The Potential Uses of Virus-Infected Symbionts to Improve Drought and Heat Stress in Trees for Improvement, Climate Change Resilience, and Restoration

Wren Mangelli

Image Credit: Wren Mangelli, 2022

The Potential Uses of Virus-Infected Symbionts to Improve Drought and Heat Stress in Trees for Improvement, Climate Change Resilience, and Restoration

by

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ABSTRACT

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With the rapid changes occurring to the climate of the boreal forest, many populations of trees are facing increased stresses and seeing lower productivity and survival. Conventional tree improvement programs take decades to come to fruition and may not provide extensive enough benefits to tackle changing conditions. Curvularia Thermal Tolerance Virus (CThTV) has the capacity to dramatically improve the heat stress resilience of its host by activating the basic stress response genes of a mutual endophytic fungus within the plant host. This novel system has the potential to be introduced into economically important trees using endophytic fungi and/or mycorrhizal species as vectors. If successful, this virus would confer significant relief from a large variety of environmental stressors, and be persistent across generations of plants on-site, without being a risk to the genetic diversity of the host population.

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INTRODUCTION

CURVULARIA THERMAL TOLERANCE VIRUS SYSTEM OVERVIEW

In 2002, Redman *et al.* discovered that the presence of an endophytic fungus, *Curvularia protuberata* Nelson, conferred significant heat tolerance to *Dichanthelium lanuginosum* (Elliott) Gould, a species of grass found at geothermal sites in Yellowstone National Park. Grasses that possessed this endophyte were able to survive in soil temperatures up to 65°C. In 2007, Marquez *et al.* conducted a follow-up study, and found that the driving force behind the thermal tolerance was a virus infecting *C. protuberata*, which they named Curvularia Thermal Tolerance Virus (CThTV). Figure 1 from Roossinck (2011) visualises the relationship and effects of CThTV and *C. protuberata* on *D. lanuginosum*.

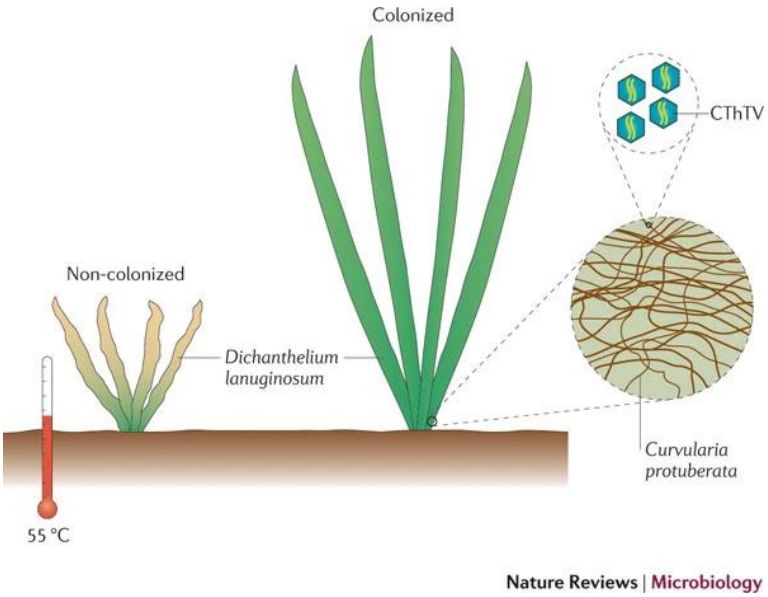

Figure 1. Effects of CThTV and *C. protuberata* colonisation of *D. lanuginosum*
Source: Roossinck (2011).

Curvularia protuberata infected with CThTV has even been found to confer heat tolerance to more plant species than its original host species, including tomatoes

(Marquez *et al.* 2007), showing that neither CThTV nor *C. protuberata* are restricted to their current niches. The use of an infectious and symbiotic agent to induce improved resilience in trees would be a novel approach to tree improvement. One of the major benefits of this approach as compared with breeding or transgenic projects would be that the genetic code of the host is not being altered, only activated. This would make the system applicable to more subjects more efficiently, and would be free from many of the concerns tied with Genetically Modified Organisms and the long-run genetic diversity loss that can be seen in crop improvement programs.

CLIMATE CHANGE INFLUENCES

With increases to global average temperatures and resultant changes to climate and weather patterns, heat and drought stresses are going to become significant challenges for forest tree species globally. The boreal forest is under significant threat from global climate changes, with 65% of the total area being rated as highly vulnerable (Gonzalez *et al.* 2010). Increased drought effects, in addition to causing tree mortality directly, also greatly reduce vigour and make trees more vulnerable to attacks by pests, pathogens, and parasites (Pichler and Oberhuber 2007). Each temperature increase of 1°C to the annual average shifts ecological zones in the Northern hemisphere northwards by 160km (Thuiller 2007). Increased temperatures are likely to cause regional shifts in the distribution of precipitation, which could result in greater drought and heat stresses for boreal species unable to migrate northwards or upwards in altitude to compensate for rapid temperature changes. Climate change models produced by the National Oceanic and Atmospheric Administration (NOAA) show the predicted alterations to average land

surface temperature, annual precipitation in winter and spring, for several different carbon emissions inputs. Crowe and Parker (2011) developed specific models to investigate the impacts of predicted climate change on the boreal forest in Ontario, and found that 41% of Ontario tree species will require assisted migration in order to survive the climate conditions predicted for 2050.

