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Adopting Novel Cultural Practices for Managing Grapevine Diseases

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ABSTRACT OF THE DISSERTATION

Adopting Novel Cultural Practices for Managing Grapevine Diseases

by

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The adverse effects of grapevine diseases must be mitigated through innovative and improved cultural techniques that are cost-effective and environmentally friendly. In this thesis, I aim to assess the effect of pruning practices on pathobiome and mycobiome of asymptomatic grapevine using both culture-based and amplicon-based Illumina sequencing approaches. We hypothesized that the severe pruning of Guyot-Arcure increases esca disease severity and incidence and provides a gateway for higher pathogen load and microbial diversity compared to the minimal pruning of Guyot Poussard. We recorded over a 3-year period the number of symptomatic and asymptomatic vines for the two training systems, including the number of vines with esca foliar symptoms, partially unproductive, and dead vines. We also selected 6 asymptomatic vines from each pruning method and provided culturing and sequencing data from 27 samples per vine. Results showed that fungi in the Phaeomoniellaceae, Togniniaceae, and Botryosphaeriaceae were the most

frequently identified. Our data supported the hypothesis that severe pruning increased the risks of esca-pathogen infections caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. and shortened vine longevity. Results also indicated that severe pruning increased microbial diversity in vines and that the pruning methods influenced mycobiome community composition. This gain in knowledge improves the pruning practice guidelines and provides cost-effective solutions to manage GTD.

Innovative approaches that maximize crop output and quality while minimizing pesticide use are also required to attain environmental sustainability. Instead of using pesticides indiscriminately, agriculture can be supported by better pesticide management, yet ensuring that we can feed a growing population. With limited approaches to address the inefficiency of pesticide delivery, it is of special interest to explore how engineered nanomaterials can be used to target delivery of pesticides. In this research, an alternative targeted nano-technology delivery approach is tested using the grapevine and *Botrytis cinerea* pathosystem. Using surface functionalization of nanoparticles with biorecognition molecules of sucrose that can be scalable and low cost, nanoparticles were delivered to fungi in culture (*in vitro*) and to grapevine on leaves (*in vivo*). Confocal images showed delivery of naked GDCD (gadolinium doped carbon dot) and fully functionalized, sucrose coated-β-cyclodextrin gadolinium doped carbon dots (suc-β-GDCD) to plant and fungal structures. One direct application of nanotechnology is the targeted delivery of agrochemicals to distant plant biocomparments which could offer alternative

management strategies to certain diseases. We discuss the strengths and weaknesses of nanoparticle application in the context of our discoveries and provide a research plan on how to move forward.

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Introduction

Grapes (*Vitis vinifera)* provide fresh, dried, and processed fruit for table, wine, and juice production, and are an economically significant commodity worldwide (Fontaine et al., 2016). However, Grapevine Trunk Diseases (GTD) and Grey Mold, caused by several pathogenic fungi, are among the most devastating grapevine diseases, posing a significant economic and commodity loss in the grape industry (Fontaine et al., 2016). Grapevine Trunk Diseases (GTDs) alone cause an estimated $260 million annually in financial losses (Gubler et al., 2005). Grey Mold, which affects a broad range of fruits and vegetables, is reported to result in losses of up to $10-100 billion worldwide (Brito et al., 2021). These diseases have pernicious effects and are incurable but manageable. Therefore, critical steps toward supporting the profitability and sustainability of grape production are essential. Climate change is an additional pressure on viticulture, and there is an immediate need for these agro-solutions and technologies to be economically and environmentally sustainable (van Leeuwen et al., 2019).

