THESIS

EFFECTS OF DROUGHT STRESS ON EARLY WHITE PINE

BLISTER RUST DEVELOPMENT IN LIMBER PINE

Submitted by

Jonathan H Bertram

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Master's Committee:

Advisor: Jane E. Stewart
Co-Advisor: Anna W. Schoettle

Brad Borlee
Troy Ocheltree

ABSTRACT

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Climate change and forest pathogens are expected to interact as incidences of drought increase and affect the disease triangle between hosts, pathogens, and the environment. Trees will become physiologically affected by drought stress and primary pathogens such as fungal biotrophs will experience drought stress as mediated through the host. White pine blister rust, caused by the non-native pathogen *Cronartium ribicola*, is a devastating fungal pathogen, and little is known about how it will perform (measured by fungal growth or disease severity) within pine hosts experiencing unusual drought.

This study aimed to address some of the unknown aspects of this interaction by performing a greenhouse drought × pathogen experiment with *Pinus flexilis* seedlings, measuring host physiology, quantifying specific aspects of pathogen performance, and looking for interactive effects. Watering treatments consisting of well-watered, mild chronic drought, or severe acute drought were applied to 432 seedlings; after 3 months, a subset of 198 seedlings were inoculated with *C. ribicola* basidiospores under ideal inoculation conditions, after which watering treatments continued for a further 9 months. Specific rust performance measurements included mycelial growth via relative rust DNA quantification and ratings of disease severity by watering treatment.

The effect of watering treatments on seedlings was characterized by water potential and chlorophyll fluorescence techniques at 10 intervals throughout the experiment. Needles

were sampled and DNA was extracted just after inoculation and just prior to the onset of visual symptoms; *C. ribicola* was detected within 81% of inoculated seedlings, with no effect of watering treatment ($p = 0.1999$). Within 9 months of inoculation, white pine blister rust disease symptoms developed on 25% of *P. flexilis* seedlings, with no effect of watering treatment. Using real-time qPCR, *C. ribicola* DNA was quantified and standardized against *P. flexilis* DNA at 14 days post-inoculation and 116 days post-inoculation, and no effect of watering treatment on relative DNA amount was observed ($p = 0.3936$, $p = 0.9347$, respectively). Within this early disease period, it was observed that inoculated seedlings were likely to have significantly lower water potential and lower chlorophyll fluorescence ($p = 0.0417$, $p = 0.0377$, respectively), even without developing visual symptoms.

The goal of this study was to identify how white pine blister rust would respond to host drought within the early infection period. The drought treatments within this experiment did not differentiate rust performance as measured by DNA techniques or visual inspection, but we were able to detect *C. ribicola* infection and colonization much sooner than if relying on visual white pine blister rust symptoms alone. Quantifying standardized rust DNA is an effective tool to measure early white pine blister rust development in pine hosts.

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1. Background

General effects of climate change will result in increased incidences of drought in the western United States (van Mantgem, 2009). Changes to seasonal weather cycles that divert precipitation will lead to chronic drought or varied drought, and the type of drought will play a role in how plant pathosystems respond. Regions that experience a continual water deficit throughout the growing season would be described as that experience chronic drought, while regions that experience varied drought will be challenged with fluctuations in the timing and severity of water deficits (IPCC, 2001). Regardless of the type, drought will act on all biotic agents within a system, including both plant pathogens and the plant host.

Within a pathosystem, droughted environments can affect both the pathogen and cause host stress, thereby distorting the relationship described as the disease triangle (McNew, 1960; used in Agrios, 2005, La Porta et al., 2008). The disease triangle describes the relationship between a host, pathogen, and the environment, and certain abiotic factors in the environment or when factors for the host can tip the balance in favor of the pathogen which results in disease (Hennon et al., 2020). Under drought, hosts endure stress that limits their performance and fitness, while also inhibiting their ability to produce defenses against pathogens. In general, water-stressed hosts have less carbon to allocate towards defensive molecules, as discussed by Chaves et al. (2002), compromising their ability to fight off pests and pathogens. However, not all pathosystems will respond in the same manner, particularly for pathogens whose success is dependent on the vitality of the host. Rust pathogens, for

example, are entirely dependent on the health of their plant host for nutrients and reproduction (Staples, 2000). Whether future climate scenarios in western forests will favor the pathogen or the host is not yet clear for many of these pathosystems, including the disease white pine blister rust.

White pine blister rust (WPBR) is the disease caused by the invasive fungal pathogen *Cronartium ribicola*, which can kill North American five-needle pines. The rust fungus *C. ribicola* alternates between two different host plants (heteroecious) while advancing through five different life stages (macrocyclic). The complex life cycle starts when an infected primary pine host produces aeciospores in spring that infect the leaves of the alternate host, *Ribes* spp. Throughout the growing season, the infected *Ribes* spp. produces orange uredinia which produce urediniospores that infect nearby alternate host leaf tissues, like neighboring *Ribes*, other leaves on the same plant, or even other parts of the same leaf (Lachmund, 1933). This reinfection stage on the alternate host continues throughout the summer, until environmental cues in the late summer—early fall trigger the formation of telia from the existing uredinia. Telia are brownish, fine, finger-like extensions that are a transitional stage, producing large teliospores, which produce basidiospores. Dispersed basidiospores infect the needles of five needled pines, if environmental conditions are appropriate for germination (Van Arsdel et al., 1956). Environmental cues are important for the advancement of the rust life stages on the alternate host, and environmental limits drive the success of primary host infection.

After needle penetration through the stomata (Lachmund, 1933), *C. ribicola* interacts with the pine, secreting effector molecules which recognize the suitability of the host (Liu et

al., 2015). Within the needle, the rust produces haustoria (Robb et al., 1974) and begins absorbing non-structural carbohydrates from the host, allowing for mycelial growth. Over several years the rust grows from the needle entry point towards the base of the needle, growing through extra-cellular spaces (see Hudgins et al., 2005), towards the bole of the tree. During the first fall 6-8 months after initial infection, pycnia can form, exuding haploid spermatia secretion that can fuse with other spermatia. After a few years of growth, the fertilized spermatia of *C. ribicola* in the stem can produce aecia and subsequently aeciospores, capable of infecting *Ribes* spp. During infection, the rust diverts resources from the tree, causes vascular occlusion, and produces wounds (cankers, blisters) (Hunt et al., 2007). If mycelial growth reaches the bole, it can girdle the tree and cause top-kill, which can take 10 years or more in mature trees but can kill seedlings in less than 2 to 3 years. After a pine is infected, the ability to produce cones in the upper crown is impacted due to top-kill from the rust, and infected pines can spread aeciospores until death (Geils et al 2010). Produced aeciospores infect the alternate hosts, which can infect other hosts or reinfect the initial primary host, multiplying the pathogen spread. High humidity is required for *C. ribicola* spore formation and infection of the pine host, and successful reinfection events that occur alongside appropriate weather patterns can create wave years, where high incidence of WPBR is found when meteorological conditions and available hosts align. However, after successful pine infection, *C. ribicola* only experiences the environment as mediated through the host, suggesting that environmentally induced physiological changes to the pine host will play an outsized role in determining the fate of the pathogen.

Pines have morphological and physiological responses to drought that can affect their development and survival. Typically, pines will divert resources towards root elongation to

aid in water acquisition, and this drought response is usually measured via the root-to-shoot ratio (Aaltonen et al., 2017; Pearson et al., 2013). Physiologically, constant water potential is maintained during drought by partial closure of stomata, lowering the water demand. If water demand is great enough, these gymnosperms can close stomata completely, preventing hydraulic failure (Klein et al., 2011). Partially closed stomata limit the input of carbon dioxide, and intercellular carbon dioxide concentration can be rate-inhibiting for photosynthesis (Gao et al., 2002). Water stress leads to the reduction of carbon compounds which are in high demand (Kozlowski, 1992), and tree maintenance of hydraulic integrity is prioritized over tree growth or synthesizing carbon-based tree defenses (Madmony et al., 2018). The de-prioritization of defense compound production can leave pines vulnerable to pathogen attack. Letts et al. (2009) documented how limber pine (*Pinus flexilis)* in the Rocky Mountains reduced photosynthetic activity in response to arid conditions and that the needles were well-suited to withstand long drought events. Reinhardt et al. (2015) noted that the conservative water-use strategy of *P. flexilis* enabled needles to continue selectively photosynthesizing long into drought, until mortality caused by hydraulic failure once soil moisture content dropped below a critical threshold. Millar et al. (2007) found that high-elevation limber pine in Sierra Nevada, California expressed high variability in year-to-year growth during periods of sustained drought and elevated temperature. They observed that drought-weakened *P. flexilis* were more likely to suffer mortality from a subsequent mountain pine beetle (*Dendroctonus ponderosae* Hopkins) attack but highlighted that the limber pine which survived were hardened against drought, as they observed no mortality in the stand after a later severe drought (Millar et al., 2007).

There are many ways that drought can affect fungal infection, largely depending on the relationship the pathogen has with the plant host and if the host is under additional duress (Desprez-Loustau et al., 2006; Hennon et al., 2020). Necrotrophs, fungal pathogens that parasitize dead or dying cells, are typically more successful on a droughted host, and a more severe drought increases access to dying cells (Taylor & Deacon, 1997). In contrast, biotrophs parasitize living cells for carbon and nutrients (Glazebrook, 2005); host stress such as drought may lead to pathogen starvation. Trees have strategies to respond to parasitism, including bolstering resources and defenses near the infection site to fight infection (endurance strategy), and attempting to starve the infection area by moving resources away (evasion strategy)(Seifi et al., 2013). However, water stress can interfere with both strategies, as droughted trees have less resources for defenses and hydraulic stress could limit the ability to mobilize nutrients away from infection. Drought-induced defense reductions are likely to increase the susceptibility of trees to certain fungal infections, but the interaction is complicated because drought can also reduce the suitability of the host (Kolb et al., 2016, Jectel et al., 2012). As rusts are obligate biotrophs, they are particularly linked to the host—they only grow on suitable hosts, some stages cannot reinfect the same host, and advancing to the next reproductive stage often only occurs under appropriate environmental conditions. These qualities make rusts an effective pathogen in which to study host stress interactions, as each rust-host pathosystem is self-contained at certain stages and limited in the ways it can respond to changing host physiology.

While these ideas have been explored with native pathogens and native hosts, it's not clear if non-native pathogens will benefit from host stress in the same manner, as non-native pathogens often succeed due to novel or specialized strategies no matter the condition of the

host (Rausher, 2001). Some non-native pathogens evade host defenses (Hudgins et al., 2005), making stress-induced host defense reductions less relevant. However, host plant stress affects resource availability not only for maintaining osmotic potential (Meier et al., 1992) and defense but also affects carbon availability for biotrophic parasitic pathogens, as illustrated by Oliva et al. (2014). A stressed plant with less supply of carbon compounds will also place a constraint on the capacity of the pathogen to reach its growth potential, especially if the compounded stresses lead to carbon starvation (Oliva et al., 2014).

The threat of WPBR is great in high elevation five-needle white pines in western forests (Tomback & Achuff, 2010), and it may be as devastating in the southern Rocky Mountains as it has been throughout North America (Geils, Hummer, & Hunt, 2010). White pine blister rust requires elevated humidity to advance through life stages (Van Arsdel, 1956), but little is known about how drought will affect disease development. *Pinus flexilis* has mechanisms for dealing with extreme conditions, particularly drought (Letts et al., 2009; Millar et al., 2007), and is attributed as having high phenotypic plasticity, expressing different phenotypes and competitiveness in response to environmental stress (Reinhardt et al., 2011; Schuster et al., 1995). Many aspects of the white pine blister rust system have not yet been studied experimentally, including what factors impact the success of this pathogen and how anticipated environmental changes will affect the impact of this ecologically important disease.

White pine blister rust is an important non-native plant pathogen that threatens five-needle pines, and there are aspects of *C. ribicola* biology that remain unknown. Susceptible pine hosts are expected to experience drought as climate change intensifies, altering the

disease triangle in this WPBR pathosystem. The full extent of this changed relationship is not yet clear, but there are several factors that are expected to play an important role in understanding the potential outcome, including the duration and intensity of drought. It is clear that white pine blister rust depends on high humidity to spread and infect new hosts (Van Arsdel et al., 1956), but it is not clear how subsequent drought will affect colonization. The consequences of host drought could lead to rust starvation, or the combined water stress and pathogen burden could accelerate decline and hasten tree mortality. As of yet, the effect of pine drought on WPBR colonization has not been studied. Ahead is a greenhouse drought experiment that aims to identify the effects of water stress on *Cronartium ribicola* within *Pinus flexilis*.

2. References

Aaltonen, H., Linden, A., Heinonsalo, J., Biasi, C., & Pumpanen, J. (2017). Effects of prolonged drought stress on Scots pine seedling carbon allocation. *Tree Physiology*, 37(4), 418–427. https://doi.org/10.1093/treephys/tpw119

Agrios, G. N. (2005). *Plant pathology*. Elsevier.

Chaves, M. M., Pereira, J. S., Maroco, J., Rodrigues, M. L., Ricardo, C. P. P., Osório, M. L., Carvalho, I., Faria, T., & Pinheiro, C. (2002). How Plants Cope with Water Stress in the Field? Photosynthesis and Growth. *Annals of Botany*, 89(7), 907–916. https://doi.org/10.1093/AOB/MCF105

Desprez-Loustau, M. L., Marçais, B., Nageleisen, L. M., Piou, D., & Vannini, A. (2006). Interactive effects of drought and pathogens in forest trees. *Annals of Forest Science*, 63(6), 597–612. https://doi.org/10.1051/forest:2006040

Gao, Q., Zhao, P., Zeng, X., Cai, X., & Shen, W. (2002). A model of stomatal conductance to quantify the relationship between leaf transpiration, microclimate and soil water stress. *Plant, Cell and Environment*, 25(11), 1373–1381. https://doi.org/10.1046/j.1365-3040.2002.00926.x

Geils, B. W., Hummer, K. E., & Hunt, R. S. (2010). White pines, Ribes, and blister rust: A review and synthesis. *Forest Pathology*, 40(3–4), 147–185. https://doi.org/10.1111/j.1439-0329.2010.00654.x

Glazebrook, J. (2005). Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. *Annual Review of Phytopathology*, 43(1), 205–227. https://doi.org/10.1146/annurev.phyto.43.040204.135923

Hennon, P. E., Frankel, S. J., Woods, A. J., Worrall, J. J., Norlander, D., Zambino, P. J., Warwell, M. V., & Shaw, C. G. (2020). A framework to evaluate climate effects on forest tree diseases. *Forest Pathology*, 50(6), 1–10. https://doi.org/10.1111/efp.12649

Hudgins, J. W., McDonald, G. I., Zambino, P. J., Klopfenstein, N. B., & Franceschi, V. R. (2005). Anatomical and cellular responses of *Pinus monticola* stem tissues to invasion by *Cronartium ribicola*. *Forest Pathology*, 35(6), 423–443. https://doi.org/10.1111/j.1439-0329.2005.00425.x

Hunt, R. S., Roke, G., & Jensen, G. D. (2007). Initiation of stem infection in western white pine by blister rust [*Cronartium ribicola*]. *Canadian Journal of Plant Pathology*, 29(1), 18–24. https://doi.org/10.1080/07060660709507433

IPCC. 2001. Climate change 2001: the scientific basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.

Jactel, H., Petit, J., Desprez-Loustau, M. L., Delzon, S., Piou, D., Battisti, A., & Koricheva, J. (2012). Drought effects on damage by forest insects and pathogens: A meta-analysis. *Global Change Biology*, 18(1), 267–276. https://doi.org/10.1111/j.1365-2486.2011.02512.x

Klein, T., Cohen, S., & Yakir, D. (2011). Hydraulic adjustments underlying drought resistance of *Pinus halepensis*. *Tree Physiology*, 31(6), 637–648. https://doi.org/10.1093/treephys/tpr047

Kolb, T. E., Fettig, C. J., Ayres, M. P., Bentz, B. J., Hicke, J. A., Mathiasen, R., Stewart, J. E., & Weed, A. S. (2016). Observed and anticipated impacts of drought on forest insects and diseases in the United States. *Forest Ecology and Management*, 380, 321–334. https://doi.org/10.1016/j.foreco.2016.04.051

Kozlowski, T. T. (1992). Carbohydrate sources and sinks in woody plants. *The botanical review*, 58(2), 107-222.

La Porta, N., Capretti, P., Thomsen, I. M., Kasanen, R., Hietala, A. M., & Von Weissenberg, K. (2008). Forest pathogens with higher damage potential due to climate change in Europe. *Canadian Journal of Plant Pathology*, 30(2), 177–195. https://doi.org/10.1080/07060661.2008.10540534

Lachmund, H. G. (1933). Mode of entrance and periods in the life cycle of *Cronartium ribicola* on *Pinus monticola*. *Journal of Agricultural Research*, 47(10), 791–806.

Letts, M. G., Nakonechny, K. N., Van Gaalen, K. E., & Smith, C. M. (2009). Physiological acclimation of *Pinus flexilis* to drought stress on contrasting slope aspects in Waterton Lakes National Park, Alberta, Canada. *Canadian Journal of Forest Research*, 39(3), 629–641. https://doi.org/10.1139/X08-206

Liu, J. J., Sturrock, R. N., Sniezko, R. A., Williams, H., Benton, R., & Zamany, A. (2015). Transcriptome analysis of the white pine blister rust pathogen *Cronartium ribicola*: De novo assembly, expression profiling, and identification of candidate effectors. *BMC Genomics*, 16(1), 1–16. https://doi.org/10.1186/s12864-015-1861-1

Madmony, A., Tognetti, R., Zamponi, L., Capretti, P., & Michelozzi, M. (2018). Monoterpene responses to interacting effects of drought stress and infection by the fungus *Heterobasidion parviporum* in two clones of Norway spruce (*Picea abies*). *Environmental and Experimental Botany*, 152(March), 137–148. https://doi.org/10.1016/j.envexpbot.2018.03.007

McNew, G. L. (1960). The nature, origin, and evolution of parasitism. *Plant pathology: an advanced treatise*, 2, 19-69. Elvesier.

Meier, C. E., Newton, R. J., Puryear, J. D., & Sen, S. (1992). Physiological responses of loblolly pine (*Pinus taeda* L.) seedlings to drought stress: osmotic adjustment and tissue elasticity. *Journal of Plant Physiology*, 140(6), 754-760.

Millar, C. I., Westfall, R. D., & Delany, D. L. (2007). Response of high-elevation limber pine (*Pinus flexilis*) to multiyear droughts and 20th-century warming, Sierra Nevada, California, USA. *Canadian Journal of Forest Research*, 37(12), 2508–2520. https://doi.org/10.1139/X07-097

Oliva, J., Stenlid, J., & Martínez-Vilalta, J. (2014). The effect of fungal pathogens on the water and carbon economy of trees: Implications for drought-induced mortality. *New Phytologist*, 203(4), 1028–1035. https://doi.org/10.1111/nph.12857

Pearson, M., Saarinen, M., Nummelin, L., Heiskanen, J., Roitto, M., Sarjala, T., & Laine, J. (2013). Tolerance of peat-grown Scots pine seedlings to waterlogging and drought: Morphological, physiological, and metabolic responses to stress*. Forest Ecology and Management*, 307, 43–53. https://doi.org/10.1016/j.foreco.2013.07.007

Rausher, M. D. (2001). Co-evolution and plant resistance to natural enemies. *Nature*, 411(6839), 857–864. https://doi.org/10.1038/35081193

Reinhardt, K., Germino, M. J., Kueppers, L. M., Domec, J. C., & Mitton, J. (2015). Linking carbon and water relations to drought-induced mortality in *Pinus flexilis* seedlings. *Tree Physiology*, 35(7), 771–782. https://doi.org/10.1093/treephys/tpv045

Robb, J., Harvey, A. E., & Shaw, M. (1975). Ultrastructure of tissue cultures of *Pinus monticola* infected by *Cronartium ribicola*. I. Prepenetration host changes. *Physiological Plant Pathology*, 5(1), 1-8

Schuster, W. S., Mitton, J. B., Yamaguchi, D. K., & Woodhouse, C. A. (1995). A comparison of limber pine (*Pinus flexilis*) ages at lower and upper treeline sites east of the continental divide in Colorado. *American Midland Naturalist*, 133(1), 101–111. https://doi.org/10.2307/2426351

Seifi, H. S., Van Bockhaven, J., Angenon, G., & Höfte, M. (2013). Glutamate metabolism in plant disease and defense: Friend or foe? *Molecular Plant-Microbe Interactions*, 26(5), 475–485. https://doi.org/10.1094/MPMI-07-12-0176-CR

Shepherd, B., Jones, B., Sissons, R., Cochrane, J., Park, J., Smith, C. M., & Stafl, N. (2018). Ten years of monitoring illustrates a cascade of effects of white pine blister rust and focuses whitebark pine restoration in the Canadian Rocky and Columbia Mountains. *Forests*, 9(3). https://doi.org/10.3390/f9030138

Staples, R. C. (2000). Research on the rust fungi during the twentieth century. *Annual review of phytopathology*, 38(1), 49-69.