Tree improvement programs have largely been focused on improved yield and economic value, and there are comparatively few programs that focus on optimizing tree improvement to counter environmental stressors like global warming or drought. One commonly used tool for addressing stress response in trees is to overexpress genes within the tree that are activated during stress induction. However, this tactic can have tradeoffs with regards to growth and yield (Polle *et al.* 2019). With predicted increases to temperature and changes to rainfall patterns, tree improvement that accounts for resilience in the face of environmental stress will become increasingly important for sustainable forestry.

EFFECTS OF STRESS

When trees experience drought, heat, or any environmental stress, due to their inability to leave a suboptimal site, they will alter their physiology to accommodate the new conditions in an effort to live sustainably on the site they inhabit. These tactics include decreasing resource use, slowing growth or stalling reproduction, increasing production of protective compounds, or recruiting microbial partners.

In heated soils, plants will alter their root exudates in both quantity and composition as a method of acclimation to the altered conditions. These root exudates result in greater soil respiration overall (de Vries *et al.* 2019). Root exudates also

improve the recruitment of beneficial rhizobacteria, which help break down those same exudates into compounds that are beneficial for plant growth (Tiziani *et al.* 2022).

Under drought stress, plants will also host greater quantities of carbohydrates and free amino acids in their tissues to help maintain osmotic pressure. As a result, they will experience greater levels of oxidative stress from reactive oxygen species (ROSs), which can lead to the degradation of cellular signaling pathways. The capacity for converting ROSs into non-toxic compounds is a critical function of any aerobic organism. With these systems degraded or absent, cells cannot survive, as seen in anaerobic bacteria exposed to oxygen (Hentges 1996). Field studies on beech have shown that leaves cannot regain their ability to regulate the antioxidant pathways after they have been significantly drought or heat stressed (Polle *et al.* 2001). If antioxidant pathways cannot be restored after disturbance, the survival rates become exponentially lower during each subsequent stressor. Additionally, if these pathways cannot be restored by the tree itself, then external remediation is the one method to recover significantly stressed individuals.

SCOPE OF INTEREST

There would be significant value in testing whether CThTV could be inoculated into tree species. Trials to find compatible fungal hosts and to test viral integration and influence, have significant potential for benefits to forest trees. CThTV has the potential to greatly reduce the oxidative stress induced by drought and heat, and could help prevent the initial cellular damage and recover populations that have experienced significant and damaging stress. If paired with an ectomycorrhizal fungal host, the benefits of the virus and the longevity of the treatment could be further enhanced. Heat and drought resilient

trees have the potential to provide benefits in industrial forestry, site reclamation, greenbelts, and even in urban environments. According to the World Commission on Environment and Development (1987), sustainable resource management is grounded on three conceptual pillars: Ecological health restoration or preservation, social approval or acceptance, and economic growth or stability. This paradigm is reflected in almost all aspects of forest management today. Improved stress tolerance would help current forests survive in a rapidly changing climate and allow trees to grow where they might otherwise be incapable such as in desert greenbelts. There is potential for improved timber yields from forests that would otherwise fail as a result of heat stress or drought, and growth improvements in populations subjected to less stress. At a social level, the preservation of forests carries significant weight and is widely accepted as a crucial component of mitigating the negative effects of climate change.

LITERATURE REVIEW

CThTV LIFE HISTORY

Viruses made of double stranded RNA (dsRNA) have a unique challenge during the infection and replication process, as dsRNA segments are not naturally produced in eukaryotic cells. This means that the virus cannot simply hijack its host cell's infrastructure to reproduce. Eukaryotic host cells even have antiviral defences that can attack dsRNAs very effectively. In order to avoid detection, dsRNA viruses will transcribe their genome into single stranded, messenger RNA which can be read by the host cells and translated into functional proteins (Figure 2). This process includes the production of a capsid, where the newly produced proteins and mRNA can be assembled and replicated in a space separate from the host cytoplasm. Once assembled, the viruses and all their components, now encased in a double layered particle, can leave the cell without detection to infect further cells (Hulo *et al.* 2011).

