal growth. For example, *Rhizopogon subaustralis* showed very little inhibition by all fungicides tested while *Thelephora terrestris* was almost universally inhibited by all fungicides tested. Therefore, we need to focus more deeply on finding alternative fungicides or pathogen control measures to ensure that only the pathogen is affected by the treatment and not other fungi. We also found evidence of fungicide resistance in certain pathogen species tested. Whether this is naturally acquired resistance, or the result of overuse of chemicals in the nursery, is unknown. If naturally acquired (ie passed through generations without loss), the finding that specific species within one genus of fungi can be completely resistant to a fungicide while another species may be susceptible indicates that we need to improve the resolution of our studies to be able to identify the specific species of a fungal disease in order for growers to know which fungicides will be most effective. We should also begin to reinforce the need for fungicide rotational use in nurseries to ensure that we minimise the risk of fungicide resistance arising in our growing operations.

These new fertiliser/fungicide/microbiome guidelines were tested by nursery partners. Results in the nursery appear to date to have copied what we observed in controlled conditions. However, growers would like to test the revised fertilisation and fungicide regimes over a number of years to ensure there is reproducibility between seasons. The growers, however, are

actively disseminating what they have been doing with other nurseries country-wide, efforts that encourage adoption of our findings across the sector.

2. Exploiting beneficial microbes to prime plant immunity and boost plant resistance against specific diseases:

Recent research has shown that one of the main ways that plant diseases establish on their hosts is through direct suppression, or manipulation of plant immune pathways. Pathogenic fungi do this through the use of proteins that enter into plant cells or bind to cell surface receptors and thereby alter how the plant perceives the fungi. Beneficial fungi, meanwhile, have been shown to improve plant resistance to pathogens through a process called immune 'priming'. In these instances, the beneficial fungus begins colonising the root which induces a cascade of signalling events in the plant that eventually lead to the production of metabolic compounds that actively repress disease-causing microbes. Our experimentation studying the impact that the addition of beneficial mycorrhizal inoculum had to Nectriaceae and *Fusarium* growth in plant roots provided specific outcomes on the best practises to control these soil-borne pathogen within nurseries. These experiments also allowed us to determine the degree to which the optimal control of one soil-borne pathogen (e.g. Fusarium) overlaps with the control of other similar but unrelated pathogens (e.g. Nectriaceae). Current analysis of our results demonstrate that addition of beneficial microbial inoculants (including both mycorrhizal and endophytic fungi) were able to either repress or arrest the establishment of both pathogens. This is a critical finding going on as it demonstrates that the pine root microbiome has as relevant a role in containerised production of pine as it has been shown under field conditions. It also highlights the importance of getting the fertilisation and fungicide dosage correct to encourage the proper establishment and functioning of beneficial microbes in the root system for optimal plant health.

Our results, however, went beyond showing that it is simply enough to add mycorrhizal fungi, but that the diversity of the pine mycobiome is critical in supporting plant growth and health. This is a topic that is increasingly gaining attention in the literature as other groups in Europe and the America's are also finding that maintaining microbiome diversity in soils is a critical aspect of sustainable plant production. A unique aspect of our work has been the finding that the link between fungal biodiversity and plant health is not necessarily resting on the diversity of mycorrhizal fungi alone, but rather the diversity of saprotrophic and endophytic fungi as well. This has guided our further research into how we can feasibly affect microbiome diversity in containerised production and we have shown that to get the balance correct, potting mix is best supplemented by a microbial inoculum, but also that these synthetic communities need to be formulated based on larger data sets that currently available. Therefore, we need to produce more fungal presence/absence datasets to further our planning for optimal microbial diversity in the roots of pines to control pathogen establishment.

3. Leverage natural fungal-fungal parasitic relationships to develop novel biocontrol strategies:

The experiments associated to control of *B. cinerea* sought to understand how we can best pair below-ground management with above-ground treatment to improve pine nursery stock systemic disease resistance. These experiments used state-of-the art methodologies that are being trialled for other pathogen systems as a means of controlling prevalent crop diseases.
While the majority of our methodologies have yet to be applied within the forestry sector in Australia, if successful, they could prove to be a more environmentally friendly, cost effective

means of controlling disease. Specifically, we sought to apply our molecular understanding of how disease is caused by a pathogen to develop novel products that could be later commercialised.

Currently, on-market biocontrol products to manage foliar pathogenic microbes rely on one fungus from the genus *Trichoderma*, a fungus that can promote plant health through direct parasitism of other disease causing fungi (a process called 'hyperparasitism'). These products are not always suited to harsh Australian conditions, and may not be effective as a result. Additionally, all of the machine learning approaches that we applied within this project constantly highlighted that *Trichoderma* application to pine was detrimental to below-ground biodiversity – an aspect that we consistently see as needed for optimal host health. Australian forests, however, host a much wider range of fungal hyperparasites than just *Trichoderma* and researchers at WSU have cultured a number of novel hyperparasites that could be used to combat *Botrytis cinerea*. This project trialled the use of these hyperparasites as biocontrol options for nurseries, one of which significantly repressed *B. cinerea.* If further research proves that this is applicable across growing regions, we would consider pursuing the necessary approval by the APVMA and then marketed to growers. Adoption of this product would be promoted as above through reports, workshops, and consultations.

We tested the role of methyl-jasmonate to control the same plant diseases. While we did not investigate the underlying reasons for the high susceptibility in Methyl-Jasmonate-treated plants, the timing of application may have played a significant role. Methyl-Jasmonate acts as a priming agent that triggers systemic immunity responses. Experimentation considering different timing of application of this hormone, or other hormone regulators, should be considered in future.