Taylor, J., & Deacon, J. W. (1997). Introduction to Modern Mycology. *Mycologia*, 73(6), 1212–1213.

Tomback, D. F., & Achuff, P. (2010). Blister rust and western forest biodiversity: Ecology, values and outlook for white pines. *Forest Pathology*, 40(3–4), 186–225. https://doi.org/10.1111/j.1439-0329.2010.00655.x

Van Arsdel, E. P., Riker, A. J., & Patton, R. F. (1956). The effects of temperature and moisture on the spread of white pine blister rust. *Phytopathology*, 46(6), 307-318.

Van Mantgem, P. J., Stephenson, N. L., Byrne, J. C., Daniels, L. D., Franklin, J. F., Fulé, P. Z., Harmon, M.E., Larson, A.J., Smith, J.M., Taylor, A.H., Veblen, T. T. (2009). Widespread increase of tree mortality rates in the western United States. *Science*, 323(5913), 521-524.

II. HOST DROUGHT DOES NOT EFFECT INFECTION OR EARLY COLONIZATION OF *CRONARTIUM RIBICOLA* IN LIMBER PINE AND EARLY COLONIZATION HAS A PHYSIOLOGICAL COST

1. Introduction

Non-native pathogens pose an ongoing threat to forest ecosystems and the variability associated with climate change has implications for invasion, infection, and colonization (IPCC, 2007; La Porta et al., 2008; Sturrock et al., 2011). On an epidemiological scale, changes in precipitation will open up invasion pathways that enable pathogens to establish and threaten new communities (Dudney et al., 2021). On a community scale, humidity-dependent infection processes will be affected by changes in meteorological conditions, with drought benefitting some pathogens and disadvantaging others (Helfer, 2014; Tomback et al., 2016). Physiologically, drought experienced by the host is likely to influence the colonization of pathogens, but most of what is known relates to native pathogens (Kolb et al., 2016). The expected climate change-induced drought in western US forests will alter key ecosystems and the performance of species like limber pine (*Pinus flexilis* James) which are important ecosystem pioneers (Brodersen et al., 2019; Moyes et al., 2013; Reinhardt et al., 2011). We have yet to fully understand how climate change and associated drought in western forests will change interactions between non-native pathogens and native hosts.

Generally, it is predicted that climate change in the western US will lead to increasing drought and increasing temperatures which will accelerate evapotranspiration (Dudney et al., 2021; Hennon et al., 2020; IPCC, 2007), with some regions chronically receiving less precipitation. The result of changes in precipitation will depend on interactions with terrain,

especially high-elevation mountains, where changes in precipitation and snowpack can vary with geographic variation and change which streams and valleys receive snowmelt. The precipitation sources for established mountainous communities may be redirected, altering watershed hydrodynamics temporally and spatially (Marston & Anderson, 1991). These effects include introducing drought to areas that do not regularly experience it and altering the severity or duration in areas the experience regular drought (Williams et al., 2007). Areas that continue to receive snowfall, but less of it, would experience chronic drought. Other areas may face acute drought, where concentrated snowfall occurs in short periods but in between precipitation events, severe drought occurs, creating wet and dry intervals. Loss of snowpack in the winter may lead to bare soil exposure, erosion, and runoff in the spring, exacerbating the volatility of rain events (Farnes, 1990). A vital component of community survival in new drought regimes will depend on, among other things, physiological plasticity of plants to withstand different types of drought, whether it be chronic or acute (Garland & Kelly, 2006), and limber pine is an important mountain species that will continue to experience drought.

Pinus flexilis is a native keystone species of high-elevation ecosystems in the mountain west and plays an outsized role in supporting and maintaining subalpine ecosystems (Schoettle & Rochelle, 2000). Individuals are able to germinate and grow in dry, rocky slopes, stabilizing soil against erosion and acting as nurse plants for successional tree species which develop into communities which offer ecological services (Baumeister & Callaway, 2006; Donnegan & Rebertus, 1999; Rebertus et al., 1991). Limber pine can tolerate harsh environments and offers environmental protection for flora and food and shelter for fauna (McCutchen, 1996; Schoettle & Negron, 2001), particularly its seed-dispersal agent, Clark's

Nutcracker (*Nucifraga columbiana* Wilson) (Tomback & Linhart, 1990). Though limber pine can be found within a wide range of elevations and habitats (Webster & Johnson, 2000), it is most competitive in extreme environments where individuals can live to be hundreds of years old (Schuster et al., 1995). The long-lived tree has drought avoidance strategies and physiological plasticity to help it succeed in xeric environments (Letts et al., 2009; Borgman et al., 2015). Limber pine is facing increasing challenges from climate change (McDowell et al., 2016), white pine blister rust (Schoettle, 2004; Schoettle et al., 2014; Smith, et al., 2013), and pests like mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Cleaver et al., 2015; Langor, 1989). It has been established that limber pine is susceptible to white pine blister rust (Hoff & Mcdonald, 1993; Jacobi et al., 2018; Schoettle et al., 2014) and the disease has been detected in Colorado (Johnson & Jacobi, 2000; Kearns & Jacobi, 2007) and it is contributing to tree mortality. White pine blister rust infections are expected to increase in limber pine (Kearns et al., 2014; Krist et al., 2015), but it is uncertain if a depression in physiological processes in limber pine caused by stress will affect rust performance after infection.

Cronartium ribicola, the non-native rust fungus that causes the lethal white pine blister rust (WPBR) disease in five-needle pines, has already spread through many forests in North America and threatens the Great Basin (Tomback & Achuff, 2010). The success of *C. ribicola* has been attributed to the lack of co-evolution, and many native North American five-needle pines have been killed by the non-native rust (Geils et al., 2010; Rausher, 2001). Annually, *C. ribicola* reproduces via asexual urediniospores which are produced on the telial host (e.g., various *Ribes* spp.) throughout the growing season. During late summer/early fall, teliospores on *Ribes* spp. give rise to basidiospores which spread to infect needles of five-

needle pine aecial hosts (e.g., *Pinus flexilis*)(Van Arsdel et al., 1956). After infections of needles, rust haustoria feed on compounds produced by the pine host (Robb et al., 1975; Voegele et al., 2001). The life cycle of *C. ribicola* is complex, requiring access to two hosts and high humidity conditions for spore production, dispersal, and infection, implicating moisture as an important factor for infection in this pathosystem (Van Arsdel et al., 1956). However, once a droughted host is infected, the performance of *C. ribicola* within is uncertain when compared with a well-watered host.

As a fungal biotroph, *C. ribicola* depends entirely on its living plant hosts to grow, which raises questions about the role of host health in disease progression (Mendgen & Hahn, 2002). The effects of drought on pine physiology are well-documented (Borgman et al., 2015; Bucholz et al., 2020; Letts et al., 2009; Meinzer et al., 2014; Millar et al., 2007). A droughted pine fixes less carbon overall, both for structural and non-structural carbohydrates and for carbon-based defensive compounds (McDowell et al., 2008). Native pathogens can often proliferate further within a stressed host that has reduced defenses (Kolb et al., 2016). Many native filamentous fungal pathogens are in an evolutionary arms race with their host and host defenses (Holub, 2006), but as a non-native pathogen, *C. ribicola* has only a limited relationship with specialized host defenses (Liu & Ekramoddoullah, 2004). The growth of *C. ribicola* would not be impeded nor aided by variations in defenses, suggesting that the changes in available carbohydrates as a food source plays a more important relative role in *C. ribicola* colonization during drought (Hudgins et al., 2005). As an obligate parasite, *C. ribicola* is limited in how it can be cultured in medium; therefore, analysis of white pine blister rust performance needs to be performed *in situ*.

Our objectives were to ascertain if stresses caused by drought and by *C. ribicola* infection would interact physiologically within *P. flexilis*. In this greenhouse study we imposed two types of drought stress on *P. flexilis* seedlings throughout the study, inoculated seedlings with *C. ribicola*, consistently monitored physiological properties of the seedlings and measured *C. ribicola* proliferation in the seedlings by quantifying *C. ribicola* DNA within the host during the early stages of colonization before the onset of signs and symptoms. Specifically, we asked if chronic or acute drought stress affects the colonization and disease progression of *C. ribicola* within *P. flexilis* seedlings. We hypothesized that the host physiological stress resulting from drought would reduce both colonization of *C. ribicola* and WPBR disease progression, and the type of drought stress would affect these parameters differently.

2. Methods

2.1. Experimental design

Two-year old limber pine seedlings were obtained from Colorado State Forest Service Nursery (Fort Collins, CO) and transplanted into 10×10×20 cm pots (Stuewe & Sons, Inc., Tangent Oregon, Oregon, USA). Soil for pine seedlings was composed by volume of 30% sand, 60% Promix HP Soil + Biofungicide & Mycorrhizae (Premier Tech Horticulture, Quakertown, PA, USA), and 10% forest soil obtained from a limber pine forest in southern Wyoming. Seedlings were grown in a greenhouse with daily average temperatures ranging from 20°C to 26°C and daily average relative humidity ranging from 41% to 74%. Prior to initiation of experiment, 576 seedlings were acclimated to greenhouse conditions with

weekly watering for three months. Initial height and stem diameter was recorded for each seedling. Seedlings were fertilized every 6 months with Osmocote Plus extended-release fertilizer (15% N, 9% P, 12% K) per manufacturer's label.

Seedlings were assigned randomly to one of three blocks (3 technical replicates) and arranged in groups on the greenhouse bench by block. The experiment began with initiation of watering treatments on the first block of seedlings; the watering treatment began for the second block 5 days later, and the watering treatment began for the third block of seedlings 9 days after the first block. All watering treatments and the inoculation treatment (inoculated or not inoculated) occurred on the same relative day-of-experiment, but the absolute day varied by block. Within each block, seedlings were assigned randomly to one of two replicate groups. Within each replicate group seedlings (n = 432) were randomly assigned to one of three watering treatments, and one of two inoculation treatments.

Cuttings of *Ribes nigrum* (~ 60 cm in length each) were rooted and grown in 2-gal pots containing HP Pro Soil + Mycorrhizae in the greenhouse and watered weekly for 6 months until a full flush of foliage was present on over 50 potted. *R. nigrum* plants were fertilized every three months with Osmocote Plus extended-release fertilizer (15% N, 9% P, 12% K). Potted plants were then transferred to three growth chambers (Conviron CMP6050, Pembina, North Dakota) prior to inoculation with *Cronartium ribicola*.

Pathogen inoculum was obtained from wild sources of white pine blister rust aeciospores in Colorado and Wyoming from limber pine during Summer of 2020.

2.2. Watering treatments

Limber pine seedlings were randomly assigned to three watering treatments within each of the two replicates within each block: well-watered, chronic drought, and acute drought. Seedlings were weighed between 0600 – 0900 weekly. The relative soil water deficit was the amount of water lost to evapotranspiration by weight as a proportion of the field capacity weight, which was recorded at the end of each watering cycle. All watering treatments were calculated from individual potted seedling field capacity weights and the individual potted weight at the end of each watering cycle. The drought treatment assigned to seedlings was imposed by weekly measurements of pot weight which were used in calculations to determine amount of water to add each week. In addition to soil water deficit, volumetric soil moisture content (5 – 10 cm deep; percent) was measured weekly on a subset (n = 48) of all treatments with a soil probe (Hydrosense; Campbell Scientific, Logan, Utah, USA). Even in the greenhouse, the water usage varied with season, and so we present watering-related responses seasonally (see Figure 1). For the first months of the experiment (days 0 – 94), seedlings were watered on a 14-day cycle. Well-watered seedlings (Treatment 1) were watered to field capacity weekly, chronic drought seedlings (Treatment 2) were watered to 85% field capacity weekly, and acute drought seedlings (Treatment 3) were watered to field capacity bi-weekly. In the winter months after inoculation (days 105 – 265), an additional 7-day dry-down period was added to each treatment (21-day cycle) to compensate for lower evapotranspiration. For the following summer months (day 266 – 365), the seedlings were returned to a 14-day watering cycle. During inoculation (days 95 – 104), all seedlings were watered to field capacity prior to entering the dew chamber, regardless of watering treatment.

Plant water potential measurements were used to quantify the physiological impact of watering treatments on seedling physiology prior to inoculation. For each sampling event, we randomly selected 36 seedlings from each of three watering treatments within each of three blocks. A total of 108 out of 576 seedlings were evaluated at each pre-inoculation sampling event. Measurements were made on current-year or one-year old fascicles collected from the main apical stem prior to watering. Water potential evaluations from a subsample of seedlings were performed biweekly before inoculation for a total of 5 sampling events, and while the sampled individuals varied with each interval, the sampling size per treatment per replicate per block remained the same (n = 3). Predawn water potentials (Ψ_{pd}) and midday water potentials (Ψ_{md}) of sampled seedlings were measured for fascicles collected between 4:30am – 6:30am and between 11:30am – 1:00pm, respectively. Fascicles were immediately sealed in an aluminum foil envelope and secured in a chilled cooler before being transported and read within 2 hours. Each fascicle was cut at the base transversely with a razor and water potential was measured with a pressure chamber (Model 600, PMS Instrument Company, Corvallis, Oregon, USA).

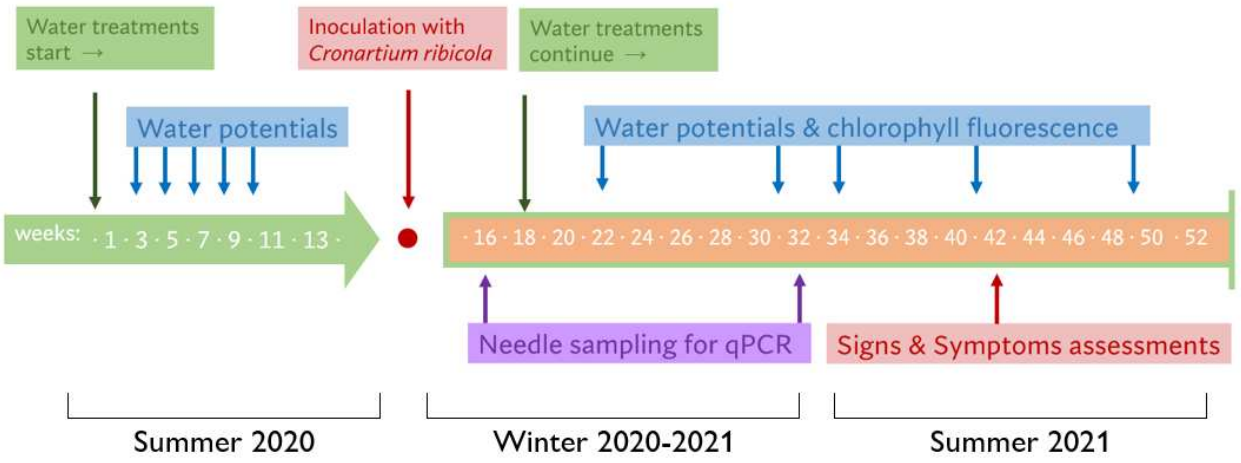


Figure 1. Experimental timeline. Watering treatments occur over 52 weeks, with the inoculation treatment occuring at week 15. Water potentials and chlorophyll fluorescence were measured at

the weeks indicated prior to and after inoculation. Needle sampling and disease assessment occurred at the weeks 16, 32 and 52, respectively.

2.3. *Cronartium ribicola* inoculation

2.3.1. Alternate host inoculation

White pine blister rust inoculum was reared on alternate host *R. nigrum* leaves *in vivo* following an established protocol (Zambino, 2019, unpublished). Aeciospores from more than 50 individual aecia were pooled and diluted in a Tween-20 (0.03% v/v) solution to a concentration of 1×10^5 spores/mL. About 25 prominent, younger leaves per *R. nigrum* plant (from n = 10 individual plants for each inoculation) were inoculated by misting approximately 100 µl of spore solution on the abaxial sides of the leaves. Following misting with a handheld sprayer, the individual potted *R. nigrum* plants were watered and covered in a plastic bag to maintain elevated humidity. Plants were placed within a dark growth chamber maintained at 60% RH with a daytime temperature of 17°C for 48 hours. In growth chamber equipment without humidity control, humidity was raised by adding water trays and wet towels to the growth chamber that were rewetted every 48 hours.

After 48 hours, the plastic bags were removed; on the third day, the daytime temperature in the growth chamber was increased to 20°C, and the illumination was set to a photoperiod of 16 h of 400 µmol m^{-2} s^{-1} light and 8 h dark. Inoculated plants were watered every three days for about six weeks during the continued reinfection by uredinia. After six weeks, the growth chamber daytime temperature was decreased to 15°C to promote the

formation of telia and basidiospores required for primary host inoculation. After seven days at a lower temperature, telia were visible throughout the infected areas on the leaf tissues.

2.3.2. Primary host inoculation

Inoculation of seedlings with *C. ribicola* occurred over three time-delayed inoculation events according to block assignment. During each inoculation event, seedlings were assigned to one of two inoculation dew chambers according to replicate assignment. At the start of the experiment, an equal number of pine seedlings from each watering treatment were randomly assigned to be inoculated (n = 66 inoculated per treatment), while the rest remained uninoculated (n = 78 uninoculated per treatment).

All seedlings to be inoculated were watered to field capacity before being placed into a dew chamber. Harvested infected *Ribes* leaves were placed between wet burlap over ice in a cooler and transferred to the dew chambers. Infected *Ribes* leaves were evenly distributed abaxial-side down on a coarse mesh rack placed directly above the pine seedlings, following the methods of Kinloch and Dupper (2002) (See arrangement in Figure 2). Pine seedlings, infected *Ribes* leaves, and blank slides (for basidiospore counts) were kept in the dark in dew chamber at 100% RH and an air temperature set at 16°C for 4 days and the seedlings were left in the chamber for an additional 24 hrs at 20°C after the *Ribes* leaves were removed.

Figure 2. Primary host inoculation system with (A) WPBR-infected *Ribes* leaves placed on upper mesh rack and *P. flexilis* seedlings to be inoculated placed underneath. (B) Blank slides with a thin coating of rubber cement dispersed among pine seedlings for spore capture and germination counts.

For each inoculation, three (3) control seedlings and blank slides with a thin coating of rubber cement were included to determine spore density and germination rate in each inoculation chamber and assay. Slides were removed and replaced every 24 hrs after the start of an inoculation; slides were stained with lactophenol cotton blue and stored 4°C until counted. From each slide, a transect of 7 images were captured from 6 transect lines at 6.3x magnification for a total 42 images per slide. A count including the total number of basidiospores and germinated basidiospores was performed for 20 of the images, selected randomly from the 42 available. A running count over the 4 days was tabulated and used to calculate spores per square centimeter and germination rate. The inoculation spore densities and germination rates are listed in Table 1 by date and chamber. The germinated

spore densities range from 1,017 – 4,212 basidiospores per square centimeter over a 4-day inoculation event, and germination ranged from 79.0 – 92.5%. After inoculation, seedlings were returned to the greenhouse and resumed their assigned watering treatment.