GTD have been associated with 133 fungal species worldwide and involve four major grapevine trunk diseases: Esca, Eutypa dieback, Botryosphaeria dieback, and Phomopsis dieback (Gramaje et al., 2018). Although symptoms can be variable, diseased vines are usually marked by wood necrosis, the presence of brown streaking or cankers, discoloration, drying and stunting of foliage, and dead spurs/ cordons/ vines (Fontaine et al., 2016). Covert symptom development makes observation and monitoring difficult for young and mature vineyards. Since there

are no curative methods or treatments for GTD, these diseases are managed exclusively by preventative strategies, among which fungicide applications to protect pruning wounds have been the most effective (Rolshausen et al., 2010). There are substantial costs associated with cultivating grapevines, of which significant financial and human resources are allocated to pest and disease management programs. Those include cultural practices, such as pruning, and the cost of chemicals associated with the management practice, such as organic vs. conventional (Gramaje et al., 2018; Casolani et al., 2022); (Mondello et al., 2018). Since banning the use of sodium arsenite in Europe and other chemicals such as carbendazim and benomyl due to their negative impact on the environment and human health, there have been drastic changes in production methods that have created favorable conditions for fungal infection (Bruez et al., 2021; Gispert et al., 2020); (Andolfi et al., 2011; Larignon et al., 1997; Mondello et al., 2018; Gramaje et al., 2018). Among these methods are pruning strategies, defined as removing plant parts to obtain horticultural objectives (i.e., growing table grapes, high-quality cognac grapes, wine grapes, etc.).

In grapevine management, pruning objectives are to control the size & form of the grapevine, optimize the production potential, and maintain a balance between vegetative growth and fruiting. However, all GTDs are linked to several wood pathogens that can infect the vines through pruning wounds in the field (Mondello et al., 2018). Growers often start protecting pruning wounds with the appearance of first GTD symptoms when it is too late. Therefore, adopting

preventative strategies at the establishment of the vineyard to limit early infection events is key (Gispert et al., 2020; Kaplan et al., 2016; Gispert et al., 2020). Some strategies to manage GTD include pruning wound protection, training system, trunk renewal practices and sanitation (Fontaine et al., 2016; Mondello et al., 2018; Gramaje et al., 2011; Gramaje et al., 2018; Gispert et al., 2020). Many growers do not protect pruning wounds due to the management costs and lack of immediate visible effect of fungicide application because of the long incubation period from infection to disease symptoms appearance (Hillis et al., 2017). Thus, implementing alternative preventative strategies that are affordable and time-efficient would provide immense benefits to the viticulture industry.

Esca, a complex disease, is one of the most cosmopolitan and destructive of the four major Grapevine Trunk Diseases and is caused mostly by *Phaeomoniella chlamydospora* and *Phaeoacremonium species*. This ancient disease has seen a dramatic surge over the last two decades, and it is of major economic concern in Europe (Gramaje et al., 2018). It affects fresh crop marketability by blemishing the fruits with “black spots,” and can also affect the sugar content and grape flavors (Gramaje et al., 2018). Most importantly, it is pathogenic to woody tissues by producing an array of cell wall degrading enzymes, and phytoalexins that cause wood necrosis and cankers, leading to grapevine decline and shortening in longevity. A recent study indicated that severely pruned vines exhibit more decay and disease than minimal-pruned vines (Lecomte et al.,

2018, 2019). In the first objective of this thesis we will continue evaluating the effects of two different pruning strategies on Esca incidence and severity. To do this, we investigated the composition and localization of pathogens associated with extensive and minimal pruning practices. Understanding the diversity and composition of microbial communities, especially fungal communities, in vine wood as it is impacted by different cultural, pruning, and training practices may lead to improved management strategies.