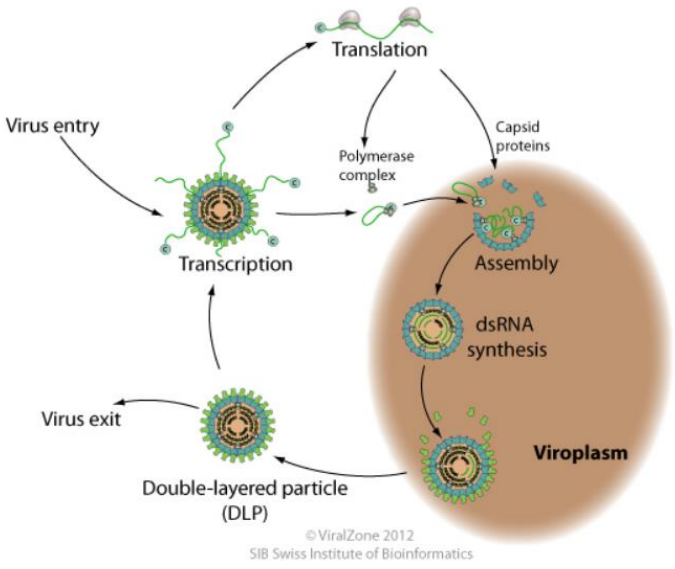

Figure 2. The dsRNA infection and replication process. Source: Hulo *et al.* (2011)

Creating a Stress Resilient System

A key feature of viruses is their ability to manipulate the genetic and biochemical machinery of their host cells. CThTV cannot create novel defences against heat stress. Instead it amplifies and optimises the existing defences of its fungal host. Additionally, because CThTV works on genes encoding for universal stress response factors like those for oxidative and osmotic stresses, there is significant potential for it to be able to colonize a diverse array of hosts. Even if viral inoculation cannot provide the same degree of benefits in trees as in panic grasses, any improvement to stress response would improve growth and survival rates.

CThTV operates by inducing heat stress related genes in *Curvularia protuberata*. These include genes for the production of trehalose, glycine betaine, taurine, melanin, glutathione, and various catalases and peroxidases, all responsible for critical biosynthesis pathways and for degrading osmoprotectants. In addition, the genes for key enzymes in melanin production are also significantly induced by CThTV. Viral infection is capable of inducing significantly greater levels of peroxidase and catalase production during the early stages of heat stress (Morsy *et al.* 2010). This action by the virus grants the fungus the ability to scavenge ROSs from its host plant's tissues, helping to maintain osmotic pressure, reduce tissue damage from water loss, and overall reduce the impacts of oxidative stress.

Trehalose

Trehalose is a nonreducing disaccharide made up of glucose molecules that is stable at high temperatures (Iturriaga *et al.* 2009). Trehalose is found in high quantities in the resurrection plant, *Selaginella lepidophylla* (Hook. & Grev.) Spring, allowing the plant to restore function to its tissues after desiccation (Zentella *et al.* 1999). Trehalose protects molecules by replacing water molecules in the hydration layers of tissues, preventing them from being damaged when water is removed (Clegg 1985). Trehalose is structurally unique among sugars, having relatively flexible bonds, which allow for novel interactions with other molecules (Donnamaria 1994). Additionally, it has a glass structure when solid, such that in its crystal form it remains amorphous, allowing it to remain intact even at very high temperatures (Crowe *et al.* 1998).

While trehalose has been seen as an important biomolecule for protecting tissues from desiccation, some studies have also disputed its influence. In yeasts, although the production of trehalose is correlated to greater osmotic stress protection, it has been seen that mutated strains unable to produce trehalose are still resistant to desiccation (Ratnakumar and Tunnacliffe 2006). However, trehalose is just one tool among many that allow organisms to survive stress conditions.

Glycine Betaine

Glycine betaine is a type of osmolyte, and is found in a wide variety of aerobic organisms. An osmolyte is an organic molecule that helps maintain the physical integrity of cells by altering the composition of aqueous solutions (Yancy 2005). Glycine betaine is a particularly efficient osmolyte that, while almost always present to some degree, will

accumulate in the tissues of organisms during periods of stress (Muñoz-Clares *et al.* 2010). Within the first few hours of heat stress, levels of betaine aldehyde dehydrogenase were increased twice as quickly in CThTV infected cultures as compared with those lacking the virus (Morsy *et al.* 2010).

Taurine

Taurine is a type of osmoprotectant, a major component of the antioxidation process of many aerobic organisms, studied intensively in mammalian cells (Huxtable 1992). It has been found in notably high quantities in marine invertebrates living around hydrothermal vents (Rosenberg *et al.* 2006) and in the desert cactus *Opuntia ficus-indica* (L.) Mill. (Stintzing *et al.* 1999). Morsy *et al.* (2010) saw that there were different levels of the enzyme taurine catabolism dioxygenase between CThTV infected and non-infected cultures of *C.protuberata*. Taurine catabolism dioxygenase is the enzyme responsible for catalysing metabolic reactions involving taurine. Having higher levels of this enzyme means that an organism can more effectively process ROSs and reduce rates of oxidative stress. In other microbes, differential degradation of taurine has also been seen to help regulate nitrogen and sulphur availability, and it is hypothesised that taurine degradation is a method of controlling carbon use during times of stress.