Inoculation efficacy was also monitored by including three 1.5-year-old limber pine seedlings in each inoculation chamber (n = 18 total) as "inoculation controls". Younger seedlings are often more easily infected than older seedlings; these seedlings were placed to monitor and estimate the probability that each of our experiment seedlings (almost 3 years old) was challenged by *C. ribicola*. The inoculation control seedlings were from the same seed lot as the experimental trees, also grown at the Colorado State Forest Service Nursery and were well-watered throughout the study. Control seedlings were rated for WPBR signs and symptoms at the same time as the treatment trees (see Section 2.3.3 below). Eighty-three percent (15 of the 18 seedlings) of the control seedlings were symptomatic for WPBR at 265 days post inoculation. Seventy-three percent (11 of 15 seedlings) of the symptomatic seedlings had severe disease (ratings of 6-8) with six having already died from rust (rating of 8). Bulked seed lots of limber pine from the southern Rocky Mountains can be expected to have on average 5% of the seedlings (range of 0 to 13.9%) to have complete resistance to WPBR and therefore to remain disease-free after inoculation (Schoettle et al., 2014). Additional testing of populations in the vicinity of where the seed lot for this study was collected has shown an average frequency of complete resistance of 23% (Schoettle, unpublished data) suggesting that the 17% of the control seedlings that were asymptomatic at the time of inspection may not have escaped infection but have complete resistance or latent disease or a mix of both. These data provide confidence that the inoculation treatments were effective at infecting and causing disease in pines.

2.3.3. Assessment of white pine blister rust signs & symptoms

Cankers and spermatia were quantified on each seedling 37 weeks (265 days) after inoculation. Inoculated seedlings were counted as symptomatic if a stem or branch had new swelling under a current or late fascicles, or if spermatia (a spore stage observed as an orange exudate) were present. Needle lesions or spots were rarely observed on any of the seedlings, even those that were visually diseased, therefore spots were not assessed. The curvature of the swelling and often an orange tint to the bark was observed visually on the stem or branch and was sometimes accompanied by the presence of spermatia. Among seedlings with signs and symptoms, the disease progression was ranked on a severity index (1 – 8) according to established methods at the USDA Forest Service Dorena Genetic Resource Center white pine blister rust resistance screening program (Kegley et al., 2012). The size of the canker and the degree of stem circumference coverage is scored: seedlings with a canker partially around the bole were rated increasing in severity from 1 – 4 and those with a bole canker that approached 100% of the circumference rated 4 – 7, based on increasing severity, and seedlings that were dead from rust rated 8. A modified severity index was used for modelling, where seedlings rated 1 – 4 were listed as less severe (0), and seedlings rated 5 – 8 were listed as more severe (1). Seedlings that had died by the end of the experiment were noted and counted, regardless of cause of death.

2.3.4. DNA Extraction of inoculated seedlings

Rust presence in seedling needles was assessed using qPCR 2 weeks and 16 weeks after inoculation. Fresh bud tissue from a field-grown limber pine (100 mg) was collected to serve as the pine standard and cultivated urediniospores served as the rust standard. Three

current-year needles were collected from each inoculated seedling; if seedling needle length was significantly less than that of the rest of the population, a fourth needle was collected. Collected needles and tissues for standards were stored at -80°C until extraction. Prior to grinding, collected needle tissues were measured (length, mm) and washed in an ethanol solution (70% v/v) to remove surface biota. Samples were then ground in liquid nitrogen with mortar and pestle to a fine powder. Samples were further ground in FastPrep-24 Sample Preparation System (M.P. Biomedicals, LLC, Santa Ana, California, USA) at 30 Hz for 1 min with two 2 mm steel beads and one 4 mm glass bead placed in each tube.

DNA extractions of ground tissue were performed following the methods of Cubero et al. (1999), using CTAB (2% cetyl trimethylammonium bromide) and chloroform. A solution of CTAB extraction buffer (0.750 mL) with polyvinyl pyridine (2%) was mixed with each tissue sample and was incubated at 70°C for 30 minutes. An equal volume of chloroform was added to each sample and inverted gently for two minutes before centrifuging at 10,000 relative centrifugal field (rcf) to separate the organic and aqueous phases. The upper aqueous phase was added to a vial containing precipitation buffer and mixed by inversion for two minutes. The samples were then centrifuged at 13,000 rcf and the resultant supernatant was disposed of. The remaining pellet was resuspended in a sodium chloride solution and inverted with chloroform for two minutes. Following centrifugation at 10,000 rcf, the upper phase was transferred to a new vial along with chilled isopropanol. After a twenty-minute incubation at 4°C, samples were centrifuged at 4°C and 13,000 rcf for twenty minutes. The supernatant was removed, and chilled ethanol (70% v/v) added to the remaining DNA pellet. The samples were then centrifuged at 4°C and 13,000 rcf for 5 minutes, before disposing of the supernatant again. The vials were centrifuged in a vacuum

for 15 minutes to evaporate residual ethanol, and the remaining DNA was resuspended in molecular grade water. Total DNA concentration and purity was subsequently measured with NanoDrop One^{C} (Thermoscientific, Waltham, Massachusetts, USA) and samples were diluted to a concentration of 20.0 ng DNA/µL. Diluted DNA extractions were stored at -20°C.

2.3.5. qPCR targeting C. ribicola *and* P. flexilis

Real-time PCR was performed using a QuantStudio 3 Real-time PCR System (Applied Biosystems, Waltham, Massachusetts, USA) using PrimeTime Gene Expression 2x Master Mix with ROX reference dye (IDT, Coralville, IA). DNA extracted from three pine needles from each inoculated seedling was each used in two separate reactions to target and quantify both a pine host gene, Agp6, (Krutovsky et al., 2004) and a *C. ribicola* pathogen gene, Crib190, using primers developed by Bergeron et al. (2019) (Table 2). Reactions were conducted in a 96-well plate, with 10 µL Master Mix, 0.50 µL forward primer, 0.50 µL reverse primer, 0.25 µL probe, 8.75 µL molecular grade water, and 2 µL of diluted sample DNA extract. All assays were performed in duplicate, including the negative control, water control, and the target standards. The *C. ribicola* standard curve consisted of 6 dilutions ranging from 0.10 to 30.0 ng/µL; the *P. flexilis* standard curve consisted of 6 dilutions ranging from 0.03 to 16.70 ng/µL. Initial denaturation was at 95°C for 15 min followed by 50 cycles of denaturation at 95°C for 15 s and annealing at 58°C for 60 s. The cycle threshold (C_t) value of the standards of known concentration were used to calculate the DNA concentration for each sample, as illustrated in Equation 1, where *m* and *b* are derived from the standards and cycle threshold (C_t) values for each target. If a sample well tested positive for *C. ribicola* DNA, the *C. ribicola* DNA

concentration was divided by the *P. flexilis* DNA concentration to determine a standardized DNA amount for each needle sample (see Equation 2).

Equation 1

$$[DNA]^{sample} = m^{standard} \times \left(C_t{}^{sample}\right) + b^{standard}$$

Equation 2

$$Standardized\ DNA\ amount = \frac{[DNA]^{Crib190}}{[DNA]^{Agp6}}$$

Table 1. Primers and probes used to assess inoculation rate and growth of *Cronartium ribicola* in *Pinus flexilis* seedlings. The forward and reverse primers and probes (IDT, Coralville, IA) targeted the Crib190 gene in *C. ribicola* (Bergeron et al., 2019) and the Agp6 gene (Krutovsky et al., 2004), developed specifically for *P. flexilis* by Jorge Ibarra Caballero & Jane E. Stewart (2021, unpublished). The associated melting temperature for each primer and amplicon length are indicated.

	Pathogen – *Cronartium ribicola*		Host – *Pinus flexilis*	
Gene	Crib190	T_m	Agp6	T_m
Forward primer	CTCCAGCTACAGTGGGTA	59.9°C	CTGCTAAACCACCCACAACT	62.4°C
Reverse primer	CCTTGTCTGTTGGTGAGGT	59.2°C	CTTGGTGGGAGCAACGG	62.4°C
Probe	ACATGGGAACGACA… …AGGACAATTTGGAC	66.0°C	TCTCAACTCCGAAGCCTCCCAC	67.4°C
Amplicon length	121 bp		134 bp	
GenBank accession no.	MH171743-MH171751		AF101785	

2.4. Post-inoculation physiology

2.4.1. Sampling

After inoculation, a subsample of pine seedlings from each watering and inoculation treatment combination were used to estimate the physiological condition of the pine hosts. For each sampling event, we randomly selected 18 seedlings from each of six treatments (3 water treatments × 2 inoculation treatments) across three time-delayed blocks. A total of 108 seedlings from the total 432 were evaluated at each post-inoculation sampling event.

2.4.2. Fascicle water potential and chlorophyll fluorescence

To quantify the effect of inoculation and continuing water treatments on seedlings, we measured F_v/F_m and water potential of fascicles at 5 intervals (see Figure 1) after WPBR inoculation. At each interval, we randomly selected three (3) seedlings from each of the six treatments from each of three blocks and two replicates within each block to measure. A subtotal of 108 out of a possible 432 seedlings were randomly selected at each interval, and while the individuals varied with each interval, the sampling size per treatment per replicate per block remained the same (n = 3).

Measurements were made on current-year or one-year old whole fascicles collected from the main apical stem, collected prior to watering. Evaluations of a subsample of seedlings were performed at 5 intervals after inoculation (December 2020 through August 2021), as illustrated in Figure 1. The predawn water potential (Ψ_{pd}) and midday water potential (Ψ_{md}) of each sampled seedling was quantified as described above for the pre-

inoculation measurements. For each randomly selected individual, three measurements of maximum quantum efficiency (F_v/F_m) were collected from fascicles after dark-adaptation; the three concurrent readings were averaged to represent the mean F_v/F_m of the individual seedling. Chlorophyll fluorescence was collected the day prior to watering between 11:00am – 1:00pm, and readings were taken from each of the subsampled trees of current-year or one-year old needles. Measurements were obtained after 40 min of dark acclimation by use of dark-adapting clips.

2.5. Statistical analysis

Statistical parameters are listed in Table 3. The Kenward-Roger degrees-of-freedom method was employed for all tests, significance was assessed at alpha = 0.05, and the Tukey method for comparing a family of 3 estimates was used as a p-value adjustment. All statistical analyses were conducted in R (RStudio 2021.09.0+351 "Ghost Orchid" Release for Windows). The responses for water potential and DNA amount tests were log-transformed, and the germinated and total basidiospore density co-variates were log-transformed to meet normality assumptions for all analyses.

A linear mixed-effect model (lme4)(Bates et al., 2015) was used to analyze for the effect of watering treatment as a predictor prior to inoculation, and watering treatment, inoculation treatment and their interaction as a predictor after inoculation on water potentials, using block, replicate, and tree as random effects. Covariates included initial seedling height and stem diameter, germinated and total basidiospore density for each inoculation, and mean daily maximum temperature and relative humidity (RH) for the week prior to measurements, chosen to reflect extrema within the greenhouse environment

during the last week of each dry-down period. Water potential response was log-transformed to meet assumptions of normality.

A linear mixed-effect model (lme4) (Bates et al., 2015) was also used for analyses of *C. ribicola* relative DNA amount. Again, block and replicate were random effects and watering treatment was the predictor for DNA amount and relative fold change with initial seedling height, initial diameter, and germinated basidiospore density for each inoculation were used as co-variates. DNA amount responses were log-transformed. A similar analysis was run for growth (log-transformed) to test for the effects of water and inoculation treatments and their interaction.

Logistic regression with random effects was used in the analysis of binary responses, including vigor, WPBR severity index, and the proportion of inoculated seedlings with WPBR symptoms. Watering treatment, inoculation treatment, and the interaction was used as predictor for vigor, and watering treatment was used as a predictor for the severity index and the proportion of symptom seedlings. Block and replicate were used as random effects and initial height, initial diameter, and germinated basidiospore density for each inoculation were used as covariates.

Beta regression was used for the chlorophyll fluorescence F_v/F_m, which as a response is restricted to an imposed unit interval (0 – 1), with watering treatment, inoculation treatment, and their interaction as a predictor. Initial seedling height, initial stem diameter, measurement week, germinated and total basidiospore density for each inoculation, and the mean daily maximum temperature and relative humidity for the week prior to measurements were used as co-variates.

Table 2. Table of predictors, co-variates, and responses. Watering treatment, inoculation treatment, and their interaction were the main predictors used in modelling. Variable types include predictors, co-variates, random effects, and responses. Grouping factors 'block' and 'replicate' account for the location of seedlings within the greenhouse, the timing of measurements, and the timings of inoculation, and are used as random effects in the linear mixed model. For each variable, the date or date range collected is indicated, either in absolute time, or days after start of experiment (d.o.e.), or days after inoculation (d.a.i.).

Variable	Description	Time period	Variable type
Watering treatment	14-day watering cycle 21-day watering cycle 14-day watering cycle	Summer '20 (week 0 – 14) Winter '20-'21 (week 16 – 32) Summer '21 (week 33 – 52)	Predictor
Inoculation treatment	WPBR Infection status (0 = uninoculated, 1 = inoculated)	d.o.e.: 100	Predictor
height	Initial seedling height (cm)	d.o.e.: 0	Co-variate
diameter	Initial seedling diameter at base cotyledon scar (mm)	d.o.e.: 0	Co-variate
T_{max}	Greenhouse temperature for prior 7 days (mean daily maximum, °C)	08/05/2020 – 09/04/2021	Co-variate
RH_{max}	Greenhouse relative humidity for prior 7 days (mean daily maximum, %)	08/05/2020 – 09/04/2021	Co-variate
$Spores_{germ}$	Germinated basidiospores (cm^{-2})	d.o.i.: 1	Co-variate
$Spores_{total}$	Total basidiospores (cm^{-2})	d.o.i.: 1	Co-variate
Ψ_{pd}, Ψ_{md}	Pre-dawn and midday fascicle water potential (bars)	Before inoc.: week 0 – 12 After inoc.: week 16 – 32 week 33 – 52	Continuous Response
F_v/F_m	Maximum quantum efficiency (restricted ratio)	After inoc.: week 16 – 32 week 33 – 52	Proportional Response
Growth	Annual terminal growth (mm/year)	d.o.i.: 265	Continuous Response
Vigor	Visual inspection of health of tree (0 = no observed signs of stress, 1 = observed signs of stress)	d.o.e.: 301 d.o.i.: 200	Binary Response
Mortality	Tree status (0 = alive, 1 = dead)	d.o.i.: 265	Binary Response
Signs	Signs & symptoms of WPBR observed on inoculated individual (0 = absent, 1 = present)	d.o.e.: 301 d.o.i.: 200	Binary Response
Amp_{Crib}	Evidence of *C. ribicola* DNA in sampled needle (0 = absent, 1 = present)	d.o.i.: 14 d.o.i.: 114	Binary Response
DNA_{Crib}	Standardized amount of *C. ribicola* DNA to *P. flexilis* DNA	d.o.i.: 14 d.o.i.: 114	Continuous Response
Block	Location and time-aligned trials: B1, B2, B3 (n = 144 seedlings per block)	B1: 08/05/2020 — 08/05/2021 B2: 08/25/2020 — 08/25/2021 B3: 09/04/2020 — 09/04/2021	Random effect
Replicate	Two replicate groups within each block (6 replicates, n = 72 seedlings per repl.)	Same as Block	Random effect
Seedling ID	Label to track each individual seedling	Before inoc.: n = 576 After inoc.: n = 432	Random effect

For counts of seedling with undetected *C. ribicola* DNA and counts of tree mortality, a Pearson's Chi-squared test was used to determine if watering treatment or watering treatment and inoculation treatment influenced each response, respectively.

3. Results

3.1. Watering treatment

The driest soil a seedling experienced during the watering cycle was expressed as minimum relative pot weight compared to the field capacity of that individual (See Figure 3 A, B, C) and was documented through measures of minimum soil moisture content (See Figure 3 D, E, F). During the first 13 weeks of water treatment in late Summer 2020, the well-watered, chronic drought, and acute drought seedlings reached mean (± standard error) minimum relative pot weights of 83.8 ± 0.3%, 75.4 ± 0.3%, and 68.8 ± 0.3%, respectively, as a proportion of field capacity, and had minimum soil moisture contents of 11.6 ± 0.4%, 9.7 ± 0.4%, and 7.6 ± 0.4%, respectively. Following *C. ribicola* inoculation in week 15, in weeks 16 – 32, (Winter 2021), well-watered, chronic drought, and acute drought seedlings reach mean minimum relative pot weights of 78.8 ± 0.7%, 72.4 ± 0.7%, and 60.1 ± 0.7%, with soil moisture contents of 13.1 ± 0.6%, 9.5 ± 0.6%, and 4.9 ± 0.6%. In weeks 33 – 52, Summer 2021, well-watered relative pot weight reached 77.5 ± 1.0%, chronic drought reached 71.6 ± 1.0%, and acute drought reached 62.7 ± 1.0%, and soil moisture content reached 10.9 ± 0.9%, 8.4 ± 0.9%, and 5.3 ± 0.9%. For minimum relative pot weight, there was no evidence of an interaction between the watering and inoculation treatments (Winter 2021 $p = 0.5449$; Summer 2021 $p = 0.5320$), but there was a clear effect of watering treatment (Summer 2020,

p = 0.0001; Winter 2021, p = 0.0001; Summer 2021, p = 0.0001). For minimum soil moisture content, there was no evidence of an interaction between the watering and inoculation treatments (Winter 2020 – 2021, p = 0.5449; Summer 2021, p = 0.5320) nor was there any evidence of an effect of inoculation (Winter 2020 – 2021, p = 0.9454; Summer 2021, p = 0.2386). There was an effect of watering treatment on the measured response of minimum soil moisture content in each period (Summer 2020, p = 0.0001; Winter 2020 – 2021, p = 0.0001; Summer 2021, p = 0.0001).

Prior to inoculation, watering treatment had a significant effect on pre-dawn (Figure 4 A, p = 0.0011) and on midday water potentials (Figure 4 B, p = 0.0001) when measured on the day when soil moisture availability was at a minimum just prior to watering. The acute droughted seedlings experienced significantly lower pre-dawn water potentials (p = 0.0010) compared to the other water treatments, and both chronic drought and acute drought experienced significantly lower midday water potentials compared to the well-watered seedlings (p = 0.0416, p = 0.0001, respectively). Prior to inoculation, the seedlings were physiologically experiencing water treatments differently, specifically with the acute droughted seedlings that experienced lower water potential.