Grey mold, Botrytis blight (Chang et al., 1997) or Botrytis Bunch Rot is caused by *B.cinerea*, a necrotrophic fungus that decay plant tissues. However, classification as a hemibiotroph has been proposed since during its short life cycle, it acts as a biotrophic organism, colonizing living plant tissues for nutrient acquisition (Cheung et al., 2020). At the start of the infection, when the fruit tissues are softer, darker circular discoloration occurs, and the mold is turning white to gray and is visible on the surface of both leaves and fruit (Williamson et al., 2007; Van Kan, 2005). The life cycle of B. cinerea includes different stages of sexual and asexual development (Fukumori et al., 2004; Cohrs et al., 2016). The sexual reproductive cycle of B. cinerea is initiated when it is subjected to unfavorable conditions (Fukumori et al., 2004; Romanazzi et al., 2014). *Botrytis cinerea* asexual conidiophores, and conidia travel through dry-wind or water droplets and attach themselves, via glycosylated proteins, to plant or fruit tissue (Cohrs et al., 2016; Roca-Couso et al., 2021). They enter the plant through natural openings or wounded tissue and begin to germinate (Cheung et al., 2020). Conidial

germination appears with a germ tube elongation from conidia, and later the development of appressoria (Cheung et al., 2020), a structure capable of penetrating the host plant tissues. *Botrytis cinerea* secretes cell wall degrading enzymes, which are necessary to penetrate the plant cell wall. Pectin methylesterase PME1, for example, breaks down pectin, a major component of a plant's cell wall (Cheung et al., 2020; Blanco-Ulate et al., 2016; Dalmais et al., 2011). Similarly, β-galactosidase helps to break down lactose within the cell wall into glucose and galactose to accelerate fruit softening and may provide carbon sources for fungal metabolism (Peng et al., 2021; Urbanek et al., 1984). *Botrytis cinerea* also breaks down the outer wax layer of the cuticle by secreting esterases which aid in the penetration of host tissue. Ultimately, plant cell death leads to disease proliferation and tissue maceration (Zhu et al., 2017). After adhering to the plant, B. cinerea must bypass the plant-secreted antifungal secondary metabolites that induce cell death in fungi (Vela-Corcía et al., 2019).

Conidia produced in the late winter or early spring are the primary source of infectious conidia in vineyards. In in-vitro experiments, the optimal temperature for B. cinerea sporulation was between 15-20°C, which begins about three days after inoculation (Ciliberti et al., 2016). In the vineyard, the minimum temperature for growth is 0°C (as seen in grape storage), the optimum is 20°C, and the maximum is 30°C (Oliveira et al., 2009; Romanazzi et al., 2014). Relative humidity of 90% has been shown to positively correlate with an increase in infection prevalence at temperatures ranging from 5-20oC, and this trend was also

observed in the days following rainfall (Ciliberti et al., 2016). Furthermore, it was shown that B. cinerea infection was significantly increased at the time of harvest when there was recorded rainfall in the days leading up to harvest time (Pertot et al., 2017). Because the fungus favors moderate temperatures and high humidity, in California, when rainfall is more common in late fall to winter, and herbaceous vegetative material is plentiful, infection risk is high (Broome 1995; McClellan et al., 1973).

Fungicides are commonly employed as chemical control measures. However, *B. cinerea* has been demonstrated to develop resistance to the most promising fungicides on the market. (Leroux et al., 2002; Fernández-Ortuño et al., 2015; Myresiotis et al., 2007; Shao et al., 2021). Shortly after mixed spray programs were developed to rotate active ingredients to limit the development of resistance. However, *B. cinerea* developed increased insensitivity to many combinations of fungicides, also called multi-drug resistance (Williamson et al., 2007). Another control method is resistance breeding, which may be the most socially accepted. Still, it ultimately leads to cultivating undesirable commercial traits like in grapevine species, where fruits develop thicker skins (Cheung et al., 2020; Herzog et al., 2015; Gabler et al., 2003). Cultural control methods for *B. cinerea* include eliminating potential sources of spores inoculum from decaying vegetation (Valdés-Gómez et al., 2008; Mundy et al., 2012). In addition, well-pruned canopies allow optimal air movement with reduced humidity and penetration of spray (Valdés-Gómez et al., 2008; Asao et al., 2019).