Melanin

Melanin is a natural defence mechanism used by fungi to protect against enzymatic digestion by other antagonistic microbes (Rosas and Casadevall 2001). Melanin also provides a viable defence against stresses ranging from simple UV exposure

(Gorbushina *et al.* 2003), to extreme radiation such as is found on space stations and Chernobyl's nuclear reactors (Dadachova and Casadevall 2008). Being able to produce melanin in response to changing conditions allows organisms to weather times of stress without needing to metabolically invest in enhanced melanin production all the time. Fungal tissues infected with CThTV saw a 100-fold increase in the production of scytalone dehydratase within the first few hours of heat stress (Morsy *et al.* 2010). Scytalone dehydratase is one of the key components of the melanin biosynthesis pathway, and was seen to be amplified ten times more in infected cultures than in cultures lacking CThTV (Morsy *et al.* 2010). This reaction to infection by CThTV signifies that the production of melanin, via enhanced production of its precursors, is one of the components that helps host fungi remain resilient to environmental stressors.

Glutathione

Glutathione is an antioxidant found in all aerobic organisms and is part of the first level of defence against oxidative cellular damage. Glutathione S-transferases (GSTs) are enzymes that are found in all aerobically respiring organisms, and play a role in reducing oxidative stress. Glutathione S-transferases feature a wide variety of functions depending on the organism they're found in and the substrates or toxins that the organism is periodically exposed to. Mammalian GSTs are the best studied due to their connections with cancer development and treatment (Ranson and Hemingway 2005). Five genes for glutathione S-transferase were amplified in the early stages of heat stress by CThTV in *C. protuberata* (Morsy *et al.* 2010). Glutathione (GSH) is a chemical scavenger of ROSs during non-enzymatic reactions (Polle *et al.* 2001). During periods of stress occurring

from low water availability, plant tissues will accumulate ROSs due to the increased retention of carbohydrates. Higher levels of GSH in an endophyte of the plant tissue would allow the symbiont to decrease ROSs of both its own tissues and those of its host.

Catalase/Peroxidase

Catalase is found in all aerobic organisms and is a crucial part of preventing cellular damage from oxygen based metabolic reactions. Catalase works to catalyse the breakdown of hydrogen peroxide into water and oxygen, working in tandem with glutathione peroxidase during the second level of protection against ROSs (Figure 3).

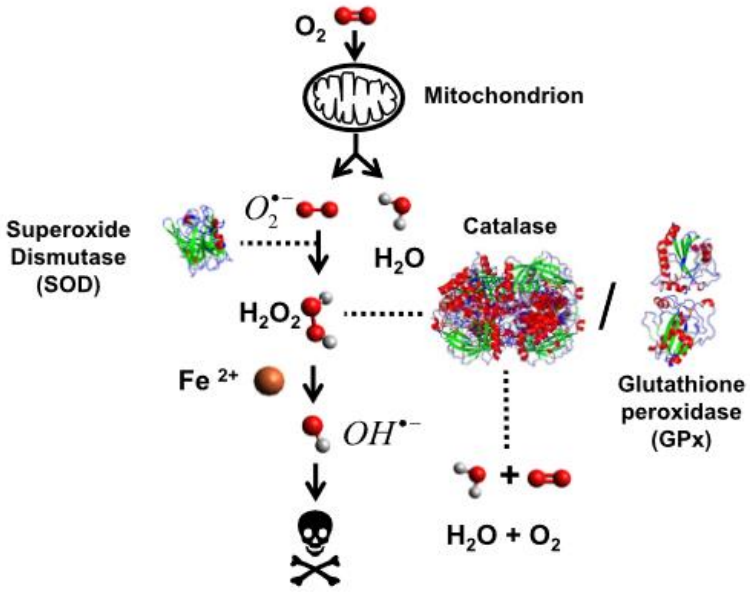

Figure 3. Functions of catalase in antioxidant reactions. Source: Tehrani and Moosavi-Movahedi (2018)

In the early stages of heat stress, as with GST, higher levels of expression of both catalases and peroxidases were seen in *C. protuberata* infected with CThTV. This upfront production of enzymes helps prevent cellular damage from sudden condition changes (Morsey *et al.* 2010), and improves resilience and survival during periods of stress.

Transmission and Spread

CThTV can be transmitted vertically through conidial spores, and horizontally via anastomosis (Marquez *et al.* 2007). This means that the virus can be spread through ascomycetous asexual reproduction, or through sexual reproduction by ascomycetes or basidiomycetes, both of which would allow effective spread between hosts. Ascomycetes and Basidiomycetes are the two largest and most studied groups of fungi. Ascomycetes produce conidia which allow them to reproduce asexually through spore dispersal. Basidiomycetes need to mate in order to produce spores. Members of both the Basidiomycota and the Ascomycota have potential as viral hosts.