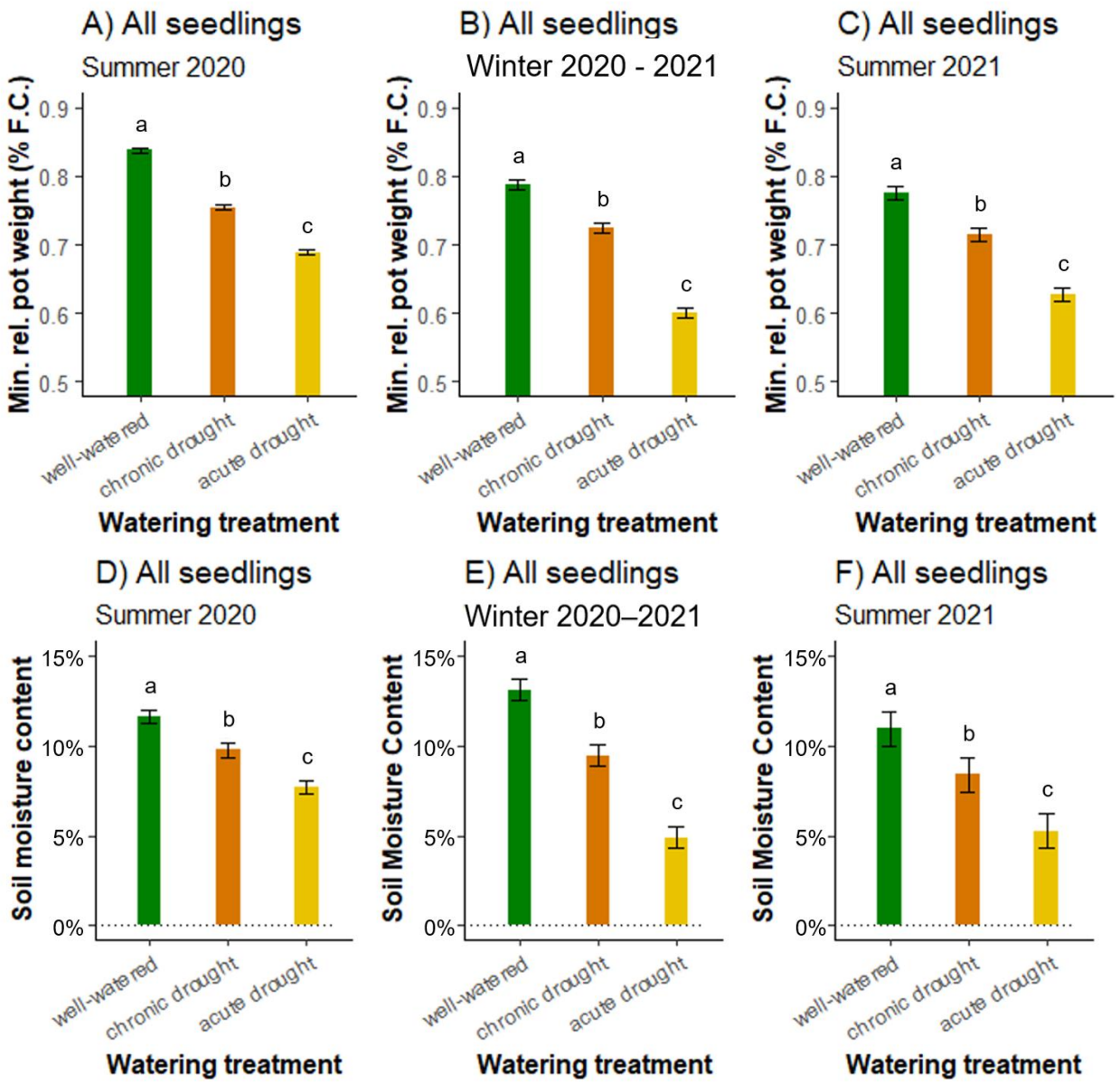


Figure 3. Mean minimum relative pot weight and mean minimum soil moisture content by watering treatment. Recorded at end of watering cycle during (A/D) late Summer 2020, 12 weeks prior to inoculation (n = 720 from each treatment over 5 sampling events), (B/E) Winter 2020 – 2021, 1– 16 weeks after inoculation (n = 288 from each treatment over 2 sampling events), and (C/F) Summer 2021, 18 – 37 weeks after inoculation (n = 432 from each treatment over 3 sampling events). Mean ± standard error as percentage of field capacity weight (% F.C.) and of soil moisture content from measurements taken prior to watering, at driest time of water cycle; significance ($\alpha = 0.05$) is noted by letters. Effect of watering treatment is significant on minimum relative pot weight in Summer 2020 (A, $p = 0.0001$), Winter 2020 – 2021 (B, $p = 0.0001$), and Summer 2021 (C, $p = 0.0001$). No evidence of effect of inoculation in Winter 2020 – 2021 ($p = 0.9454$) or Summer 2021 ($p = 0.2386$) or interaction with watering treatment and inoculation treatment (Winter 2020 – 2021, $p = 0.5449$; Summer 2021, $p = 0.5320$). Effect of watering treatment is significant on minimum soil moisture content in Summer 2020 (D, $p = 0.0001$), Winter 2020 – 2021 (E, $p = 0.0001$), and Summer 2021 (F, $p = 0.0001$). No evidence of effect of inoculation in Winter 2020 – 2021 ($p = 0.9454$) or Summer 2021 ($p = 0.2386$) or interaction with watering treatment and inoculation treatment (Winter 2020 – 2021, $p = 0.5449$; Summer 2021, $p = 0.5320$).

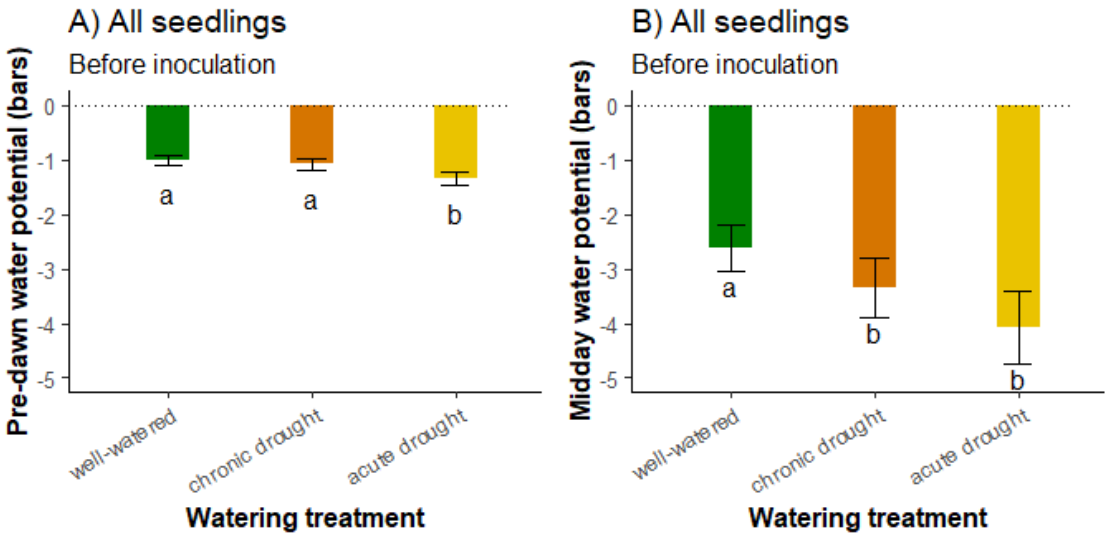


Figure 4. Fascicle water potentials of a randomly selected subsampling of limber seedlings by watering treatment during Summer 2020, a period of 12 weeks of drought treatments prior to *C. ribicola* inoculation. Mean ± standard error (in bars) from measurements taken prior to watering, at driest time of water cycle; significance ($\alpha = 0.05$) is noted by letters. A) Pre-dawn water potential (n = 180 from each treatment over 5 sampling events); effect of acute drought is significantly different from well-watered ($p = 0.0010$) and to chronic drought ($p = 0.0225$) seedlings. B) Midday water potential (n = 180 from each treatment over 5 sampling events); effect of acute drought is significantly different from well-watered seedlings ($p = 0.0001$) and the effect of chronic drought is significantly differed from well-watered seedlings ($p = 0.0416$).

3.2. *Cronartium ribicola* Inoculation

Inoculated seedlings were classified as being symptomatic if characteristic visual signs and symptoms of white pine blister rust, including cankers and spermatia were observed. Overall, 25% of the inoculated seedlings were visually symptomatic 265 days after inoculation, irrespective of watering treatment (Figure 5 A, $p = 0.9559$). Among the symptomatic seedlings, disease severity was similar across watering treatments (Figure 5 B, $p = 0.3830$). After 365 days of drought treatments and 265 days after inoculation, 4.6% of the seedlings died (20/432 seedlings, Table 4), with a significant effect of watering treatment on mortality (χ^2 test; $p = 0.0002$). Mortality was 3.5x greater in the acute drought treatment compared to the well-water treatment and lowest mortality was observed in the chronic drought treatment (Table 4A). There was no evidence of an effect of *C. ribicola* inoculation

on mortality (Table 4B, χ^2 test; p = 0.2204). Within the timeframe of this study, most of the dead seedlings were lost prior to the general onset of visual WPBR symptoms. Based on visual observations, 18/432 seedlings appeared to suffer drought-related mortality, while only 2/432 seedlings had severe symptoms of WPBR before dying.

All inoculated seedlings were subsampled for DNA extractions at 14 days and 116 days post-inoculation; extracted needles were subjected to qPCR assay targeting both *C. ribicola* DNA and *P. flexilis* DNA. DNA of *C. ribicola* was detected in 58% and 60% of all the inoculated seedlings 14 days and 116 days after inoculation, respectively (Table 5 A). Over both sampling periods, *C. ribicola* DNA was not detected in 19% of the seedlings irrespective of watering treatment (Table 5 A). Needle sampling did not detect *C. ribicola* DNA in 16% of the symptomatic seedlings (Table 5 B), despite the seedlings displaying unambiguous visual signs and symptoms of white pine blister rust disease. Like the result for all the inoculated seedlings, the watering treatments did not affect the detection of *C. ribicola* DNA in visually symptomatic seedlings.

At 14 days after inoculation, watering treatment did not significantly affect the amount of *C. ribicola* DNA in seedlings (ng/ng *P. flexilis* DNA) for all inoculated seedlings (p = 0.9917) or for symptomatic seedlings (p = 0.3936; Figure 6 A, B) using standards of known quantity for DNA quantification. Likewise, at 116 days after inoculation, no significant effect of watering treatment on *C. ribicola* DNA quantity was identified among all seedlings (p = 0.9347) and symptomatic seedlings (p = 0.8820; Figure 6 C, D).

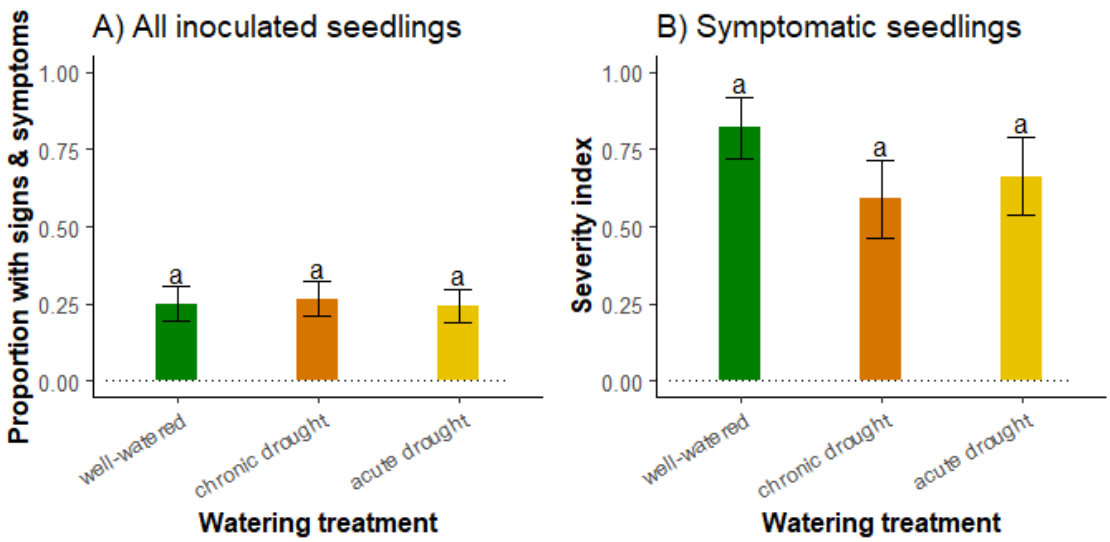


Figure 5. Signs and symptoms of WPBR and disease severity 265 days after inoculation (See pg. 24 for assessment methods). (A) The proportion of limber pine seedlings with signs and symptoms of white pine blister rust disease by watering treatment (n = 66 per treatment; p = 0.9559). (B) The mean severity index (0 – less severe, 1 – more severe) among those diseased and symptomatic seedlings by watering treatment (p = 0.2691).

Table 3. Seedling mortality by watering treatment 265 days after inoculation. A) Significant effect of watering treatment on seedling mortality (χ^2 test, p = 0.0002). B) No significant effect of inoculation treatment on mortality (χ^2 test, p = 0.2204).

A) Water treatment	Total number of seedlings	Mortality		χ^2
		Actual	Expected	
Well-watered	144	4	7	
Chronic drought	144	1	7	* p = 0.0002
Acute drought	144	15	7	
B) Inoculation treatment	Total number of seedlings	Mortality		χ^2
		Actual	Expected	
Uninoculated	220	14	11	p = 0.2204
Inoculated	192	6	9	

Table 4. Presence of *Cronartium ribicola* DNA within needles of seedlings 14 and 116 days after inoculation among watering treatments. *Cronartium ribicola* DNA was detected by qPCR from DNA of three needles per seedling. The number of seedlings without *C. ribicola* DNA detection at either timepoint is included. (A) Among all inoculated seedlings (n = 66 per watering treatment), there was no effect of watering treatment on absence of detection (χ^2 test, p = 0.1999). (B) Among only seedlings later characterized as being diseased and symptomatic (n = 16, 18, 15 for each respective treatment), no effect of treatment on the absence of detection (χ^2 test, p = 0.4266, approximation may be incorrect due to small sample size).

A) All inoculated seedlings	Total inoculated	Number of sampled seedlings with *C. ribicola* DNA presence		*C. ribicola* DNA not detected at
Water treatment	seedlings	day 14	day 116	either sampling
Well-watered	66	44	42	13
Chronic drought	66	46	36	8
Acute drought	66	39	41	16
	198 seedlings	**81%**		**19%**
B) Symptomatic seedlings	Total symptomatic	Number of sampled seedlings with *C. ribicola* DNA presence		*C. ribicola* DNA not detected at
Water treatment	seedlings	day 14	day 116	either sampling
Well-watered	16	13	14	2
Chronic drought	18	12	9	2
Acute drought	15	10	8	4
	49 seedlings	**84%**		**16%**

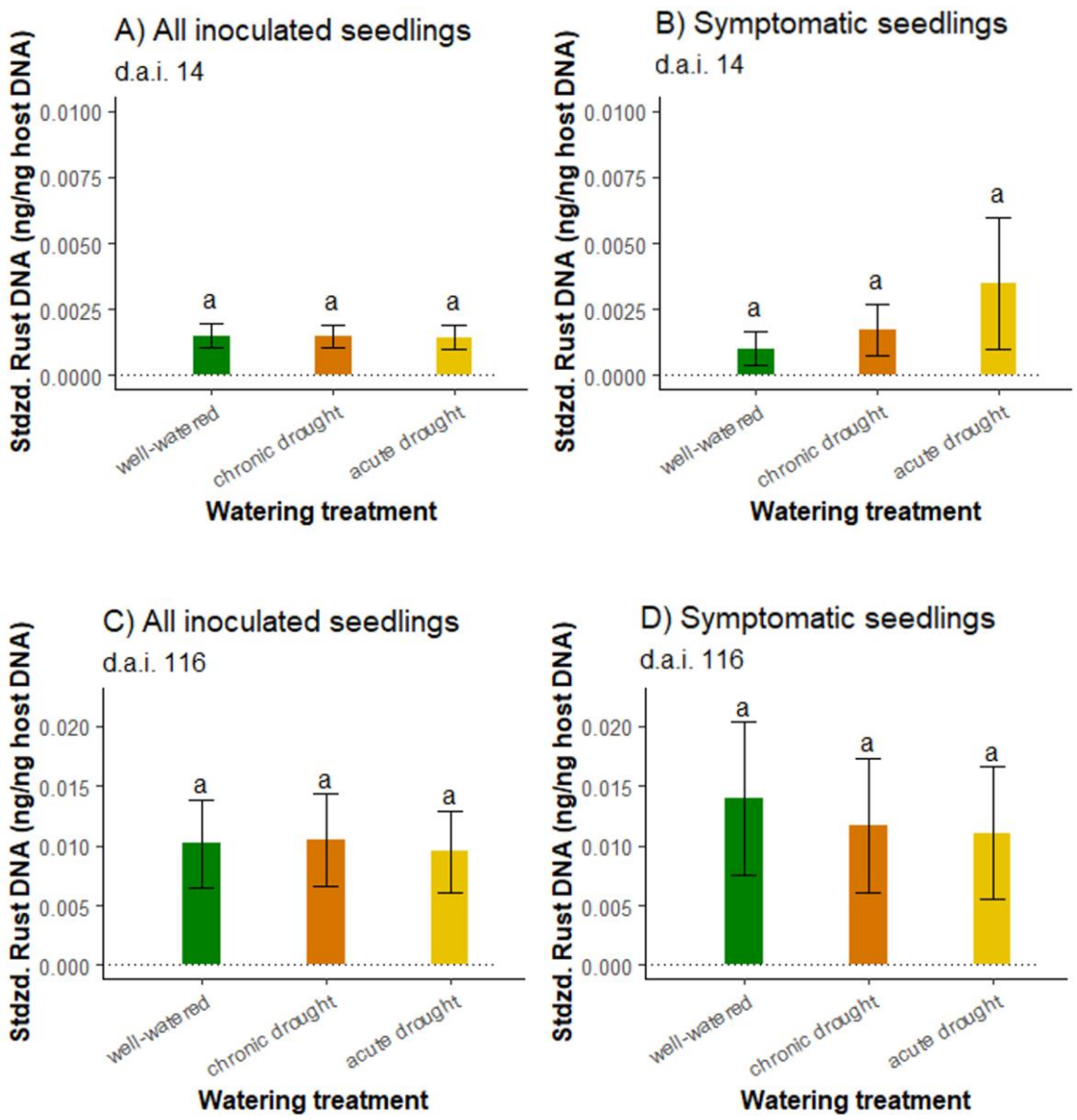


Figure 6. Standardized *C. ribicola* DNA amount (ng *C. ribicola* DNA/ng host pine DNA) by watering treatment (well-watered, chronic drought, acute drought) in (A) all inoculated seedling samples with detectable *C. ribicola* DNA at d.a.i. 14 (n = 44, 46, 39) and (B) only symptomatic inoculated seedlings with detectable *C. ribicola* DNA at d.a.i. 14 (n = 13, 12, 10) and in (C) all inoculated seedling samples with detectable *C. ribicola* DNA (n = 42, 36, 41) and (D) only symptomatic inoculated seedlings with detectable *C. ribicola* DNA (n = 14, 9, 8) at d.a.i. 116. Mean ± standard error of 3 pooled needles from each sampled seedling. In each, no significant effect of treatment on DNA amount; means are not significantly different ($\alpha = 0.05$, lowercase 'a').

3.3. Post-inoculation physiology

We monitored the continued effect of drought and inoculation by sampling and measuring quantitative physiological traits and making qualitative observations of *P. flexilis* seedlings. Vigor was visually assessed for all seedlings to estimate the effects of watering and inoculation treatments (Figure 7). There was no interaction between the watering and inoculation treatments for seedling vigor ($p = 0.1299$). Both the watering ($p = 0.0037$) and inoculation ($p < 0.0001$) treatments had a significant effect on seedling vigor (Figure 7A). Seedlings experiencing acute drought had lower vigor than those in the well-watered and chronic drought treatments, and inoculated seedlings had lower vigor than uninoculated seedlings (Figure 7B).