While pesticides boost crop yield and quality, they are also concerns to the environment and human health (Yadav et al., 2020; Rani et al., 2021). Pesticide overuse and indiscriminate application are particularly concerning because only a small percent of the millions of metric tons of these agrochemicals reach the intended biological target (Huang et al., 2020; Schreinemachers et al., 2020; Kalia et al., 2011; Dhawan et al., 2013). This makes agricultural practices one of the greatest pressures on the planet's natural resources resulting in groundwater, soil, and air pollution, deforestation, and increased greenhouse gas emissions (Oenema et al., 2001; Evans et al., 2019). In addition, agrochemicals build up in the environment and can enter the food chain through biomagnification, causing harm to life (Bhadouria et al., 2020; Kyei-Baffour et al., 1993). The key to addressing these inefficiencies is precisely delivering the agrochemicals to their intended targets in plants, where they will be most effective. New technologies and delivery strategies must be more efficient and attend to rotation schedules for IPM. This means precision technologies used to deliver pesticides must also be tunable, resilient against factors affecting pesticide behavior and breakdown, and able to be used in various application tools (An et al., 2022; Lowry et al., 2019).

Nanobiotechnologies are possible because of the nanoparticles' tunable physical and chemical properties (Heath 2015; Resham et al., 2015). In addition, some engineered nanomaterials can enable a higher delivery efficiency of chemical and biomolecular cargoes in plants (Baker et al., 2019). For example, the size and charge of nanomaterials can influence their foliar delivery efficiency to

plant cells and organelles, including stomata guard cells and chloroplasts (Avellan et al., 2021; Hu et al., 2020; Newkirk et al., 2021; Avila-Quezada et al., 2022). Recently, Santana et al., (2022, 2020), demonstrated that engineered nanomaterials could be led by a guiding peptide that targets chemical cargoes to plant organelles. Plant biorecognition approaches to target nanomaterials to plant-specific cells and organelles have been recently exploited for delivery to plant stomata, trichomes, and chloroplasts (Santana et al., 2020; Spielman-Sun et al., 2020). These advances are meaningful and pave the pathway for nanobiotechnology to better serve agricultural demands of disease mitigation. Strategies that allow for more precise control over biomolecule or agrochemical delivery to pathogens in the plant are needed. Because plants can absorb pesticides through leaves, nanotechnologies must then successfully enter the plant through the leaves and enter spaces that pathogens occupy (i.e., phloem, mesophyll) (Husted et al., 2023). Endocytosis has been comprehensively reported both *in vivo* and *in vitro* and appears to be the primary mode of action for NP in leaf uptake (Liu et al., 2009; Torney et al., 2007; Husted et al., 2023).

Most recently, unpublished research by Jeon et al., (2022), targeted the delivery of suc-β-GdCDs (sucrose coated-β-cyclodextrin CDs) to the plant phloem, a vascular tissue that transports sugars and signaling molecules. Functionalized surfaces of nanocarriers with sucrose enabled targeted delivery to the phloem, which enhances long-distance translocation. It was hypothesized that the chemical affinity of sucrose molecules to sugar membrane transporters on the phloem cells

enhances the uptake of sucrose-coated β-CDs. Results show the distribution of fluorescent chemical cargoes into the leaf vascular tissue *in vivo* was significantly improved by the suc-β-CDs and made it possible for targeted root nanoparticle delivery, with roughly 70% of phloem-loaded nanoparticles reaching these belowground root organs. Along with the use of β-cyclodextrin as a molecular basket, the use of sugars (i.e., sucrose) as a biorecognition is novel. Oparka (Oparka et al., 2000) reported functionalizing molecules with sucrose to enhance uptake into the phloem through sucrose-uptake transporters. Leveraging this mechanism would be practical use in nanobiotechnology, providing an opportunity to guide nanomaterials with agrochemical cargoes by plant biorecognition. In the second chapter of this thesis, I tested a novel agrochemical delivery platform for functionalized nanoparticles coated in sucrose (as a biorecognition molecule) to *Botrytis Cinerea*, the pathogen responsible for Grey Mold in grapevine.