Work in our lab has determined that pathogenic fungal manipulation of host immunity can be interrupted by applying a spray of double-stranded interfering RNA (dsiRNA), thereby boosting plant disease resistance. In this project, we used previous research to design and test a range of dsiRNA sprays that will repress pathogenesis. Unfortunately, the pathogen continued to grow and there was no evidence of attempting to accesss root colonisation. However, there was promise of one of the dsiRNA products that could be further studied at different concentrations to see if there was a 'sweet spot' for fungal and host aspects.

Conclusions

This project was established to develop new options to boost the biodiversity of beneficial soil and root-associated fungi in pine nursery production to improve nursery pathogen control in a more sustainable and environmentally conscious manner. This was achieved through our findings that:

- Reduced fertilization by ≤50% achieved the desired plant growth and health within both controlled environment growth and in a nursery setting
- Fungicides currently being used across the partners were not be as effective as desired for controlling target pathogens. This was possibly due to some of the pathogens isolated from nurseries showing traits consistent with fungicide resistance.
- Fungicides used across the partners had very detrimental effects to mycorrhizal fungi (e.g. phosphorus acid containing formulations).
- We have developed a testing pipeline and list of effective fungicides for use that have reduced impact on beneficial microbes and equal or improved control of target pathogens
- Commercially available amino acid fertilizer increased the biodiversity of the root microbiome in nursery grown seedlings
- Addition of benefical microbial inoculants improved plant health in both nursery and controlled conditions, likely through direct competition between the beneficial microbiomes added and disease-causing pathogens
- As a general relationship, increases in microbiome diversity were found to correlate strongly and significantly to repression of pathogen populations in the roots of pine.
- We have successfully piloted novel, environmentally conscious treatments that better control *Botrytis cinerea*

Our nursery partners have already begun to adapt their nursery management practices based on these findings with reduction in fertigation in radiata pine nurseries and altered fungicide choice in both radiata pine and Southern pine hybrid nurseries.

Recommendations

The originally proposed project had three objectives. Here we synthesize our key recommendations to industry based on our findings. These are:

Objective 1: Determine the optimal combination of in-nursery applications of fertilisation, fungicide, and beneficial microbes (e.g. mycorrhiza) to enhance seedling natural immunity against soilborne pathogens and to increase health/survival while minimising inputs and validate these best practises against an identified priority pathogen within forestry nurseries.

Recommendations for Objective 1:

- Current nursery levels of fertilisation can be reduced:
    - Slow-release fertiliser can be decreased between 25-50% without significant impact on plant health. Medium-term release fertilisers had the best impact on microbiome diversity while longer-term release profiles did not appear to impart benefit during the nursery phase of growth.
    - Fertigation can be reduced by 25% and improve plant health.
- Mineral fertiliser should be partially replaced with more complex forms of nutrition (ie. Amino acids)
    - Amino acid-based fertiliser use can be implemented to increase the diversity of the root microbiome
    - Amino acid-based fertiliser discourages root pathogen growth for *Fusarium* and *Ilyonectria*
- Use of Biochar + *Trichoderma* had nominal impact in our experimental analyses, and in some cases had negative impacts on microbiome diversity and to plant health and growth. Therefore future research is needed to understand if these treatments should be used of if they should be discontinued
- Fungicide choice currently in rotation at the partner nurseries must be reviewed as testing suggests that the majority do not appear to effectively control target pathogens, that the specific species of the pathogen affects fungicide choice (e.g. *Ilyonectria palmarum* was far more fungicide resistant than *Ilyonectria capensis*) and/or that they have negative impacts on the growth of beneficial microbes
    - For *Fusarium* control, the best active ingredients found were fluazinam, azoxystrobin, and a mixture of cyprodinil and fludioxonil.
    - For treating *Dactylonectria/Ilyonectria*, the best active ingredients found were N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide, carbendazim, and tebuconazole.
- A rotation of these active compounds should be used to avoid fungicide resistance in nurseries

Objective 2: Determine how optimised fertilisation and belowground microbial populations can affect plant immunity and ameliorate above ground control methods of foliar based pathogens.

Recommendations for Objective 2:

- Our current data would suggest that rotational use of fungicides with the active ingredients Pyrimethanil and Iprodione are likely to be the most beneficial at controlling *B. cinerea* in nursery settings.

- Under controlled conditions, use of methyl jasmonate as an immune inducer after detection of *B. cinerea* infection is not recommended as it overstimulates plant defense and leads to increased death.
- Use of the hyperparasite trialled showed promise with significant reduction in disease. Further research in the application of hyperparasite control in nursery production conditions is recommended.
- Amino acid-based fertilisers reduced disease incidence, showing impact of nutrient source also can be a novel control for foliar pathogens
- Inoculation of cuttings with a beneficial mycorrhizal inoculum slurry is not recommended as a sole control option for *B. cinerea* infection.

Objective 3: Determine the level of improvement conferred by using optimised fertilisation x mycorrhization x fungicide treatments on in-nursery pathogen control and early seedling survival post-planting.

Recommendations for Objective 3:

- Addition of mycorrhizal inoculum is recommended as it reduces the establishment of fungal root pathogens in nursery growth
- Appropriate fungicides should be used as mycorrhizal inoculum with the addition of broad spectrum fungicides (e.g. phosphorous acid) nullifies the protective effect of mycorrhizal inoculation.
- Combined reduction of fertiliser use by 25% with the use of fungicides as listed above at 75% label recommended dose is recommended in radiata pine growth as the dual strategy synergistically works to generate seedlings with equivalent plant establishment and improved survival in field
- In Southern Pine hybrid, fertilisation and fungicide treatments should be varied depending on planting date to reduce the number of topping events prior to field planting (i.e. lower nutrition during early sowings and higher nutrition for late sowing to manage growth). Ideally no more than one topping should be used to prevent loss of post-planting vigour.

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