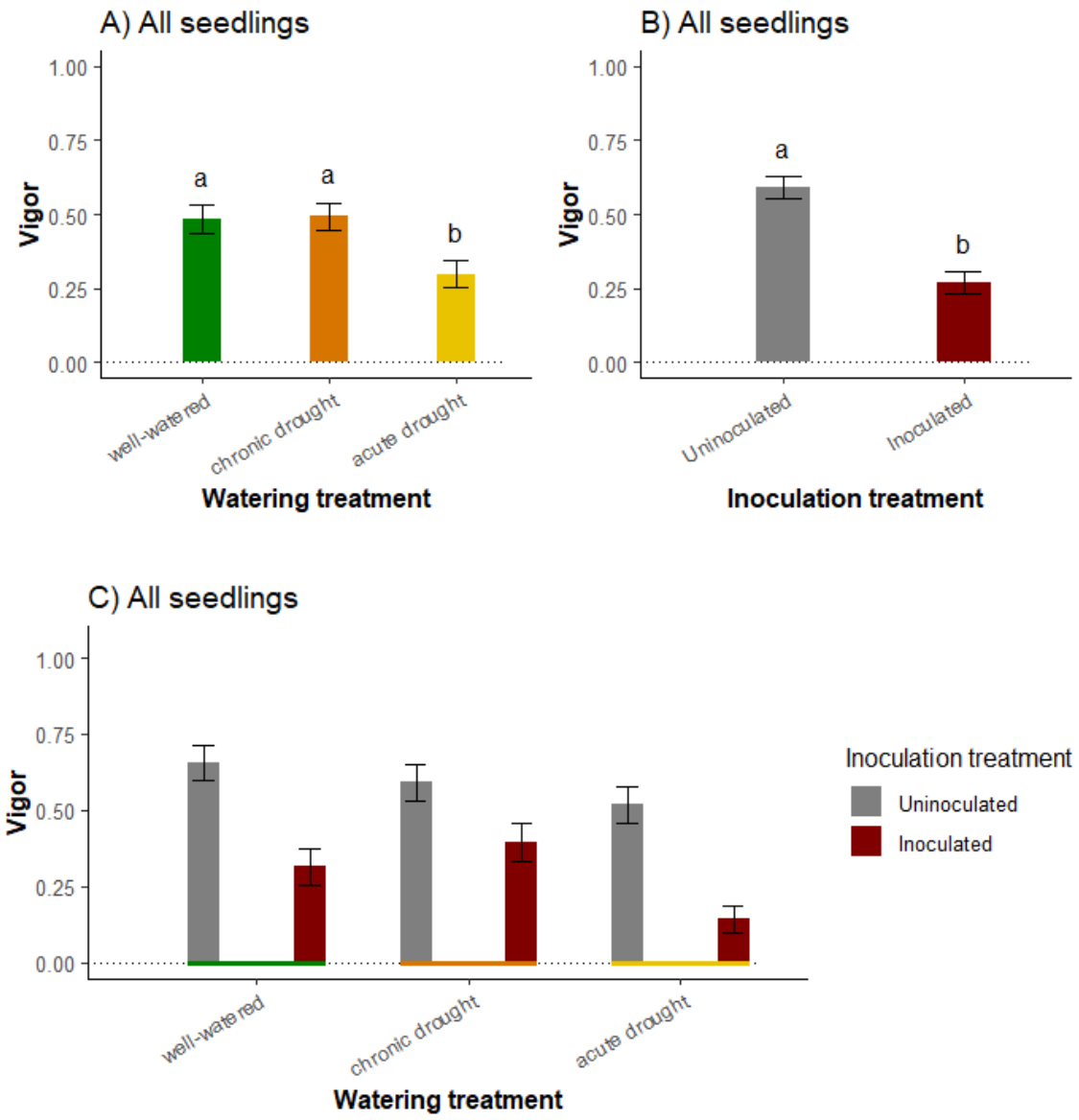
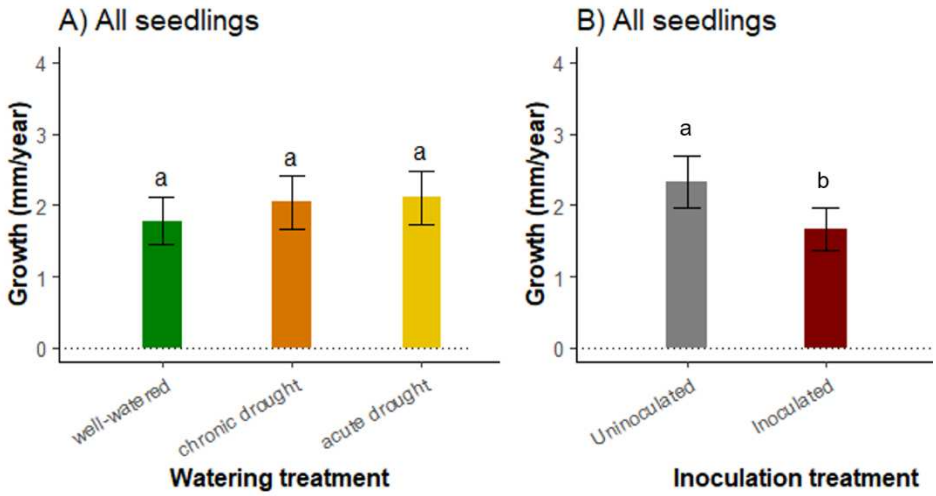


Figure 7. Seedling vigor of all limber pine seedlings (0 = unhealthy, 1 = healthy) by A) watering treatment (well-watered, chronic drought, acute drought, n = 144) at d.a.i. 200, B) inoculation treatment (uninoculated n = 234, inoculated n = 198) at d.a.i. 200, and C) both watering and inoculation treatment. Mean ± standard error; significance ($\alpha = 0.05$) noted by letters: A) Effect of acute drought is significant compared to well-watered ($p = 0.0113$) and compared to chronic drought ($p = 0.0061$). B) Effect of inoculated is significant compared to uninoculated ($p < 0.0001$). C) Effect is only significant by inoculation treatment, and there is no significant effect of interaction between watering and inoculation treatment ($p = 0.1299$).

There was also no significant effect of watering treatment on terminal growth ($p = 0.3751$), but a significant effect of inoculation treatment on growth ($p = 0.0310$), with inoculated seedlings growing less than the uninoculated seedlings (Figure 8 A, B). There was

no interaction between the treatments on terminal growth (Figure 8 C, p = 0.0838). No interaction was found among the watering and inoculation treatments for pre-dawn or midday water potentials measured in Winter 2021 (0-112 days after inoculation; p = 0.1268; p = 0.3837, respectively) or Summer 2021 (113-253 days after inoculation) time periods after inoculation (p = 0.6605 and p = 0.8432, respectively). The watering treatments continued to affect pre-dawn water potential of the seedlings following inoculation in the Winter 2020 – 2021 (p < 0.0001) and Summer 2021 (p < 0.0001) but there was no effect of inoculation treatment detected in either period (p = 0.6389, p = 0.5172, respectively; Figure 8). Measures of midday water potential indicated a significant effect of watering treatment on response for both Winter 2020 – 2021 (p < 0.0001) and Summer 2021 (p < 0.0001) with seedlings in the acute drought treatment experiencing greater water stress than well-watered and chronic drought treatments. There was no effect of inoculation treatment on midday water potential for Winter 2020 – 2021 (p = 0.6190;). However, in Summer 2021, inoculation had a significant effect on midday water potential (p = 0.0417) as inoculated seedlings had lower midday water potentials than uninoculated seedlings ().



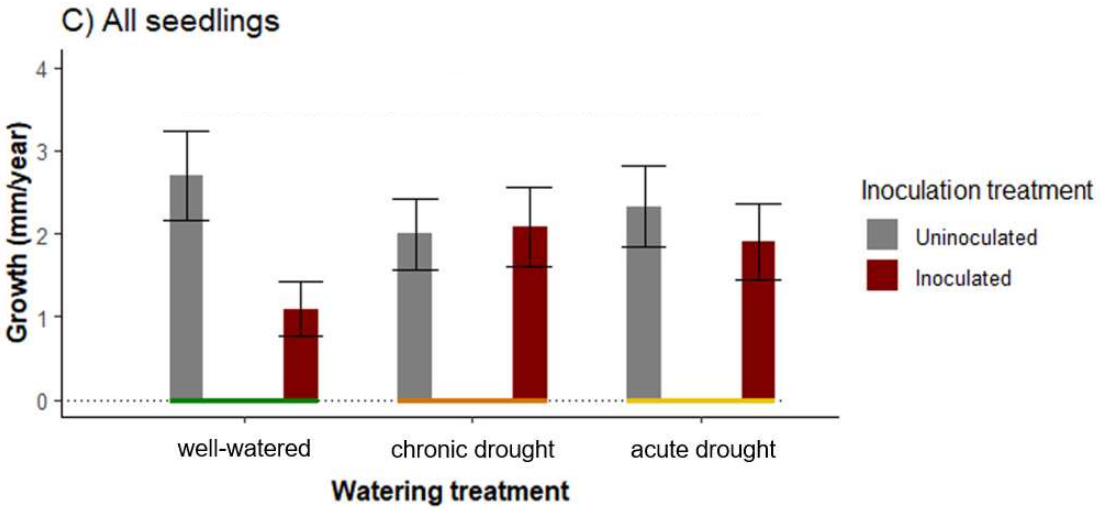
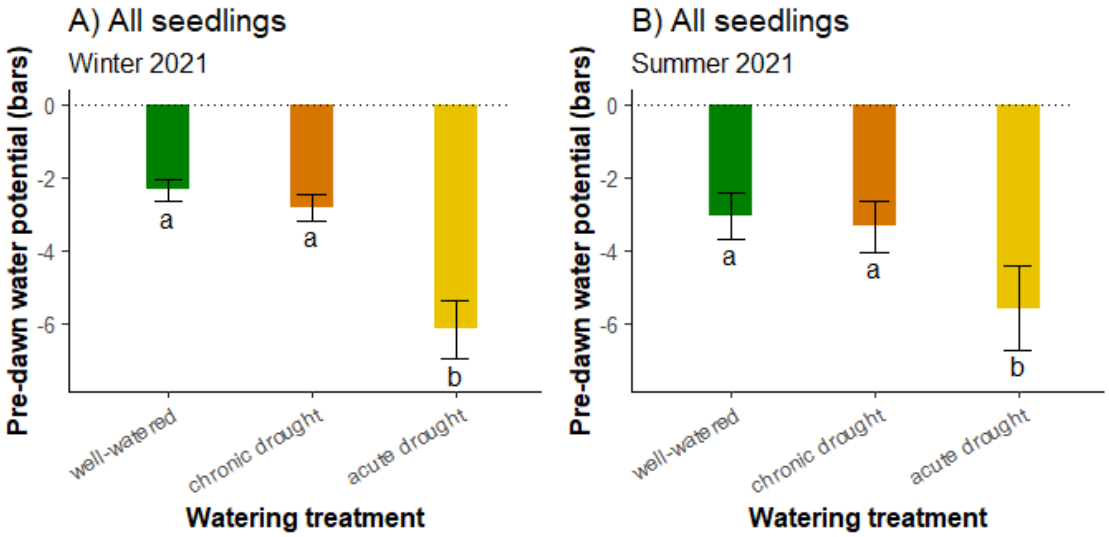


Figure 8. Annual mean terminal growth of all limber pine seedlings by A) watering treatment (well-watered, chronic drought, acute drought) and B) inoculation treatment (uninoculated, inoculated) and C) both treatments after one year of experiment (d.a.i. 265). Mean ± standard error; significance (α = 0.05) noted by letters. A) Watering treatment (n = 144 each treatment) had no significant effect on growth. B) Inoculation treatment (n = 220, 192) had a significant effect on growth (p = 0.0310). C) Effect is only significant for inoculation treatment, and though this effect is more pronounced among well-watered seedlings than drought, interaction of both treatments is not significant (p = 0.0838).



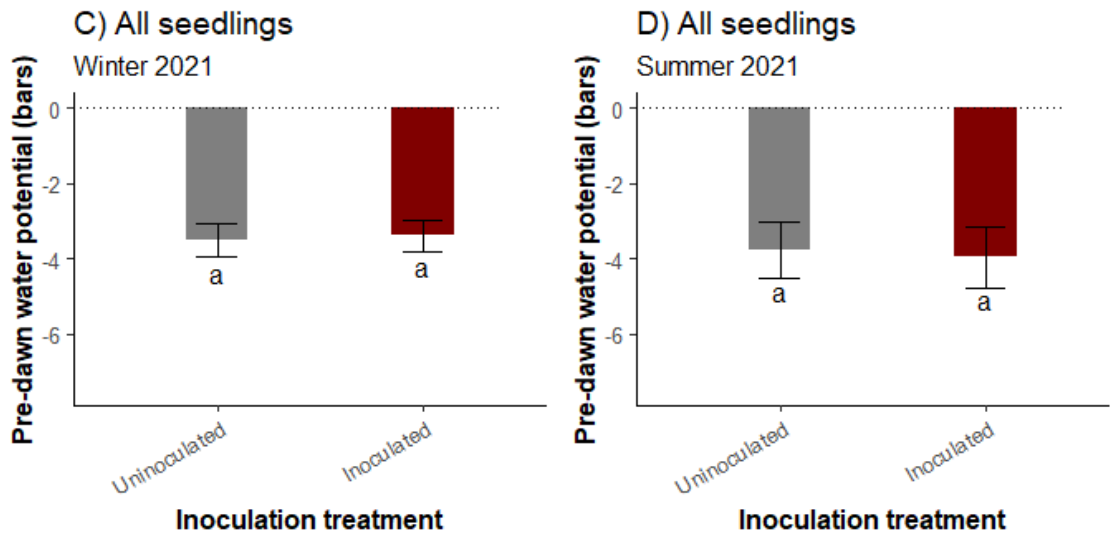
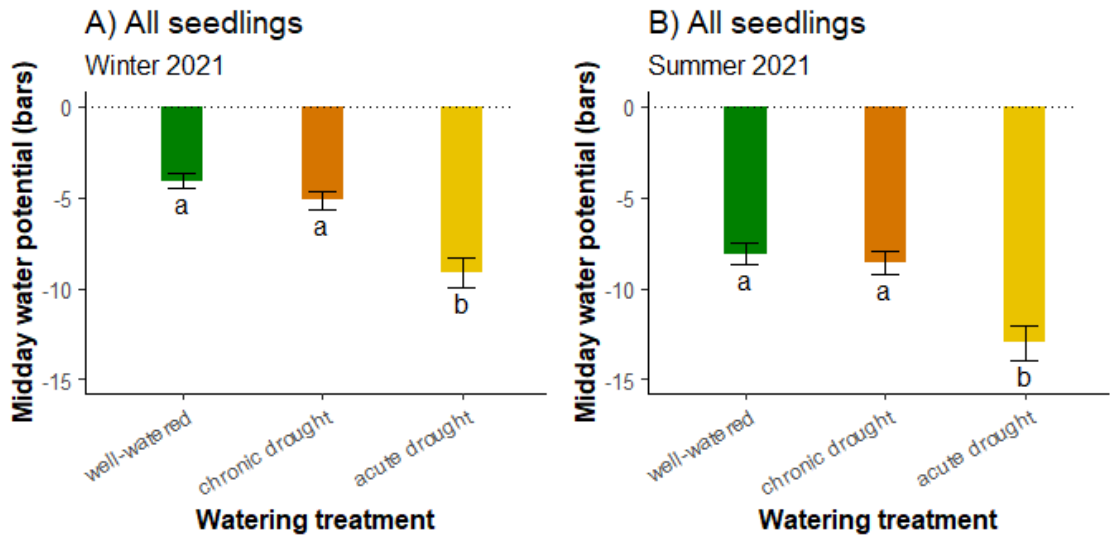


Figure 9. Pre-dawn fascicle water potentials of a subsampling of all limber seedlings by watering treatment (well-watered, chronic drought, acute drought) from (A) Winter 2021, 1– 16 weeks after inoculation (n = 72 from each treatment over 2 sampling events), and (B) Summer 2021, 18 – 37 weeks after inoculation (n = 108 from each treatment over 3 sampling events). Significance indicated by letters. (A) Effect of acute drought is significant when compared to well-watered ($p < 0.0001$) and to chronic drought ($p < 0.0001$) in Winter 2021, and (B) effect of acute drought is significant in Summer 2021, compared to well-watered ($p < 0.0001$) and chronic drought ($p < 0.0001$). Pre-dawn water potentials for the same subsampling of limber seedlings by inoculation treatment (uninoculated, inoculated). (C) Winter 2021, 1 – 16 weeks after inoculation (n = 108 from each treatment over 2 sampling events), and (D) Summer 2021 for weeks 18 – 37 after inoculation (n = 162 from each treatment over 3 sampling events). There was no significant effect of inoculation on pre-dawn water potential (C) $p = 0.6688$; D) $p = 0.5172$); means are not significantly different ($\alpha = 0.05$, lowercase 'a'). Mean ± standard error from measurements taken prior to watering, at driest time of water cycle.



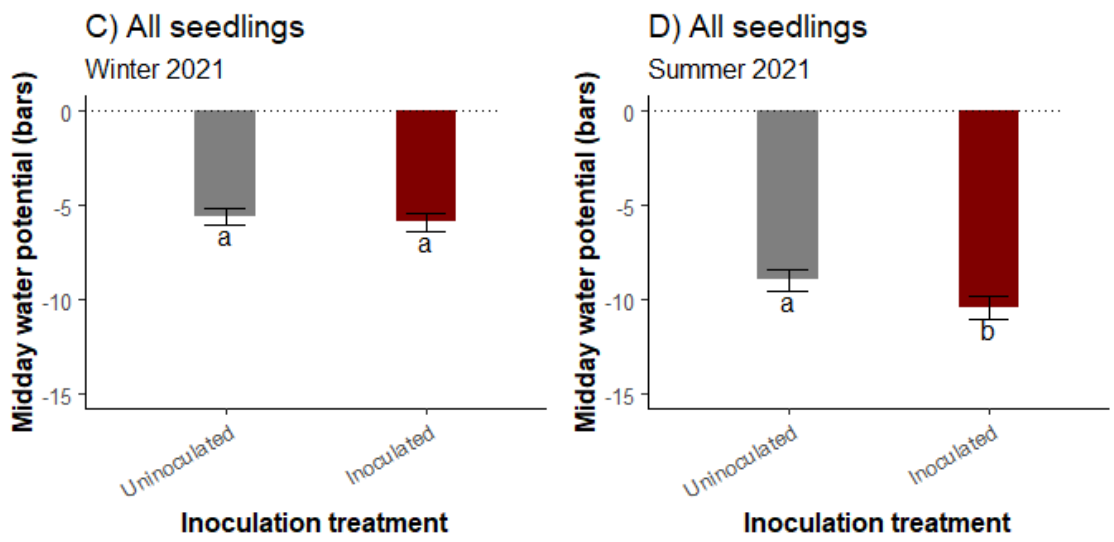


Figure 10. Midday fascicle water potentials of a subsampling of all limber seedlings by watering treatment (well-watered, chronic drought, acute drought) from (A) Winter 2021, 1– 16 weeks after inoculation (n = 72 from each treatment over 2 sampling events), and (B) Summer 2021, 18 – 37 weeks after inoculation (n = 108 from each treatment over 3 sampling events). Significance indicated by letters. (A) Effect of acute drought is significant when compared to well-watered ($p < 0.0001$) and to chronic drought ($p < 0.0001$) in Winter 2021, and (B) effect of acute drought is significant in Summer 2021, compared to well-watered ($p < 0.0001$) and chronic drought ($p < 0.0001$). Midday water potentials for the same subsampling of limber seedlings by inoculation treatment (uninoculated, inoculated). (C) Winter 2021, 1 – 16 weeks after inoculation (n = 108 from each treatment over 2 sampling events), and (D) Summer 2021 for weeks 18 – 37 after inoculation (n = 162 from each treatment over 3 sampling events). (C) There was no significant effect of inoculation on midday water potential ($p = 0.6190$). (D) There was a significant effect of inoculation on midday water potentials during Summer 2021 ($p = 0.0417$). Mean ± standard error from measurements taken prior to watering, at driest time of water cycle.

Chlorophyll fluorescence (F_v/F_m), measured in parallel with post-inoculation water potentials, to quantify the effects of the treatments on limber pine seedling physiology (Figure 11). When comparing all seedlings, there was no interaction between the watering and inoculation treatments on chlorophyll fluorescence (Winter 2021, $p = 0.7039$; Summer 2021 $p = 0.8903$). In both Winter 2021 and Summer 2021, there was a significant effect of watering treatment on chlorophyll fluorescence ($p < 0.0001$), with the acute drought treatment having significant lower F_v/F_m than the well-watered (3.2% lower in Winter 2021; 1.4% lower in Summer 2021) and lower F_v/F_m than the chronic drought (2.7% lower in Winter 2021; 2.1% lower in Summer 2021) There was no effect of inoculation treatment on

F_v/F_m in Winter 2021 (p = 0.3101), but there was a significant effect in Summer 2021 (p = 0.0378), with inoculated seedlings having reduced F_v/F_m compared to uninoculated seedlings (0.6% lower).

The watering treatment physiological impacts on the subset of visually symptomatic diseased seedlings was less compared to the physiological impacts identified among all seedlings. Within the visually diseased subset (Figure 12), watering treatment was not a significant predictor for the response of Winter 2021 pre-dawn water potential (p = 0.2314), or Winter 2021 midday water potential (p = 0.2716), and there was nearly an effect in Summer 2021 midday water potential (p = 0.0596). Within pre-dawn water potential measurements in Summer 2021, watering treatment remains a significant predictor (p = 0.0078), with the symptomatic acute droughted seedlings having a lower pre-dawn water potential than both the symptomatic well-watered (p = 0.0097) and symptomatic chronic droughted seedlings (p = 0.0240). Chlorophyll fluorescence among visually diseased seedlings was significantly affected by watering treatment in Winter 2021 (p = 0.0039), but watering treatment was not a significant predictor of F_v/F_m measurements in Summer 2021 (p = 0.056). The tests performed on each variable and response as well as Pearson's p-value are presented in Table 6.