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Chapter 1

Grapevine Pruning Strategy Affects Esca Disease Symptomatology, Wood Mycobiome and Pathobiome

Introduction

Grapevines are cultivated for their delicious fresh fruit, dried fruit, wine, and other spirits, driving production to over 77 million tons yearly and providing a $68 billion production value (Alston et al., 2019; Casolani et al., 2022). Grapevine Trunk Diseases (GTD) are a significant impediment to grape production globally (Bois et al., 2017; Gramaje et al., 2018). GTD are caused by a complex of taxonomically unrelated fungal pathogens, and vines are often compounded with mixed infections (Bertsch et al., 2013; Gramaje et al., 2018).

Among all the GTD, esca is considered a serious threat to European and Mediterranean vineyards (Lecomte et al., 2019). Surveys of French vineyards between 2003 and 2013 indicated that GTD incidence increased nationally from 3 to 13% with esca disease being a major concern (Lecomte et al., 2018). In addition to reducing vineyard longevity, esca affects the yield of productive vines and the quality of the fruit (Dewasme et al., 2022). Foliar symptom expression has been shown to be erratic, likely because of inconsistent precipitations amounts from year to year in the late spring to early summer (Dewasme et al., 2022; Kraus et al., 2019). Esca wood symptoms include black spots in cross-section that appear as

streaking in the longitudinal section but can also be surrounded with pink to brown wood discoloration and in older vines, white rot is also common (Mugnai et al., 1999; Surico, 2009). Foliar symptoms associated with this disease include tiger stripe leaf pattern, wilting, and apoplexy ranging from a few leaves to the entire canopy (Lecomte et al., 2018; Mugnai et al., 1999). The disease also manifests on fruits with the appearance of 'black measles' on berries (Mugnai et al., 1999). The Ascomycota *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum* (syn. *P. minimum*), and Basidiomycota *Fomitiporia mediterranea* are among the major causal agents of esca (Lecomte et al., 2018; Mugnai et al., 1999). However, recent studies that have deployed high throughput sequencing methods to profile the mycobiome (collection of fungal taxa) in symptomatic and asymptomatic esca affected vines, have identified several other GTD pathogens such as the causal agents of Eutypa dieback and Bot canker (Bruez et al., 2014; Del Frari et al., 2019) that could perhaps explain to some level the erratic expression of esca symptoms.

Trees and woody plants are also affected by the fungal species *Botryosphaeriaceae*. These include nut trees, pine trees, walnut trees, and even grapevines. The most common symptom of infection is cankers, where necrotic lesions appear on trunks, branches, and pruning wounds (Moral et al., 2019). The pathogen causes necrosis by cutting off the water supply to the leaves of the plant, causing chlorosis or the yellowing of leaves (Dong et al., 2021). The most common

Botryosphaeriaceae species reproduces asexually, and spores can remain dormant throughout unfavorable environmental conditions within diseased or necrotic tissue (Dong et al., 2021). The growth of *Botryosphaeriaceae* and the production of cankers on woody species is positively correlated to warm temperatures and high humidity, especially during rainfall. Because of this, pruning during rainfall is not advised in order to reduce wound size and the likelihood of cankers (Pitt et al., 2010; Sánchez-Hernández et al., 2002). Current chemical controls include QoI fungicides, which are widely accepted as being affected in preventing infection by dormant spores embedded within infected tissue/debris. However, efficacy depends on the plant species (Moral et al., 2019).