PLANT VIRUS GENOMICS

The first plant virus to be studied was also the first virus to be discovered. The Tobacco Mosaic Virus (TMV), was identified by Dmitri Ivanovsky in 1892 as a filterable pathogen and identified by Martinus Beijerinck as a "virus", a novel term at the time, in 1898 (Beijerinck 1898). The first mycovirus was discovered by Gandy and Hollings in 1962, infecting cultivated *Agaricus brunnescens* Peck.

There is an important distinction to be made between plant viruses and mycoviruses in plants. Plant viruses infect plant cells directly and mycoviruses infect fungi that subsequently interact with the target plants. The majority of disease inducing plant viruses are single-stranded RNA (ssRNA) viruses, reverse transcribing viruses, or single stranded DNA (ssDNA) viruses (Koonin *et al.* 2020), whereas mycoviruses are almost always double-stranded RNA (dsRNA) viruses (Lemke and Nash 1974). Infection of fungi by mycoviruses usually causes alterations to host morphology, metabolite

production, or virulence, depending on the type of mycovirus and the type of host (Nuss and Koltin 1990), however, these symptoms would only be visible if the fungal host has distinct enough morphology for the alterations to be seen.

There exists a precedent for horizontal gene transfer from viruses to eukaryotes, especially amongst pararetroviruses. Pararetroviruses are circular DNA viruses that are unable to integrate into host genomes (Hohn *et al.* 2008) due to the lack of an integrase gene (Hohn and Rothnie 2013). However, evidence for genome integration amongst partitiviruses, which are double stranded RNA viruses has also been seen. The work of Mann *et al.* (2003) and Lindell *et al.* (2004) both display how viruses can act as effective vectors for genetic material, either by transferring their own genetic code or by vectoring genetic materials across multiple hosts. The ability of viruses to induce genetic flow and introduce unique genetic elements to a wide range of disparate organisms increases the genetic plasticity of the hosts and can allow for unique interactions to occur (Stobbe and Roossinck 2014).

For the RNA-dependent RNA polymerase gene, there has been widespread horizontal gene transfer from dsRNA viruses to their eukaryotic host cells, a phenomenon which had historically only been thought to occur from DNA based retroviruses, and very rarely in RNA viruses (Geuking *et al.* 2009). RNA-dependent RNA polymerase genes have homologs in nearly all kingdoms of Eukarya (Liu *et al.* 2010). This supports the idea that CThTV may have compatibility and effectiveness across multiple fungal hosts and ideally, within multiple plant species. In some cases, plant immune system functions can be linked to viral infections and subsequent genomic integration. Endogenous pararetroviral elements, viral components found within the host genome, have the

potential for providing viral resistance in plants through homology dependent gene silencing (Jakowitsch *et al.* 1999). This has also been observed for short interfering RNAs (siRNAs), one of the major components involved in host-pathogen interactions and defence against nucleic acids (Voinnet 2005). These siRNAs can either originate from endogenous dsRNAs, or from exogenous viruses (Chapman and Carrington 2007).

FUNGAL HOST CANDIDATES

Fungi have long been genetically manipulated and exploited for a wide range of reasons, notably in the industrial production of proteins (Mackenzie *et al.* 2004), antibiotics (Brakhage and Caruso 2004) and lipids (Sancholle *et al.* 2004). Members of the basidiomycota division have been demonstrated to be difficult to modify genetically with traditional methods involving protoplast production. Because basidiomycetous species do not produce conidia, it is more difficult to induce spore production in a laboratory setting, and thus more difficult to produce and regenerate protoplasts. Additionally, ectomycorrhizal fungi and other obligate symbionts are difficult or impossible to grow in culture because they are obligate biotrophs and can't easily survive outside of a host association. (Kemppainen *et al.* 2011). However, modification with CThTV does not require the use of protoplast production, and selective media are available for culturing ectomycorrhizal fungi.

CThTV cannot be directly inoculated into plants because as a dsRNA virus, it is largely incompatible with plant genomes. To have active effects at the genetic level, the virus needs to be inoculated into a fungal host first, then introduced into the target plant via fungal infection.

Endophytes

Endophytes are organisms that live symbiotically and /or asymptomatically within the tissues or cells of other organisms (Pimentel *et al.* 2011). Fungal endophytes are divided into 4 classes based on their distinct life cycles. Class 1 endophytes are largely host specific to the Poaceae grass family and are vertically transmitted (Rodriguez *et al.* 2009). Class 2 endophytes are highly diverse, can colonise all parts of the plant above and below ground, can be vertically or horizontally transmitted, and are the predominant endophytes for plants living in high stress environments (Rodriguez *et al.* 2009). Class 3 endophytes are composed of a diverse group of foliar endophytes, many in the Basidiomycota division, that are horizontally transmitted (Rodriguez *et al.* 2009). Class 4 endophytes are a diverse group of root restricted endophytes, characterised by melanized hyphae (Sieber and Grunig 2006).