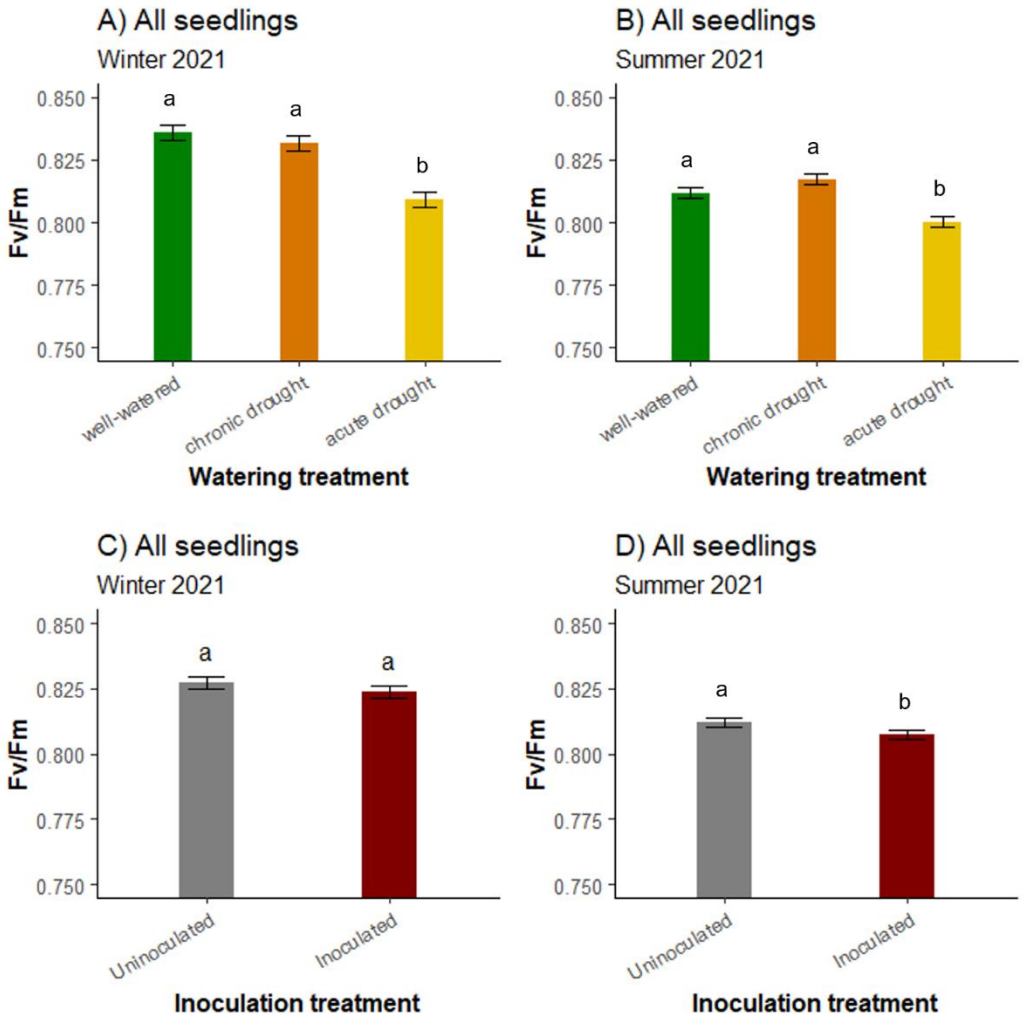


Figure 11. Chlorophyll fluorescence (F_v/F_m) of a subsampling of all limber pine seedlings by watering treatment (well-watered, chronic drought, acute drought) and inoculation treatment (uninoculated, inoculated) from (A) Winter 2021, 1 – 16 weeks after inoculation (n = 72 from each treatment over 2 sampling events), and (B) Summer 2021, 18 – 37 weeks after inoculation (n = 108 from each treatment over 3 sampling events) and (C) Winter 2021, 1 – 16 weeks after inoculation (n = 108 from each treatment over 2 sampling events), and (D) Summer 2021, 18 – 37 weeks after inoculation (n = 162 from each treatment over 3 sampling events). Mean ± standard error from measurements taken prior to watering, at driest time of water cycle. Significance ($\alpha = 0.05$) is noted by letters. (A) effect of acute drought is significant compared to well-watered ($p < 0.0001$) and compared to chronic drought ($p < 0.0001$) in Winter 2021. (B) There was a significant effect of acute drought compared to well-watered ($p = 0.0002$) and compared to chronic drought ($p < 0.0001$) in Summer 2021. (C) No significant effect of inoculation on chlorophyll fluorescence in Winter 2021 ($p = 0.3094$.). (D) There was a significant effect of inoculation on chlorophyll fluorescence during Summer 2021 ($p = 0.0377$).

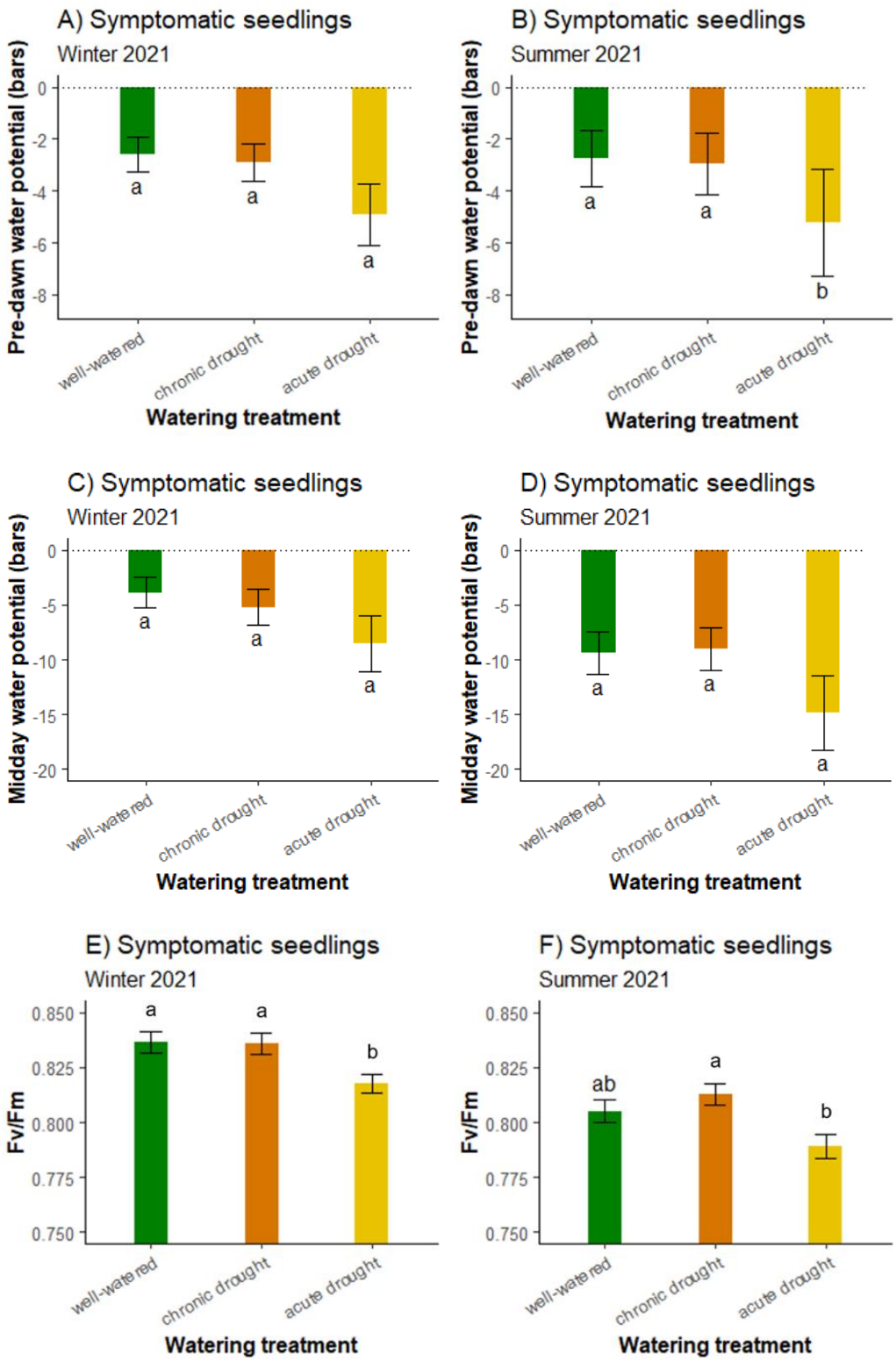


Figure 12. Pre-dawn water potential, midday water potential, and chlorophyll fluorescence in a subsampling of limber pine seedlings that exhibited symptoms WPBR by watering treatment from Winter 2021 (A, C, E), 1 – 16 weeks after inoculation (n = 11, 10, 10 from each treatment over 2 sampling events), and Summer 2021 (B, D, F), 18 – 37 weeks after inoculation (n = 26, 22, 20 from each

treatment over 3 sampling events). Mean ± standard error from measurements taken prior to watering, at driest time of water cycle. Significance ($\alpha = 0.05$) is noted by letters. (A) There is a significant effect of water in Winter 2021 ($p = 0.0001$) and (B) there is a significant effect of acute drought on pre-dawn water potential compared to well-watered ($p < 0.0001$) and chronic drought ($p < 0.0001$) in Summer 2021. There is an effect of watering treatment on midday water potential in (C) Winter 2021 ($p = 0.0010$) and (D) Summer 2021 ($p = 0.0020$). (E) Effect of acute drought is significant compared well-watered ($p < 0.0001$) and compared chronic drought ($p < 0.0001$) in Winter 2021. (F) There was an effect of acute drought compared to chronic drought in Summer 2021 ($p = 0.0001$).

Table 5. Main effect significance table. The listed response and the associated Pearson's p-value of water, inoculation, and interaction for all tests ($\alpha = 0.05$; LMM = linear mixed model, χ^2 = Chi-squared, LR = logistic regression mixed model, BR = Beta regression). Observations from Summer (Su) or Winter (Wi) in 2020 – 2021, or 14 and 116 days after inoculation with *C. ribicola*.

				p-value		
Ref.	Response	time	test	water	inoc	water × inoc
Fig3	RPW	Su '20	LMM	0.0001	—	—
		Wi '20 – '21		0.0001	0.9454	0.5449
		Su '21		0.0001	0.2386	0.5320
Fig3	SMC	Su '20	LMM	0.0001	—	—
		Wi '20 – '21		0.0001	0.5456	0.9891
		Su '21		0.0001	0.6658	0.4557
Fig4	Ψ_{pd}	Su '20	LMM	0.0011	—	—
	Ψ_{md}			0.0001		
Tab4	Mort	dai265	χ^2	0.0002	0.2204	—
Tab5	DNA Absence	dai14 or dai116	χ^2	0.1999	—	—
Fig6	DNA amt	dai14	LMM	0.9917	—	—
	DNA amt	dai116		0.9347		
Fig7	DNA $2^{\Delta Ct}$	dai14	LMM	0.7878	—	—
	DNA $2^{\Delta Ct}$	dai116		0.4325		
Fig8	Vigor	dai200	LR	0.0037	0.0001	0.1299
Fig9	Growth	dai265	LMM	0.3751	0.0310	0.0838
Fig10	Ψ_{pd}	Wi '21	LMM	0.0001	0.6389	0.1268
		Su '21		0.0001	0.5172	0.6605
Fig11	Ψ_{md}	Wi '21	LMM	0.0001	0.6190	0.3837
		Su '21		0.0001	0.0417	0.8432
Fig12	F_v/F_m	Wi '21	BR	0.0001	0.3101	0.7039
		Su '21		0.0001	0.0378	0.8903
Fig13	$\Psi_{pd}^{symp.}$	Wi '21	LMM	0.2314	—	—
		Su '21		0.0078		
	$\Psi_{md}^{symp.}$	Wi '21	LMM	0.2716	—	—
		Su '21		0.0596		
	$F_v/F_m^{symp.}$	Wi '21	BR	0.0039	—	—
		Su '21		0.0056		

4. Discussion

We did not find strong evidence of a drought-related reduction of rust colonization within *P. flexilis* seedlings during early disease development. Overall, we have evidence that our implemented drought stress treatments resulted in physiologically stressed limber pine seedlings, as we saw a significant impact from the acute drought stress on the pine seedling water potential and photosynthesis-related electron transport. We also were successful in inoculating the seedlings, and we were able to detect infection in the majority (81%) of seedlings. Regardless of drought treatment, *C. ribicola* infection resulted in a decrease in water potential and electron transport in the seedlings. Drought treatments did not mitigate or intensify *C. ribicola* infection or colonization of the seedlings, as measured by visual WPBR symptoms and amount of pathogen DNA within needle samples during the early stages of disease we assessed. We did, however, observe trends that suggest water stress-related variation in *C. ribicola* performance that may have become more pronounced had the experiment continued. Trends included a slightly higher disease severity for well-watered seedlings (see Figure 5), and slightly less DNA detection in needle sampling of acutely droughted seedlings (see Table 5).

Though we expected to see a reduction of disease due to drought stressors, the combination of effects was largely absent from our results. None of the WPBR disease progression metrics varied significantly across water treatments, including our observations of cankers and spermatia, detectable pathogen DNA, or relative amount of pathogen DNA. Though not statistically significant, infected seedling in both drought treatments tended to have reduced development of cankers and spermatia (Figure 5), and these variations in

severity may have become significant had the study continued, and a longer study would inform more about this variation. Among the seedlings that developed visual WPBR symptoms there was slightly higher, although not statistically significant, colonization within the acute drought treatment compared to the symptomatic well-watered seedlings (Figure 6 B). Induced water stress has been associated with decreases in mycelial growth and spore production in fungal-foliar pathosystems (Ayres, 1977), but few studies have tested this on obligate fungal pathogens and measured disease development under drought stress. What has been studied indicates that drought decreases carbon intake and this in turn may limit carbon availability to fungal pathogens (Desprez-Loustau et al., 2006), but this effect has not been examined in pine rusts. We observed a change in seedling vigor from drought that was typically expressed as yellowing or browning of needle tips. While not directly measured in this study, the overall impact of the inoculation to host health may be due to the additional concentrated carbon and nutrient sink toll on the limber pine seedling needles in the early infection period.

The droughted seedlings had different pre-inoculation physiological responses among watering treatments. The varied ongoing drought responses caused lasting impacts to the seedings that could have ultimately affected the pine's ability to source carbon. When initially faced with drought, pines will often increase production of soluble sugars in the short term and direct this carbon towards root elongation as drought continues. Under mild water stress, pines typically utilize drought avoidance strategies such as lowering stomatal conductance to limit transpiration and perform photosynthesis at a lower rate (Gao et al., 2002; Klein et al., 2011). However, in prolonged severe acute drought, pines will respond by closing stomata as water potentials fall below a threshold, inhibiting water loss while halting

photosynthesis and carbon assimilation (Aaltonen et al., 2017; Brodribb et al., 2014; Meinzer et al., 2014). Within an infected host, rust pathogens often act as a carbon sink, diverting and depleting host resources (Voegele et al., 2001). Our measurements of chlorophyll fluorescence (Figure 11) indicate that the photosynthesis process was impaired in acute droughted seedlings, limiting the potential supply of carbon. However, we observed comparable levels of *C. ribicola* colonization in both droughted and well-watered treatments.

The severely reduced water potential observed in pine seedlings in our acute drought treatment can be associated with changes observed in the physiological traits of other pine species (Burghardt & Riederer, 2003; Reinhardt et al., 2015), including stomatal closure, reduction in carbon assimilation, increase root-to-shoot ratio, and xylem embolism (Adams et al., 2017; Garcia-forner et al., 2016). Due to our experimental conditions, we established a drought history within our seedlings which likely changed the prospective rust environment. Similar to what has been noted in other pine seedlings (Aaltonen et al., 2017; Kozlowski, 1992), seedlings under chronic drought likely acclimated to the sub-optimal water regime, as impact of the watering treatment decreased over time and damage to the light-absorbing photosystem II was indistinguishable from the well-watered group.

We saw no significant drought-related variation in the level of *C. ribicola* colonization or WPBR disease severity. The percent of infected seedlings (about 62%) (assessed via qPCR) across watering treatments was similar and thus were not significantly influenced by drought. Visual signs and symptoms of WPBR also developed similarly in infected seedlings regardless of watering treatment; there was no reduction in canker development due to drought. We observed reduced maximum quantum efficiency (F_v/F_m) and lower water

potential prior to the onset of visual symptoms, and these effects were observed even in seedlings that did not develop visual WPBR symptoms. We were able to quantify the amount of fungal DNA in needle samples, but we didn't find that watering treatments affected the DNA quantity or predicted disease severity. Quantitative PCR has been used to estimate the relative amount of fungal DNA in host *Triticum aestivum* (winter wheat) infected with pathogen *Puccinia recondite* (brown rust) (Tischler et al., 2018). Tischler et al. (2018) employed this method along with high performance liquid chromatography and a specific measure of chlorophyll fluorescence to compare how each method assessed disease severity and found that the measure of relative fungal DNA amount correlated with their other disease severity metrics. These observations were identified using brown rust, a native and endemic pathogen with a co-evolutionary history with the wheat host. White pine blister rust, on the other hand, is a non-native pathogen and lacks that host evolutionary relationship. In our system, we were able to document mycelial growth, but we were not able to differentiate disease severity by watering treatment. A native pathogen that is contending with host defenses might have a stronger relationship with mycelial tissue growth and host drought while a non-native might perform the same regardless of the condition of the host defense, which may have led to our observed differences.

We observed that seedlings that did not develop visual WPBR symptoms still were affected by WBPR with lower maximum quantum efficiency and lower water potential than not infected seedlings. These results suggest that *C. ribicola* likely has a higher presence in 5-needle pines than has been detected by visual observations alone. In 2009, Smith et al. (2013) found evidence of symptomatic WPBR infection on 43% of living limber pine trees across 85 plots near Alberta, Canada. Cleaver et al. (2015) found symptomatic evidence of

WPBR on 26% of limber pine trees surveyed from plots in Colorado, Wyoming, and Montana and were able to attribute WPBR to 67% of declining limber pine. However, inclusion of asymptomatic trees may better represent the state of a stand and might be possible by designing an experiment to determine the proportion of infected pine that harbor *C. ribicola* without presenting early visual symptoms, though it should be noted that ascertaining infection state in the field and assessing sampling requirements would be extremely difficult. *Cronartium ribicola* within asymptomatic hosts may be awaiting appropriate environmental conditions before sporulating and causing symptoms, and the asymptomatic impact to physiology documented here may be an unattributed contribution to limber pine decline in surveys. Management decisions to allocate resources based on plot incidence may be underestimating areas in need.

We were able to detect *C. ribicola* DNA within 81% of sampled individuals (across treatments) that did not develop visual symptoms within the timeframe of this study (265 days). In all the inoculated seedlings, both visually symptomatic and asymptomatic, we observed physiological changes in the form of lower water potentials (D) and reduced chlorophyll fluorescence (Figure 11 D). The physiological impact observed prior to the onset of any visual symptoms and was evident in both those seedlings that remained visually asymptomatic and those that developed WPBR (Figure 11). There are two likely explanations for the hosts harboring *C. ribicola* but not expressing visual symptoms of WPBR: the rust was awaiting an environmental cue to advance to a sporulating stage, or the individual host was expressing some form of resistance. It is not uncommon for resistance to be associated with a physiological cost: resistant (*Cr2*) *Pinus monticola* needles expressed higher levels of resource-demanding proteins than susceptible needles when challenged

with *C. ribicola* (Zamany et al., 2012) and resistant *P. strobus* seedlings upregulated costly disease resistance proteins when infected with *C. ribicola* (Smith et al., 2006). In *P. flexilis*, Vogan & Schoettle (2016) found that resistance to active *C. ribicola* infection came at the expense of growth.