Wounds are the main gateway for GTD pathogens to infect vines. Thus, grapevines are especially susceptible to GTD infection during pruning because of the number of wounds made on a single vine. Wound susceptibility mainly depends on the time of pruning and the time lapse between pruning and infection event (Munkvold et al., 1995). Temperature and rain have shown to influence wound healing process and as a result the window of susceptibility as well as the quality and quantity of pathogen inoculum (Eskalen et al., 2007; Martínez-Diz et al., 2021). There are no curative methods or treatments for GTD, and these diseases are managed exclusively by preventative strategies among which fungicide application to protect pruning wounds have been the most effective

(Rolshausen et al., 2010). However, growers often start protecting pruning wounds with the appearance of first GTD symptoms when it is too late. Pruning wound treatments practices must be adopted early on, at the establishment of the vineyard to have yield benefits (Gispert et al., 2020); (Kaplan et al., 2016). The banning of sodium arsenite in Europe, that was long registered for protecting grapevine pruning wounds, left growers with no alternatives, and likely resulted in a surge of GTD incidence (Bruez et al., 2021). Alternatively, many growers do not protect pruning wounds at all due to the management costs and lack of immediate visible effect of fungicide application because of the incubation period from the infection point to the disease symptoms appearance (Hillis et al., 2017). Thus, implementing alternative preventative strategies that are cost affordable and time efficient would provide large benefits to the viticulture industry.

Vine training and pruning have been studied as a strategy to reduce GTD. In viticulture, pruning objectives are to balance vine productivity and fruit quality. Attaining this goal while reducing the number and size of pruning wounds would minimize the point of entry for vascular GTD pathogens. 'Vertical Shoot Positioned' system or 'VSP' that has been broadly adopted in all wine-growing regions globally, is considered an intensive pruning system that is more conducive to GTD compared to minimal pruning (Gubler et al., 2005; Kraus et al., 2019, 2022; Travadon et al., 2016). However, there are different types of VSP training systems.

The ’Guyot Poussard’ system, similar to cordon pruned vines, trains longer mature arms with few large cuts close to the main trunk and was reported to reduce esca disease (Lafon, 1921). In contrast, the ‘Guyot Simple’, similar to cane-pruned vines, trains one spur on one side each vine and of one cane on the other side but creates larger cuts close to the trunk head, and was described to be highly conducive of GTD (Lecomte et al., 2018). Following a survey from French vineyards, Lecomte et al., (2018) observed that vine training forms with long arms (cordons) decline less rapidly due to esca than training forms with no or short arms. However, those observations were not supported with studies in the United States (Gubler et al., 2005) and Australia (Henderson et al., 2021) that measured lower Eutypa dieback incidence and severity in cane- vs. cordon-pruned vineyards. Henderson et al., (2021) concluded that the spur pruning resulted in wounds that were individually smaller than those made by cane pruning, but the larger number of cuts made per vine resulted in a greater wound area per vine. Thus, limiting the total surface wound area per vine should be considered when pruning vines to limit GTD incidence. While all these comparative studies on pruning strategies have measured disease incidence and/or severity, only a few attempted to determine in what capacity they impacted the microbial community diversity and composition of the wood endosphere (Kraus et al., 2022; Travadon et al., 2016).

In this study, we conducted a comparative analysis of two pruning strategies, Guyot Arcure and Guyot Poussard, adopted in most of the Cognac vineyards of France. The goal was to evaluate, over a three-year period in a mature commercial vineyard (over 40 years old), the effect of vine pruning on esca symptomatology, including foliar and wood symptoms and vine death. In addition, we aimed at measuring how these pruning practices affected pathobiome and mycobiome using both culture-based and amplicon-based Illumina sequencing approaches. We hypothesized that the severe pruning of Guyot-Arcure increases esca disease severity and incidence and provides a gateway for higher pathogen load and microbial diversity compared to Guyot Poussard. The information generated from this study will help make educated recommendations to growers on practical ways to prevent GTD.