Endophytes of multiple plant species have been shown to confer stress resistance in their hosts. For example, *Beauveria bassiana* (Bals.-Criv.) Vuill. is capable of inducing drought resistance in red oak seedlings by stimulating root growth (Ferus *et al.* 2019).

Trichoderma harzianum Rifai, has been seen to improve seedling emergence and growth traits, and can be found within the roots of its host after soil inoculation (Klefield and Chet 1992), and is host to the curvulavirus Trichoderma harzianum bipartite mycovirus 1. This species may also have the potential to act as a viral vector.

Ectomycorrhiza

Ectomycorrhizal (EcM) species have long been investigated for their ability to improve plant growth and reduce the impacts of various environmental stressors. Studies have shown that EcM fungi can improve resilience to drought (Parke *et al.* 1983; Gehring *et al.* 2017), heat, and salinity (Yin *et al.* 2020). Viral infection of ectomycorrhizal fungi however, has been poorly studied. This is likely due to the difficulties with culturing obligate biotrophic species. However, a few instances of mycoviruses of mycorrhizal species have been observed and sequenced.

CThTV is part of the family Curvulaviridae, which includes viruses that infect *Lactarius rufus* (Scop.) Fr. and *L. tabidus* Fr. which are common in boreal ecosystems and ectomycorrhizal with pines (Sutela and Vainio 2020). While some species of *Lactarius* are host specific, there are several which function as host generalists. Most *Lactarius* members are late stage ectomycorrhizae formers and less suited for study in seedlings however (Hutchison 1999). A species of *Lactarius*, symbiotic with both conifers and hardwoods, and native to the Canadian boreal would be an ideal candidate for testing.

Thelephora terrestris Ehrh. (common fibre vase, earthfan fungus) is an ectomycorrhizal fungus most commonly associated with pines, but is able to form mycorrhizae with a wide number of coniferous and hardwood tree species. It is a pioneer species and shows vigorous growth that easily outcompetes many other ectomycorrhizal species (Colpaert 1999). Petrzik *et al.* (2016) sequenced T. terrestris virus 1 from *T.terrestris*, where interestingly, the virus is capable of infecting and using mites as vectors. This ability to use mites as vectors is an excellent example of the host flexibility of dsRNA viruses across kingdoms. While there are no documented genetic relationships

between T. terrestris virus 1 and CThTV, it would still be worth investigating whether CThTV and *T. terrestris* would be compatible. Being a pioneer species, *T. terrestris* would possess a host of stress tolerant traits that would allow it to survive in uncolonized areas. As such, CThTV may be able to act on those pathways and produce the same effects seen in *C. protuberata*.

METHODS

PROPOSED STUDY DESIGN

A proposed design for this study would involve greenhouse trials utilising at least one conifer species and broadleaf species, and for each tree species selected, at least one fungal endophyte and ectomycorrhizal species, with a virus free control for each trial, and a set of controls with neither fungal nor viral inoculation. This breakdown is shown in Table 1 below. The metric for determining efficacy of viral treatment would be seedling survival and growth rate under heated soil conditions and/or simulated drought, which would be compared to survival rates under normal growing conditions. Each test would be run for a span of several months to allow sufficient opportunity for symbionts to colonize and influence their hosts.

Table 1. Outline of treatments for full scope study.

Normal Growing Conditions								
Conifer	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
Broadleaf	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
Stressed Growing Conditions								
Conifer	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
Broadleaf	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus
	Endo1	Endo2	Ecto 1	Ecto 2	Endo 1 + virus	Endo2 + virus	Ecto 1 + virus	Ecto 2 + virus

For each treatment, 10 replicates would be required to meet standards for determining statistical significance, resulting in a total seedling count of 640.

CULTURE METHODS

Marquez *et al.* (2007) used a liquid potato dextrose medium supplemented with the antibiotics ampicillin, streptomycin and tetracycline. Potato dextrose medium is a relatively universal medium that is also easy and affordable to prepare. The antibiotics are a standard procedure that help lower the rates of bacterial contamination of cultures. This would be a viable medium to test for use with selected endophytes. There are a few different media routinely used with ectomycorrhizal fungi. Modified Melin-Norkrans medium agar has been used to grow late stage mycorrhizal formers like *Boletus edulis* Bull. (Marx 1969). Modified Pridham-Gottlieb medium has been used to culture *Pisolithus tinctorius* (Scop.) Rauschert and *Thelephora terrestris* Ehrh., and is very effective at biomass production (Litchfield and Lawhon 1981).