The visually asymptomatic seedlings in our study led us to consider disease resistance, but resistance alone would not account for all our observations. Although we did not control the source familial seedstock of our seedlings, it is likely that major gene resistance (*Cr4*) was present in the seed source used in our study (Schoettle et al., 2014), and if *Cr4* was expressed in inoculated seedlings that did not visually develop disease, there was likely an associated physiological cost (Liu et al., 2016; Vogan & Schoettle, 2016). While the exact mechanisms have yet to be identified, seedlings with *Cr4* do not develop stem symptoms from infection (Schoettle et al., 2014), yet *C. ribicola* still proliferates within infected needles (Stone et al., 2011). However, the proportion of seedlings that remained visually asymptomatic 265 days post inoculation and had detectable *C. ribicola* DNA was 59%, which is far greater than the expected frequency of the *Cr4* in the seed lot (Schoettle et al., 2014) Consequently, it is highly unlikely that all the visually asymptomatic seedlings in this study carried the *Cr4* resistance gene.

In the phenology of WPBR disease development on the pines, this study addresses just the early stages, and we expect that more seedlings would have developed visual symptoms had the experiment continued. The seedlings within our lot harboring rust without visual symptoms were still expressing physiological symptoms from infection, which is contrary to the traditional meaning of a latent infection. Typically, a pathogen is

considered latent if it has infected a host but has not yet caused symptoms, awaiting physiological triggers that lead to virulence (Mussell, 1980). However, a latent infection that is not causing damage to the host must eventually become virulent, or it is indistinguishable from a non-pathogenic endophyte (Carroll, 1986). As we detected non-visual physiological symptoms resulting from infection, along with mycelial growth, we cannot classify these infections as latent, but perhaps can be classified as a vegetative state associated with maintenance and obtaining resources (Rolland et al., 2006). These physiological impacts could contribute to stand decline even in the absence of visual WPBR symptoms.

Quantifying early fungal colonization by standardizing pathogen gene amount to host gene amount is a new technique within the WPBR pathosystem. This technique was facilitated by the identification and sequencing of a single-copy *C. ribicola* gene (Crib190)(Bergeron et al., 2019). For each DNA extract we targeted and quantified Crib190 and a single-copy *P. flexilis* gene (Agp6) and standardized by relating the pathogen DNA amount as a proportion of the host DNA amount. Each target was quantified via its respective standard curve, and we assumed that DNA from different tissue sources is relatable or scalable (that is, within pine needle tissue is genetically comparable to bud tissue, and within rust, mycelial tissue is genetically comparable to urediniospore tissue). Early relative pathogen DNA amount can be useful in assessing cellular growth rate; for example, qPCR and a known standard curve has been used to estimate fungal biomass within maize tissue infected with *Aspergillus flavus* to identify pathogen resistance in hosts (Mitema et al., 2019). Other studies have also used qPCR to identify and quantify the amount of pathogen within host tissue. Divon et al. (2012) used qPCR and a known standard curve to quantify the amount *Fusarium langsethiae* in oat tissue prior to the onset of disease

symptoms. We anticipated that drought stress would impact mycelial growth, but we were not able to detect that relationship based on measured relative fungal DNA in our experiment.

We were able to report that both *C. ribicola* infection and drought stress independently affected seedling water relations and photosynthesis. We have documented physiological cost of *C. ribicola* infection to *P. flexilis* hosts which will likely be useful in forest management. While many of the biotrophic needs of *C. ribicola* would appear to be influenced by drought-related host stress, we saw no variation in disease progression as a function of watering treatment. Advanced hyphal colonization can interfere with vascular function (Jacobs et al., 2009), and may have been the reason for our Summer 2021 observations of reduced water potentials.

In general, it is to the advantage of fungal biotrophs to limit the functional cost to their pine host, as the rusts are reliant on the host for nutrients and impacts to water relations will ultimately disrupt the nutrient source of the parasitic pathogen (Forward, 1932). In a study of a native pathosystem in western forests, *P. contorta* infected with *Endocronartium harknessii* and symptomatic with stem galls did not have an increased risk of xylem cavitation in the early stages of infection, despite the presence of fungal tissue within the stem, allowing the pine host to continue transpiring and photosynthesizing (Wolken et al., 2009). White pine blister rust is highly lethal in limber pines, but persistent spread of WPBR is dependent on hosts surviving for multiple annual growing cycles. We have documented growing *C. ribicola* within visually asymptomatic limber pines, which are likely awaiting appropriate environmental cues to advance to the next reproductive stage. Absence of visual

symptoms has contributed to existent problems with field surveying for WPBR in whitebark pine (Shanahan et al., 2021) and highlight a need for improvements to systematic surveying. Field surveys are likely underestimating the number of trees that are hosting *C. ribicola*.

While the experiment was executed as intended, there were several areas where alterations may have led to more definitive results. The primary method of early pathogen detection, needle sampling, was not performed on enough needles to accurately assess the infection status of the seedling, as we did not detect *C. ribicola* DNA in about 16% of seedlings that later became visually symptomatic for WPBR. We deliberately sampled what we estimated would be sufficient needle tissue for our inoculation and experimental conditions, while considering trade-offs such as the cost of defoliation during the ongoing experiment. We were reluctant to remove more needles for sampling, as the seedlings varied in size and foliage density, and needle removal would proportionally reduce photosynthesis capacity and could alter the disease trajectory of the pathogen. However, a higher sampling quota could have informed the true infection status of each tree. Other traits that would have been informative within this experiment could have included a study of carbohydrates, an examination of gas exchange, and a comparison of root-to-shoot ratio. As our trees were grown in pots and sourced from a nursery at 3 years old, much of the initial inherent variation among the seedlings was outside of experimental control and limits the utility of comparing traits such as belowground biomass. Without controlling the familial seedstock for the hosts, we were unable to predict and attribute major gene resistance as a factor for disease inhibition within our experiment.

5. Conclusion

Non-native pathogens continue to threaten species and ecosystems, particularly as effects of climate change allow ongoing spread. The relationship between fungal infection and drought varies with pathosystems, and few studies have examined how non-natives such as *C. ribicola* will perform when they inevitably parasitize a droughted host. This study successfully droughted and inoculated susceptible pine hosts with *C. ribicola* in an effort to highlight how the rust would respond and identified an unexpected number of visually asymptomatic infections that physiologically challenged the pine host. Though rust performance was not differentiated by drought in this timeframe, this study introduces a *C. ribicola* relative DNA quantification method that would confirm this result in a study of longer duration.

6. References

Aaltonen, H., Linden, A., Heinonsalo, J., Biasi, C., & Pumpanen, J. (2017). Effects of prolonged drought stress on Scots pine seedling carbon allocation. *Tree Physiology*, 37(4), 418–427. https://doi.org/10.1093/treephys/tpw119

Adams, H., Zeppel, M. J. B, Anderegg, W. R. L., Hartmann, H., Landhäusser, S. M., Tissue, D. T., Huxman, T. E., Hudson, P. J., Franz, T. E., Allen, C. D., Anderegg, L. D. L., Barron-Gafford, G. A., Beerling, D. J., Breshears, D. D., Brodribb, T. J., Bugmann, H., Cobb, R. C., Collins, A. D., Dickman, L. T., & McDowell, N. G. (2017). A multi-species synthesis of physiological mechanisms in drought-induced tree mortality. *Nature Ecology and Evolution*, 1(9), 1285–1291. https://doi.org/10.1038/s41559-017-0248-x

Ayres, P. G. (1977). Effect of water potential of pea leaves on spore production by *Erysiphe pisi* (powdery mildew). *Transactions of the British Mycological Society*, 68(1), 97–100. https://doi.org/10.1016/s0007-1536(77)80158-7

Bates, D., Mächler, M., Bolker, B. M., & Walker, S. C. (2015). Fitting linear mixed-effects models using *lme4*. *Journal of Statistical Software*, 67(1). https://doi.org/10.18637/jss.v067.i01

Baumeister, D., & Callaway, R. M. (2006). Facilitation by *Pinus flexilis* during succession: A hierarchy of mechanisms benefits other plant species. *Ecology*, 87(7), 1816–1830. https://doi.org/10.1890/0012-9658(2006)87[1816:FBPFDS]2.0.CO;2

Bergeron, M. J., Feau, N., Stewart, D., Tanguay, P., & Hamelin, R. C. (2019). Genome-enhanced detection and identification of fungal pathogens responsible for pine and poplar rust diseases. *PLoS ONE*, 14(2), 1–20. https://doi.org/10.1371/journal.pone.0210952

Borgman, E. M., Schoettle, A. W., & Angert, A. L. (2015). Assessing the potential for maladaptation during active management of limber pine populations: a common garden study detects genetic differentiation in response to soil moisture in the Southern Rocky Mountains. *Canadian Journal of Forest Research*, 45(4), 496–505. https://doi.org/10.1139/cjfr-2014-0399

Brodersen, C. R., Germino, M. J., Johnson, D. M., Reinhardt, K., Smith, W. K., Resler, L. M., Bader, M. Y., Sala, A., Kueppers, L. M., Broll, G., Cairns, D. M., Holtmeier, F.-K., & Wieser, G. (2019). Seedling Survival at Timberline Is Critical to Conifer Mountain Forest Elevation and Extent. *Frontiers in Forests and Global Change*, 2(April). https://doi.org/10.3389/ffgc.2019.00009

Brodribb, T. J., McAdam, S. A. M., Jordan, G. J., & Martins, S. C. V. (2014). Conifer species adapt to low-rainfall climates by following one of two divergent pathways.

Proceedings of the National Academy of Sciences of the United States of America, 111(40), 14489–14493. https://doi.org/10.1073/pnas.1407930111

Bucholz, E. R., Waring, K. M., Kolb, T. E., Swenson, J. K., & Whipple, A. V. (2020). Water relations and drought response of *Pinus strobiformis*. *Canadian Journal of Forest Research*, 50(9), 905–916. https://doi.org/10.1139/cjfr-2019-0423

Burghardt, M., & Riederer, M. (2003). Ecophysiological relevance of cuticular transpiration of deciduous and evergreen plants in relation to stomatal closure and leaf water potential. *Journal of Experimental Botany*, 54(389), 1941–1949. https://doi.org/10.1093/jxb/erg195

Cleaver, C. M., Jacobi, W. R., Burns, K. S., & Means, R. E. (2015). Limber pine in the central and southern Rocky Mountains: Stand conditions and interactions with blister rust, mistletoe, and bark beetles. *Forest Ecology and Management*, 358, 139–153. https://doi.org/10.1016/j.foreco.2015.09.010

Cubero, O. F., Crespo, A., Fatehi, J., & Bridge, P. D. (1999). Plant Systematics and Evolution DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Systematic Evolution*, 216, 243–249.

Desprez-Loustau, M. L., Marçais, B., Nageleisen, L. M., Piou, D., & Vannini, A. (2006). Interactive effects of drought and pathogens in forest trees. *Annals of Forest Science*, 63(6), 597–612. https://doi.org/10.1051/forest:2006040

Donnegan, J. A., & Rebertus, A. J. (1999). Rates and mechanisms of subalpine forest succession along an environmental gradient. *Ecology*, 80(4), 1370–1384. https://doi.org/10.1890/0012-9658(1999)080[1370:RAMOSF]2.0.CO;2

Dudney, J., Willing, C. E., Das, A. J., Latimer, A. M., Nesmith, J. C. B., & Battles, J. J. (2021). Nonlinear shifts in infectious rust disease due to climate change. *Nature Communications*, 12(1). https://doi.org/10.1038/s41467-021-25182-6

Dugyala, S., Borowicz, P., & Acevedo, M. (2015). Rapid protocol for visualization of rust fungi structures using fluorochrome Uvitex 2B. *Plant Methods*, 11(1). https://doi.org/10.1186/s13007-015-0096-0

Forward, D. F. (1932). The influence of altered host metabolism upon modification of the infection type with *Puccinia graminis* tritici. *Phytopathology*, 22, 493–555.

Gao, Q., Zhao, P., Zeng, X., Cai, X., & Shen, W. (2002). A model of stomatal conductance to quantify the relationship between leaf transpiration, microclimate and soil water stress. *Plant, Cell and Environment*, 25(11), 1373–1381. https://doi.org/10.1046/j.1365-3040.2002.00926.x

Garcia-forner, N., Sala, A., Biel, C., Savé, R., & Martínez-vilalta, J. (2016). Individual traits as determinants of time to death under extreme drought in *Pinus sylvestris* L . 1196–1209. https://doi.org/10.1093/treephys/tpw040

Garland, T., and Kelly, S.A. 2006. Phenotypic plasticity and experimental evolution. *Journal of Experimental Biology*. 209, 2344–2361. https://doi.org/10.1242/jeb.02244. PMID:1673181

Geils, B. W., Hummer, K. E., & Hunt, R. S. (2010). White pines, Ribes, and blister rust: A review and synthesis. *Forest Pathology*, 40(3–4), 147–185. https://doi.org/10.1111/j.1439-0329.2010.00654.x

Helfer, S. (2014). Rust fungi and global change. *New Phytologist*, 201(3), 770–780. https://doi.org/10.1111/nph.12570

Hennon, P. E., Frankel, S. J., Woods, A. J., Worrall, J. J., Norlander, D., Zambino, P. J., Warwell, M. V., & Shaw, C. G. (2020). A framework to evaluate climate effects on forest tree diseases. *Forest Pathology*, 50(6), 1–10. https://doi.org/10.1111/efp.12649

Hoff, R. J., & Mcdonald, G. I. (1993). Variation of virulence of white pine blister rust. *European Journal of Forest Pathology*, 23(2), 103–109. https://doi.org/10.1111/j.1439-0329.1993.tb00811.x

Holub, E. B. (2006). Evolution of parasitic symbioses between plants and filamentous microorganisms. *Current Opinion in Plant Biology*, 9(4), 397–405. https://doi.org/10.1016/j.pbi.2006.05.011

Hudgins, J. W., McDonald, G. I., Zambino, P. J., Klopfenstein, N. B., & Franceschi, V. R. (2005). Anatomical and cellular responses of *Pinus monticola* stem tissues to invasion by *Cronartium ribicola*. *Forest Pathology*, 35(6), 423–443. https://doi.org/10.1111/j.1439-0329.2005.00425.x

Jacobi, W. R., Kearns, H. S. J., Kegley, A., Savin, D. P., Danchok, R., & Sniezko, R. A. (2018). Comparative Look at Rust Infection and Resistance in Limber Pine (*Pinus flexilis*) and Rocky Mountain Bristlecone Pine (*P. aristata*) Following Artificial Inoculation at Three Inoculum Densities. *In R. M. R. S. US Department of Agriculture, Forest Service* (Ed.), In: Schoettle, Anna W.; Sniezko, Richard A.; Kliejunas, John T., eds. *Proceedings of the IUFRO joint conference*: Genetics of five-needle pines, rusts of forest trees, and Strobusphere (pp. 151–157).

Jacobs, J. J., Burnes, T. A., David, A. J., & Blanchette, R. A. (2009). Histopathology of primary needles and mortality associated with white pine blister rust in resistant and susceptible *Pinus strobus*. *Forest Pathology*, 39(6), 361–376. https://doi.org/10.1111/j.1439-0329.2009.00591.x

Johnson, D. W., & Jacobi, W. R. (2000). First Report of White Pine Blister Rust in Colorado. *Plant Disease*, 84(5), 595–595. https://doi.org/10.1094/pdis.2000.84.5.595d

Kearns, H.S.J., Jacobi, W. R., Reich, R. M., Flynn, R. L., Burns, K. S., & Geils, B. W. (2014). Risk of white pine blister rust to limber pine in Colorado and Wyoming, USA. *Forest Pathology*, 44(1), 21–38. https://doi.org/10.1111/efp.12065

Kearns, H.S.J., & Jacobi, W. R. (2007). The distribution and incidence of white pine blister rust in central and southeastern Wyoming and northern Colorado. *Canadian Journal of Forest Research*, 37(2), 462–472. https://doi.org/10.1139/X06-231

Kegley, Angelia; Sniezko, Richard A.; Danchok, Robert; Savin, Douglas P. 2012. Blister rust resistance among 19 families of whitebark pine, Pinus albicaulis, from Oregon and Washington – early results from an artificial inoculation trial. *In: Sniezko, Richard A.; Yanchuk, Alvin D.; Kliejunas, John T.; Palmieri, Katharine M.; Alexander, Janice M.; Frankel, Susan J., tech. coords. Proceedings of the fourth international workshop on the genetics of host-parasite interactions in forestry: Disease and insect resistance in forest trees. Gen. Tech. Rep. PSW-GTR-240. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture*. pp. 311-315. https://www.fs.fed.us/psw/publications/documents/psw_gtr240/psw_gtr240_311.pdf

Kinloch, B. B., & Dupper, G. E. (1999). Evidence of cytoplasmic inheritance of virulence in *Cronartium ribicola* to major gene resistance in sugar pine. *Phytopathology*, 89(3), 192–196. https://doi.org/10.1094/PHYTO.1999.89.3.192

Klein, T., Cohen, S., & Yakir, D. (2011). Hydraulic adjustments underlying drought resistance of *Pinus halepensis*. *Tree Physiology*, 31(6), 637–648. https://doi.org/10.1093/treephys/tpr047

Kolb, T. E., Fettig, C. J., Ayres, M. P., Bentz, B. J., Hicke, J. A., Mathiasen, R., Stewart, J. E., & Weed, A. S. (2016). Observed and anticipated impacts of drought on forest insects and diseases in the United States. *Forest Ecology and Management*, 380, 321–334. https://doi.org/10.1016/j.foreco.2016.04.051

Kozlowski, T. T. (1992). Carbohydrate sources and sinks in woody plants. *The Botanical Review*, 58(2), 107-222

Krist, F. J., Ellenwood, J. R., Woods, M. E., McMaran, A. J., Cowardin, J. P., Ryerson, D. E., Sapio, F. J., Zweifler, M. O., & Romero, S. A. (2015). 2013 – 2027 National insect and disease forest risk assessment: summary and data access. *Forest Health Monitoring: National Status, Trends and Analysis*, 2014. General Technical Report SRS-209, 87–92.

Krutovsky, K. V., Troggio, M., Brown, G. R., Jermstad, K. D., & Neale, D. B. (2004). Comparative mapping in the *Pinaceae*. *Genetics*, 168(1), 447–461. https://doi.org/10.1534/genetics.104.028381

La Porta, N., Capretti, P., Thomsen, I. M., Kasanen, R., Hietala, A. M., & Von Weissenberg, K. (2008). Forest pathogens with higher damage potential due to climate change in

Europe. *Canadian Journal of Plant Pathology*, 30(2), 177–195. https://doi.org/10.1080/07060661.2008.10540534

Landhäusser, S. M., Chow, P. S., Turin Dickman, L., Furze, M. E., Kuhlman, I., Schmid, S., Wiesenbauer, J., Wild, B., Gleixner, G., Hartmann, H., Hoch, G., McDowell, N. G., Richardson, A. D., Richter, A., & Adams, H. D. (2018). Standardized protocols and procedures can precisely and accurately quantify non-structural carbohydrates. *Tree Physiology*, 38(12), 1764–1778. https://doi.org/10.1093/treephys/tpy118

Langor, D. W. (1989). Host effects on the phenology, development, and mortality of field populations of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (*Coleoptera*: *Scolytidae*). *The Canadian Entomologist*, 121(2), 149–157. https://doi.org/10.4039/Ent121149-2

Letts, M. G., Nakonechny, K. N., Van Gaalen, K. E., & Smith, C. M. (2009). Physiological acclimation of *Pinus flexilis* to drought stress on contrasting slope aspects in Waterton Lakes National Park, Alberta, Canada. *Canadian Journal of Forest Research*, 39(3), 629–641. https://doi.org/10.1139/X08-206

Liu, J.J., & Ekramoddoullah, A.K.M. (2004). Isolation, genetic variation and expression of TIR-NBS-LRR resistance gene analogs from western white pine (*Pinus monticola* Dougl. *ex*. D. Don.). *Molecular Genetics and Genomics*, 270(5), 432–441. https://doi.org/10.1007/s00438-003-0940-1

Liu, J.J., Schoettle, A. W., Sniezko, R. A., Sturrock, R. N., Zamany, A., Williams, H., Ha, A., Chan, D., Danchok, B., Savin, D. P., & Kegley, A. (2016). Genetic mapping of *Pinus flexilis* major gene (*Cr*4) for resistance to white pine blister rust using transcriptome-based SNP genotyping. *BMC Genomics*, 17(1), 1–12. https://doi.org/10.1186/s12864-016-3079-2

Marston, & Anderson, J. (1991). Watersheds and vegetation of the Greater Yellowstone ecosystem. *Conservation Biology*, 5(3), 338–346. https://doi.org/10.1111/j.1523-1739.1991.tb00147.x

McCutchen, H. E. (1996). Limber pine and bears. *Great Basin Naturalist*, 56(1), 90–92.