Materials and methods

Vineyard Characteristics

We selected two adjacent vineyards with contrasting training systems located near Cognac in the Charente region of southwestern France. Vineyards cv. 'Ugni Blanc' on 101-14 (41B) rootstocks were planted in 1972 and 1973 and trained as ‘Guyot-Arcure’ and ‘Guyot-Poussard’, respectively (See Supplemental Figure 1A). Vineyards were adjoining, and it can assumed that the soil and climate are similar

for both. Climate is those regions are temperate, relatively wet (average precipitation Sept. – Dec. ~19%-33%) and influenced by the vicinity of the Atlantic Ocean, with 4 marked seasons. The Guyot-Poussard training system consists of horizontal cordons with fewer small-to-medium-sized pruning wounds primarily concentrated on the top of the cordons, allowing the permanent bilateral flow of sap under the cordons of the vine. In contrast, the 'Guyot-Arcure' training system consists of V-shaped cordons that are often renewed and restored, leading to extensive pruning. The position of pruning wounds is not controlled and can disrupt a consistent sap flow that can lead to changes in sap routes (Lafon, 1921). The two vineyards were surveyed for three consecutive years from 2018-2020 and the number of asymptomatic vines and symptomatic vines for both foliar and wood symptoms were recorded, as well as the number of dead vines. A direct comparison between grapevine training systems and the incidence of symptomatic vs. asymptomatic grapevine trunk disease was done using a Chi-square statistical analysis. A total of twelve asymptomatic vines, with six of each training type, were selected for further assessment. Whole vines were uprooted in the dormant season, numbered, and stored in a cold room at 4 ° C while awaiting processing.

Image analysis of wood decay

The vines were cut longitudinally with an upright electric chainsaw and sectioned into several pieces for easy handling and photographed using a digital camera. The percentage of the necrotic surface was then evaluated from these photos using the Image J software Fiji version 2.14.0 (Schindelin et al., 2012) by calculating the ratio of the areas of the necrotic area to that of the total area of the vine (Supplemental Figure 2). To do this, the images were cleaned of impurities (e.g., markings made by the saw used to make longitudinal cuts) and the image was scaled to 5mm to standardize all images. To crop the image out of the background, each wood piece was traced manually, and this procedure was replicated three times for accuracy. Thereafter, a threshold for the necrotic regions was created (shown in red in Supplemental Figure 2), and additional tracing necrosis on the wood was manually performed as needed. A binary image was then created, resulting in black background and white marking for necrotic tissue, and used to calculate percent necrosis by dividing the necrotic area by the total area of the wood section. The percentage of the necrotic area was calculated for the trunk and both arms separately, then the arms and trunk were averaged. The analysis of the distribution of values is then carried out using nonparametric Kruskal-Wallis tests with the package using R software version 2.8.0 (Fox, Zadoks, and Gaskins 2005).

Grapevine processing

For each of the 12 grapevines, a total of 27 samples were collected that consisted of nine samples per cordon and trunk (Supplemental Figure 1B). The length of the trunk and the arms were measured and sampled at 20%, 50%, and 80% of the length of the cordons and trunk. For each spatial location, three wood samples were collected from the top, middle, and bottom sections of the cordon and the left, center, and right sides of the trunk (about 1 cm near the bark). Samples were collected with a disinfected wood chisel by sanitizing with 70% ethanol and heated between each cut. For each sample, approximately 2g of wood was collected for molecular biology and microbiological studies. Samples for molecular biology were stored at -20°C, and microbiology samples were processed within the same day.