MOLECULAR METHODS FOR FUNGI

Identification of an introduced fungal endophyte at the genetic level is crucial, since many fungi, especially those that are not fruiting or producing conidia, can be exceptionally difficult to identify accurately from morphology. A host of symbiotic endophytes (Petrini 1996) and opportunistic wood decay fungi (Carroll 1988) are also frequently present in the plant tissue and need to be distinguished from each other and the introduced endophyte.

At the species level or finer, the Internal Transcribed Spacer (ITS) region, is widely regarded as the best method for genetic identification (Schoch *et al.* 2011). This region has been shown to have the highest levels of specificity in terms of identification as compared to other conventional barcoding methods. For fungi, amplification of the DNA that codes for ribosomal proteins is more effective for identification purposes than amplification of other genes (Schoch *et al.* 2011). The conserved mitochondrial region used for animal identification is not useful for fungi, due to large regions of interspecies homology and therefore insufficient variability. The genes that code for the large ribosomal subunit region has fairly good resolution for some groups like the Basidiomycota, but loses effectiveness for early evolved lineages and ascomycetous yeasts. The small subunit ribosomal region showed poor resolution in fungi at all species level (Schoch *et al.* 2011).

Many commercial extraction kits for DNA use silica membranes built into specialised microcentrifuge tubes, in tandem with guanidinium thiocyanate-based buffers as chaotropic agents. Post extraction, one of the most effective and frequently used methods for DNA purification is using a silica matrix in tandem with a chaotropic agent in order to bind DNA to the matrix to pull it out of the solution containing contaminants (Boom *et al.* 1990). Guanidinium thiocyanate extraction with a silica matrix is an efficient and effective method of extraction that would be ideal for use in this study.

The Polymerase Chain Reaction (PCR) is an enzymatic and molecular method used to amplify a specific section of primer defined DNA through specific cycles of heating and cooling. (Mullis *et al.* 1985, Saiki *et al.* 1988). Modern PCR is usually conducted with Taq polymerase, which is a DNA polymerase taken from the bacterium

Thermus aquaticus Brock & Freeze (Taq), which can be found in hot springs and hydrothermal vents. Polymerase from *T. aquaticus* is effective for PCR reactions because due to the intense heat of its natural environment, its polymerase is resistant to heat denaturation, and doesn't need to be replaced between PCR cycles (Chien *et al.* 1976). PCR amplifies a specific segment of DNA which can be identified with sequencing or viewed through gel electrophoresis. This makes PCR amplification an essential tool for identifying and evaluating extracted fungal DNA from plant tissue.

There are a variety of compounds that can inhibit downstream processing of genetic samples. DNA isolation from plant tissues can be challenging due to the presence of secondary metabolites like polysaccharides or phenols present in the tissues, which will inhibit Taq polymerase action during PCR (Friar 2005). Deposition of melanin in the cell walls also significantly reduces the efficiency of extraction. This commonly occurs in older cultures, but may also present issues with extraction from naturally melanized or otherwise protected hyphae (Karakousis *et al.* 2006), as is the case with *Curvularia protuberata*. Since melanin has been seen to be amplified in cultures containing CThTV, this could be a significant obstacle. The issues posed by these contaminants can often be solved with effective DNA purification after extraction.

PCR inhibition can be reduced in a few different ways. Sample dilution is a simple yet effective method of improving DNA yield pre-PCR. (Radstrom *et al.* 2004). Betaine makes it easier to amplify sections of DNA with quantities of guanine and cytosine, which are more resistant to heat denaturation. Betaine also improves the thermal stability of proteins, can reduce false PCR negatives, and can reduce the effects of some types of contaminants (Weissensteiner and Lanchbury 1996). Aluminum ammonium

sulphate ($AlNH_4(SO_4)_2$) has been shown to be highly effective at removing PCR inhibitors from soil, without any significant DNA yield losses (Braid *et al.* 2003).

MOLECULAR METHODS FOR dsRNA VIRUSES

RNA extraction and subsequent PCR detection are one set of methods for detecting the presence of specific, non-symptom producing viruses like CThTV. Detection of viruses is made more complicated by their RNA based genomes.

RNA requires a different methodology for extraction, and processing than does DNA. Firstly, RNA is more easily broken down or altered than DNA, and most cells contain very high quantities of RNase, which will rapidly degrade sample RNA if not accounted for properly (Farrel 2005). Double stranded RNA (dsRNA) however, is resistant to endonucleases and can be expected to be reasonably well preserved during the extraction process (Diaz-ruiz and Kaper 1978).

There are 2 classes of RNA extraction methods. The first involves using a suite of chemicals to simultaneously disrupt the plasma membrane and organelles, while also neutralising the RNase released in the process of membrane lysis. The second technique works by solubilizing the plasma membrane while maintaining nuclear membrane integrity (Farrel 2005).