McDowell, N. G., Williams, A. P., Xu, C., Pockman, W. T., Dickman, L. T., Sevanto, S., Pangle, R., Limousin, J., Plaut, J., Mackay, D. S., Ogee, J., Domec, J. C., Allen, C. D., Fisher, R. A., Jiang, X., Muss, J. D., Breshears, D. D., Rauscher, S. A., & Koven, C. (2016). Multi-scale predictions of massive conifer mortality due to chronic temperature rise. *Nature Climate Change*, 6(3), 295–300. https://doi.org/10.1038/nclimate2873

McDowell, N., Pockman, W. T., Allen, C. D., Breshears, D. D., Cobb, N., Kolb, T., Plaut, J., Sperry, J., West, A., Williams, D. G., & Yepez, E. A. (2008). Mechanisms of plant survival and mortality during drought: Why do some plants survive while others succumb to drought? *New Phytologist*, 178(4), 719–739. https://doi.org/10.1111/j.1469-8137.2008.02436.x

Meinzer, F. C., Woodruff, D. R., Marias, D. E., McCulloh, K. A., & Sevanto, S. (2014). Dynamics of leaf water relations components in co-occurring iso- and anisohydric conifer species. *Plant Cell and Environment*, 37(11), 2577–2586. https://doi.org/10.1111/pce.12327

Mendgen, K., & Hahn, M. (2002). Plant infection and the establishment of fungal biotrophy. *Trends in Plant Science*, 7(8), 352–356. https://doi.org/10.1016/S1360-1385(02)02297-5

Millar, C. I., Westfall, R. D., & Delany, D. L. (2007). Response of high-elevation limber pine (*Pinus flexilis*) to multiyear droughts and 20th-century warming, Sierra Nevada, California, USA. *Canadian Journal of Forest Research*, 37(12), 2508–2520. https://doi.org/10.1139/X07-097

Mitema, A., Okoth, S., & Rafudeen, S. M. (2019). The development of a qPCR assay to measure *Aspergillus flavus* biomass in maize and the use of a Biocontrol Strategy to Limit Aflatoxin Production. *Toxins*, 11(3). https://doi.org/10.3390/toxins11030179

Moyes, A. B., Castanha, C., Germino, M. J., & Kueppers, L. M. (2013). Warming and the dependence of limber pine (*Pinus flexilis*) establishment on summer soil moisture within and above its current elevation range. *Oecologia*, 171(1), 271–282. https://doi.org/10.1007/s00442-012-2410-0

Mussell, H. (1980). Chapter 3 - Tolerance to Disease (J. G. Horsefall & E. Cowling (eds.); pp. 39–52). Academic Press. https://doi.org/10.1016/B978-0-12-356405-4.50010-1

Pearson, M., Saarinen, M., Nummelin, L., Heiskanen, J., Roitto, M., Sarjala, T., & Laine, J. (2013). Tolerance of peat-grown Scots pine seedlings to waterlogging and drought: Morphological, physiological, and metabolic responses to stress. *Forest Ecology and Management*, 307, 43–53. https://doi.org/10.1016/j.foreco.2013.07.007

Rausher, M. D. (2001). Co-evolution and plant resistance to natural enemies. *Nature*, 411(6839), 857–864. https://doi.org/10.1038/35081193

Rebertus, A. J., Burns, B. R., & Veblen, T. T. (1991). Stand dynamics of *Pinus flexilis* - dominated subalpine forests in the Colorado Front Range. *Journal of Vegetation Science*, 2(4), 445–458. https://doi.org/10.2307/3236026

Reinhardt, K., Castanha, C., Germino, M. J., & Kueppers, L. M. (2011). Ecophysiological variation in two provenances of *Pinus flexilis* seedlings across an elevation gradient from forest to alpine. *Tree Physiology*, 31(6), 615–625. https://doi.org/10.1093/treephys/tpr055

Reinhardt, K., Germino, M. J., Kueppers, L. M., Domec, J. C., & Mitton, J. (2015). Linking carbon and water relations to drought-induced mortality in *Pinus flexilis* seedlings. *Tree Physiology*, 35(7), 771–782. https://doi.org/10.1093/treephys/tpv045

Robb, J., Harvey, A. E., & Shaw, M. (1975). Ultrastructure of tissue cultures of *Pinus monticola* infected by *Cronartium ribicola*. II. Penetration and post-penetration. *Physiological Plant Pathology*, 5(1), 9–12. https://doi.org/10.1016/0048-4059(75)90065-X

Rolland, F., Baena-Gonzalez, E., & Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review of Plant Biology*, 57(1), 675–709. https://doi.org/10.1146/annurev.arplant.57.032905.105441

Schoettle, AW (2004) Ecological roles of five-needle pines in Colorado: Potential consequences of their loss. *In: Sniezko, Richard A.; Samman, Safiya; Schlarbaum, Scott E.; Kriebel, Howard B., eds. 2004. Breeding and genetic resources of five-needle pines: growth, adaptability and pest resistance; 2001 July 23–27; Medford, OR, USA. IUFRO Working Party 2.02.15. Proceedings RMRS-P-32. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station.* pp 124-135. https://www.fs.usda.gov/treesearch/pubs/52826

Schoettle, A. W., & Negron, J. F. (2001). First report of two cone and seed insects on *Pinus flexilis*. *Western North American Naturalist*, 61(2), 252–254.

Schoettle, A. W., & Rochelle, S. G. (2000). Morphological variation of *Pinus flexilis* (*Pinaceae*), a bird-dispersed pine, across a range of elevations. *American Journal of Botany*, 87(12), 1797–1806. https://doi.org/10.2307/2656832

Schoettle, A. W., Sniezko, R. A., Kegley, A., & Burns, K. S. (2014). White pine blister rust resistance in limber pine: Evidence for a major gene. *Phytopathology*, 104(2), 163–173. https://doi.org/10.1094/PHYTO-04-13-0092-R

Schuster, W. S., Mitton, J. B., Yamaguchi, D. K., & Woodhouse, C. A. (1995). A comparison of limber pine (*Pinus flexilis*) ages at lower and upper treeline sites east of the continental divide in Colorado. *American Midland Naturalist*, 133(1), 101–111. https://doi.org/10.2307/2426351

Seifi, H. S., Van Bockhaven, J., Angenon, G., & Höfte, M. (2013). Glutamate metabolism in plant disease and defense: Friend or foe? *Molecular Plant-Microbe Interactions*, 26(5), 475–485. https://doi.org/10.1094/MPMI-07-12-0176-CR

Shanahan, E., Wright, W. J., & Irvine, K. M. (2021). Adaptive monitoring in action: Reconsidering design-based estimators reveals underestimation of whitebark pine disease prevalence in the Greater Yellowstone Ecosystem. *Journal of Applied Ecology*, 58(5), 1079–1089. https://doi.org/10.1111/1365-2664.13837

Smith, C. M., Gillies, C., & Stuart-Smith, J. (2013). Changes in white pine blister rust infection and mortality in whitebark pine over time. *Canadian Journal of Forest Research*, 43(10), 919–928. https://doi.org/10.1139/cjfr-2013-0072

Smith, J. A., Blanchette, R. A., Burnes, T. A., Jacobs, J. J., Higgins, L. A., Witthuhn, B. A., David, A. J., & Gillman, J. H. (2006). Proteomic comparison of needles from blister rust-resistant and susceptible *Pinus strobus* seedlings reveals upregulation of putative disease resistance proteins. *Molecular Plant-Microbe Interactions*, 19(2), 150–160. https://doi.org/10.1094/MPMI-19-0150

Staples, R. C. (2000). Research on the rust fungi during the twentieth century. *Annual review of phytopathology*, 38(1), 49-69

Stone, J., Schoettle, A., Sniezko, R., Kegley, A. (2011). Histological Observations on Needle Colonization by *Cronartium ribicola* in Susceptible and Resistant Seedlings of Whitebark Pine and Limber Pine. *In: Keane, Robert E.; Tomback, Diana F.; Murray, Michael P.; Smith, Cyndi M., eds. The Future of High-Elevation, Five-Needle White Pines in Western North America: Proceedings of the High Five Symposium, 28-30 June 2010; Missoula, MT. Proceedings RMRS-P-63. Fort Collins, CO: US Department of Agriculture, Forest Service, Rocky Mountain Research Station.* Vol. 63, p. 243. https://www.fs.usda.gov/treesearch/pubs/38233

Sturrock, R. N., Frankel, S. J., Brown, A. V., Hennon, P. E., Kliejunas, J. T., Lewis, K. J., Worrall, J. J., & Woods, A. J. (2011). Climate change and forest diseases. *Plant Pathology*, 60(1), 133–149. https://doi.org/10.1111/j.1365-3059.2010.02406.x

Tischler, Y. K., Thiessen, E., & Hartung, E. (2018). Early optical detection of infection with brown rust in winter wheat by chlorophyll fluorescence excitation spectra. *Computers and Electronics in Agriculture*, 146, 77–85. https://doi.org/10.1016/j.compag.2018.01.026

Tomback, D. F., & Achuff, P. (2010). Blister rust and western forest biodiversity: Ecology, values and outlook for white pines. *Forest Pathology*, 40(3–4), 186–225. https://doi.org/10.1111/j.1439-0329.2010.00655.x

Tomback, D.F., & Linhart, Y. B. (1990). The evolution of bird-dispersed pines. *Evolutionary Ecology*, 4(3), 185–219. https://doi.org/10.1007/BF02214330

Tomback, D.F., Resler, L. M., Keane, R. E., Pansing, E. R., Andrade, A. J., & Wagner, A. C. (2016). Community structure, biodiversity, and ecosystem services in treeline whitebark pine communities: Potential impacts from a non-native pathogen. *Forests*, 7(1), 1–22. https://doi.org/10.3390/f7010021

Van Arsdel, E. P., Riker, A. J., & Patton, R. F. (1956). The effects of temperature and moisture on the spread of White Pine blister rust. *Phytopathology*, 46(6), 307-318.

Voegele, R. T., Struck, C., Hahn, M., & Mendgen, K. (2001). The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*. *Proceedings of the National Academy of Sciences of the United States of America*, 98(14), 8133–8138. https://doi.org/10.1073/pnas.131186798

Vogan, P. J., & Schoettle, A. W. (2016). Carbon costs of constitutive and expressed resistance to a non-native pathogen in limber pine. *PLoS ONE*, 11(10), 1–18. https://doi.org/10.1371/journal.pone.0162913

Webster, K. L., & Johnson, E. A. (2000). The importance of regional dynamics in local populations of limber pine (*Pinus flexilis*). *Ecoscience*, 7(2), 175–182. https://doi.org/10.1080/11956860.2000.11682586

Williams, J. W., Jackson, S. T., & Kutzbach, J. E. (2007). Projected distributions of novel and disappearing climates by 2100 AD. *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), 5738–5742. https://doi.org/10.1073/pnas.0606292104

Wolken, J. M., Blenis, P. V., & Dyck, M. F. (2009). Whole-tree water relations of western gall rust infected lodgepole pine trees. *Canadian Journal of Plant Pathology*, 31(3), 330–339. https://doi.org/10.1080/07060660909507607

Zamany, A., Liu, J. J., & Ekramoddoullah, A. K. M. (2012). Comparative proteomic profiles of *Pinus monticola* needles during early compatible and incompatible interactions with *Cronartium ribicola*. *Planta*, 236(6), 1725–1746. https://doi.org/10.1007/s00425-012-1715-x

III. FUTURE PROSPECTS FOR WHITE PINE BLISTER RUST AND HOST EFFECTS

1. Reflection

A greenhouse experiment was performed to study the infection of droughted *Pinus flexilis* seedlings with *Cronartium ribicola*. In this drought × pathogen experiment, precisely controlled irrigation amounts were given to seedlings and a controlled rust inoculation was performed to maximize infection. The primary goal of this study was to assess the host physiology and measure the performance of the rust, as the pathosystem endured drought and infection. Though not included in this study, other rust performance and pine physiological traits were considered. The effect of rust and drought on resource availability was attempted by monitoring non-structural carbohydrates within needles via enzymatic assay. Additionally, the development of fungal tissue within the needle was to be assessed via staining and fluorescent microscopy. Both techniques were successful in limited number, but too time- and resource-intensive to perform comprehensively for a study of this size. However, an inclusion of these techniques in future studies would be beneficial, as discussed below.

In this study, *Cronartium ribicola* was inoculated onto *Pinus flexilis*, which was well-watered and droughted in two distinct ways, acute and chronic. I did not observe variation in rust performance in infection levels nor continued growth of the pathogen within the first nine months post infection. Evidence of infection was observed by quantifying external visual symptoms of cankers and spermatia. However, infection influenced pine physiology, even among pines without visual symptoms—because of the additional methods used to assess rust performance, I was also able to detect *C. ribicola* infection in seedlings without

external visual symptoms. Quantifying the relative amount of rust DNA helped us ascertain that mycelial growth was occurring in sampled tissue without regard to drought, and that mycelial growth was occurring in pines without visual symptoms. While monitoring host physiology for drought, physiological changes were also observed as a function of inoculation, and this included hosts that contained rust DNA but never exhibited visual symptoms. These early significant infection costs to host performance were suspected but heretofore unconfirmed effects of inoculation. I was able to drought the hosts to the point of physiological differentiation, as measured by two metrics, and I was able to infect our hosts, confirming the successful premise of the experiment.

Within the bounds of this study, I did not observe that our verified host changes had an effect on the performance of the rust, by measuring visual symptoms or by quantifying sampled tissue for rust DNA. The interactive effect was expected for a number of reasons, including dependency of the infection process on humidity, and pathogen dependence on host resources, which drought should have affected. There are two primary reasons that prevent us from concluding that droughted hosts have no effect on rust development in this pathosystem. First, the experimental window only examined the first nine months of infection, and WPBR is a slow-growing disease that can take years to kill the pine host, and the infection load and the age of the host are large determinants in the time it takes for WPBR to become severe or kill (Geils et al., 2010). However, both of these factors were controlled as infection load was maximized during inoculation and all seedlings were of the same age. Second, rust performance was only measured by a few factors that may have not captured the scope of disease impact to the host. Metrics of mortality or severity of cankers or blisters varied insignificantly among seedlings within the early time frame and may have been more

conclusive after a longer period of colonization. Our method of quantifying relative rust DNA was highly dependent on encountering rust during blind sampling, which fell short in a few instances where I did not detect rust DNA in trees that later became clearly symptomatic. A few modifications to our protocol may have beneficial results, primarily by increasing needle sampling for DNA detection.

Applying chronic or acute drought, I anticipated being able to stress the host enough to affect the rust pathogen but did not observe that effect in this experiment. If the experiment extended longer, it might reveal drought-related variation in canker or spermatia formation; a duration that lasted until a certain percentage of WPBR-induced tree mortality or top-kill occurred would allow the experiment to be tailored to the individual hosts. The morphological variation among limber pine created a wide range of host substrates for *C. ribicola*; seedlings of the same age varied in height, stem diameter, number of branches, number of needles, and in size of needles. This study randomized individuals within treatments and included height and diameter morphology traits as factors, and water treatments were tailored to each individual to account for needle variation. However, including additional morphology-based classification or stratification as a factor in experimental design may have illuminated further aspects of drought survival and WPBR vulnerability. Subsampling pine tissue for rust DNA worked conceptually and provided valuable insight into the number of infected trees not exhibiting symptoms. However, collecting more needles from an individual or subsampling at additional timepoints would have increased the opportunities of encountering the pathogen. Sampling needles for pathogen DNA comes with the cost of destructively removing photosynthetic tissues from each individual and defoliation may disproportionately hinder individuals. Considerations

should be made for the total number of needles on an individual and the size of individual needles, as both varied noticeably in this greenhouse study of seedlings of the same age. Another consequence of additional needle sampling is the act of removing fungal tissue, which lowers the pathogen burden and can lessen the effect of disease, effectively diluting later experimental observations. Therefore, intervals of needle sampling for DNA should be minimized and infrequent until a comprehensive destructive sampling of needles can occur, and if so, relative rust DNA can be an effective way of elucidating host effects on rust performance.

An unexpected result from this study was the impact of inoculation on host physiology, which was observed regardless of whether the seedling developed cankers, spermatia, or other visual signs and symptoms of white pine blister rust. There are multiple possible explanations for the cost to host physiology, particularly those without visual symptoms. Vogan & Schoettle (2016) observed a cost to growth in limber pine seedlings that were challenged with *C. ribicola*, if the seedlings expressed major gene resistance (*Cr4*). If major gene resistance was present among seedlings in this study, it would align with our physiological observations, both of which directly affect carbon acquisition. However, I observed this affect among a greater proportion of seedlings than can be explained by major gene resistance alone, which is expected to occur at a lower frequency (Schoettle et al., 2014). Strictly controlling the familial seedstock would aid in attributing the effect of expressed resistance on infection. Another explanation for the physiological cost of infection includes the likelihood that mycelial tissue occluded stomata or vascular tissue, physically inhibiting carbon assimilation or nutrient transport. The degree of occlusion could be determined using histopathological methods, such as staining needle tissue with a chitin-sensitive UV

dye and imaging with fluorescent microscopy (Dugyala et al., 2015). Additionally, the mycelial tissue was a carbon sink and could have diverted carbon from osmolytes or photosynthetic maintenance, explaining our observed lower water potential and lower chlorophyll fluorescence, respectively. Corroboration of this would be important and could be performed by standardized measurements of non-structural carbohydrates within needles (Landhäusser et al., 2018) before and during infection, sampling at regular intervals throughout the first year of rust growth. Whether the effect is localized to the infection area or is dispersed throughout the seedling could be assessed by selective masking during the inoculation process. This could be achieved by masking the lower half of some seedlings in a way that prevented basidiospores from reaching those needles, while others remain fully exposed as controls. During the ongoing infection, needles could be sampled from both top and bottom of fully infected, half-infected, and control seedlings and assessed for non-structural carbohydrates. Ideally, this would quantify the strength of *C. ribicola* as a carbon sink, which would be important in defining the pathogenicity of white pine blister rust.

2. References

Dugyala, S., Borowicz, P., & Acevedo, M. (2015). Rapid protocol for visualization of rust fungi structures using fluorochrome Uvitex 2B. *Plant Methods*, 11(1). https://doi.org/10.1186/s13007-015-0096-0

Geils, B. W., Hummer, K. E., & Hunt, R. S. (2010). White pines, Ribes, and blister rust: A review and synthesis. *Forest Pathology*, 40(3–4), 147–185. https://doi.org/10.1111/j.1439-0329.2010.00654.x

Landhäusser, S. M., Chow, P. S., Turin Dickman, L., Furze, M. E., Kuhlman, I., Schmid, S., Wiesenbauer, J., Wild, B., Gleixner, G., Hartmann, H., Hoch, G., McDowell, N. G., Richardson, A. D., Richter, A., & Adams, H. D. (2018). Standardized protocols and procedures can precisely and accurately quantify non-structural carbohydrates. *Tree Physiology*, 38(12), 1764–1778. https://doi.org/10.1093/treephys/tpy118

Schoettle, A. W., Sniezko, R. A., Kegley, A., & Burns, K. S. (2014). White pine blister rust resistance in limber pine: Evidence for a major gene. *Phytopathology*, 104(2), 163–173. https://doi.org/10.1094/PHYTO-04-13-0092-R

Vogan, P. J., & Schoettle, A. W. (2016). Carbon costs of constitutive and expressed resistance to a non-native pathogen in limber pine. *PLoS ONE*, 11(10), 1–18. https://doi.org/10.1371/journal.pone.0162913