Barber (1966) was the first to extract dsRNA molecules, using chromatographic separation to pull out soluble RNA and leave ribosomal RNA behind. The addition of ethanol in specific concentrations relative to sodium chloride (NaCl) concentrations in the nucleic acid solution allows for non-ionic bonding to a cellulose column. Ribosomal RNA required lower ethanol concentrations for binding to the cellulose column.

Diaz-ruiz and Kaper (1978) used a simple salt fractionation method to extract dsRNA from plant tissue, but used lithium chloride (LiCl) instead of sodium chloride (NaCl). Viral nucleic acids and plant cellular nucleic acids have different solubilities in LiCl, which allows them to be separated even when extracted from the same tissue sample.

Krajacic *et al.* (2007) used a traditional phenol-chloroform extraction method, but followed up with a High-Performance Liquid Chromatography. The usage of 3-N,N-diethylamino-2-hydroxypropyl (DEAE) monoliths, a type of weak anion-exchange monolith produced by Convective Interaction Media, was shown to be a vast improvement upon the usage of cellulose columns for the purification process. The DEAE monolith method is faster, and doesn't require the use of gel electrophoresis to confirm the presence of dsRNA, nor does it require the use of DNAse.

Non-isotopic dot-blot hybridization with digoxigenin-labelled probes has also been shown to be an effective diagnostic method for viruses extracted from plant tissue (Olmos *et al.* 2007)

Standard PCR methods are only capable of amplifying segments of DNA, and will not work for RNA. However, extracted RNA can be amplified during PCR if it is converted into DNA first. This methodology is called RT-PCR, or Reverse Transcription PCR. Reverse transcriptases were discovered by Howard Temin, Satoshi Mizutani, David Baltimore in 1970 (Temin and Mizutani 1970; Baltimore 1970).

One methodology for amplifying dsRNA from viruses collected from tissues is to denature the dsRNA at 95°C in mixture with primers, water, and mineral oil. After denaturation, standard PCR buffers can be added, along with RNase inhibitors and

Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Zhang *et al.* 1998). Reverse transcriptase works by creating a strand of complementary DNA to the template RNA strands. From there, primers can be annealed to the newly synthesised DNA segments.

DISCUSSION

With an ideal outcome from these trials, and with widespread application, the effects of these inoculations would have dramatic effects on forest productivity, restoration efforts, and survival rates under a changing climate. Because CThTV targets the basic units of the stress response, it can be applicable to a wide number of tree species given a tailored vector, and can provide benefits under a wide variety of conditions. Any growing condition that results in oxidative stress (heat, cold, drought, heavy metal toxicity) can have its impacts mitigated with the help of this virus. Additionally, because the virus is non-pathogenic and doesn't integrate with its host genome, there are minimal risks to genetic safety associated with its use in uncontrolled growing spaces.

There are particular benefits to improving the survival of newly planted seedlings. The conditions of recently disturbed planting sites are harsh, with high sun exposure, unreliable water availability, and many seedlings also suffer from planting shock, which is a compound condition involving the physical stress of being transplanted in addition to the stress of adapting to an environment they were not grown in. As such, rates of seedling mortality are very high, and this issue is currently accommodated by the planting of more seedlings. If inoculation with CThTV can improve the growing conditions of newly planted seedlings to a high enough degree, this would not only improve the success rate of replanting, but may also allow forest managers to confidently prescribe lower planting numbers, thereby saving money and resources on a broad scale.

The degree to which the virus may improve tree survival rates under stressed conditions will influence the potential range of uses that these inoculated seedlings can be

used for. Even modest improvements to stress tolerance would improve growth traits in natural forest settings. With significant improvements, these seedlings could see uses in heavily contaminated mine sites, desertified ecosystems, areas with significant salt buildup, or even in urban environments.

There are a number of policies in place that regulate seed and seedling distribution in industrial settings. To plant inoculated seedlings in the wild or in a plantation, approval must be obtained from the Canadian Food Inspection Agency, under their legislation for "Plants with Novel Traits". This legislation outlines the application and approval process for both imported and native modified plants, in addition to procedures for managing non-compliance and other safety measures.

While the use of viruses in forestry would be a novel practice, there is significant research in other areas of biology, agriculture, and biotechnology that suggest that it is worthwhile to test these possibilities. A study like this would be valuable not just for expanding scientific knowledge on the nature of viruses, fungi, plant symbiosis and stress responses, but could have critical ecological and economic benefits.

CONCLUSION

The ability of CThTV to protect both its host and its host's host from baseline stress related damage makes it an exceptional tool in forestry. With adequate testing and success, this virus could provide much needed resilience for trees facing the effects of climate change. The success of this system would also allow for the expansion of regeneration projects in disturbed environments. The tools and knowledge are available to test this system, and with funding and support, answers could be found and advances made that would greatly improve forest resilience at large.

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