Culture-dependent analysis

Wood samples (~3x5x2 mm) from all 27 data points on a vine were disinfected for 15 seconds with 3% calcium hypochlorite, rinsed with sterile water, and dried on filter paper. For each sample, the wood fragments were placed on a malt-agar medium (5 fragments per petri dish) and incubated at room temperature, and fungal development was observed over a six-week period. Taxonomic classification was done at the family level based on cultural and morphological characteristics for the Botryosphaeriaceae(Phillips et al., 2013), Diaporthaceae

(van Niekerk et al., 2005), Diatrypaceae (Trouillas and Gubler 2010), Nectriaceae (Chaverri et al., 2011; Gräfenhan et al., 2011), Phaeomoniellaceae (Chen et al., 2022)Togniniaceae (Gramaje et al., 2015) and the Basidiomycota *Fomitiporia* ("Basidiomycetous Pathogens on Grapevine: A New Species from Australia-Fomitiporia Australiensis" n.d.). The Identity of *Phaeoacremonium aleophilum* (Phaeomoniellaceae) and *Phaeomoniella chlamydospora* (Phaeomoniellaceae) was verified by PCR using primer pairs PalQr [CGTCATCCAAGATGCC-GAATAAAG] PalQf [CGGTGGGGTTTTTACGTCTA-CAG] and PchQr [CCATTGTAGCTGTTCCAAGATCAG]- PchQf [CTCTGGTGTGTAAGTTCAATC-GACTC], respectively, targeting the b-tubulin DNA region (Pouzoulet et al., 2013). The presence or absence of each fungal group was recorded for the 27 data points on a single vine. The distribution of the values of the number of fungal families recovered from the trunk and cordon samples was analyzed by ANOVA tests or nonparametric Kruskal-Wallis tests with the Rcmdr package of the R software version 2.8.0 (Fox, Zadoks, and Gaskins 2005). The statistical tests were carried out according to the training system (Arcure vs. Poussard) and was presented separately for trunk and cordons.

Culture-independent analysis

All 27 wood samples from a single vine were also flash-frozen in liquid nitrogen and ground to powder with MM300 grinder (Retsch, Haan, Germany). DNA was

extracted from 60 mg aliquots of wood powder using the Indvisorb Spin Plant Mini Kit (Eurobio, France) according to the manufacturer's instructions. The purity of the extracted DNA was evaluated with NanoDrop One (Thermo Fisher Scientific) and quantified with Qubit 2.0 (Thermo Fisher Scientific). Fungal ribosomal ITS region was amplified using the forward (AAAACT- TTCAACAACGGATC) and reverse (TYCCTACCTGATCCGAGGTC) GTAA primers designed by Morales-Crus et al., (2018). The 25-µl PCR reaction mix contained 1 ng of DNA template, Apex 2x Taq DNA Polymerase Master Mix solution (Genesee Scientific), 0.4 µM of each primer. PCR program (Veriti thermal cycler, Thermo Fisher Scientific) was as follows: initial denaturation at 95 °C for 3 min, followed by 37 cycles at 95 °C for 45s, 55 °C for 1 min., and 72 °C for 1 min., and a final extension at 72 °C for 10 min. Following PCR, amplicon size and uniqueness were verified using gel electrophoresis. The PCR products were cleaned using 1X Ampure XP magnetic beads (Agencourt, Beckman Coulter). DNA concentration was determined for each purified amplicon using Qubit 2.0 (Thermo Fisher Scientific). For the single isolate validation, amplicons were sequenced with Sanger (DNA Sequencing Facility, University of California, Davis). For high-throughput sequencing, equimolar amounts of all barcoded amplicons were pooled into a single sample, the total concentration of which was determined. Five hundred nanograms of pooled DNA were then end-repaired, A-tailed and single-index adapter ligated

(Kapa LTP library prep kit, Kapa Biosystems). After adapter ligation, the library was finished with two consecutive 1X Ampure XP magnetic beads (Agencourt, Beckman Coulter) cleanups. The size distribution of the library was determined with the Bioanalyzer (Agilent Technologies) and submitted for sequencing in 250-bp paired-end mode on an Illumina MiSeq (UC Davis, Genome Center DNA Technologies Core).

Computational analysis

Trimmomatic v 0.39 was used to initially clean the sequencing reads with a sliding window of 4:19 and a minimum length of 150. The R v4.1.2 (Team 2021) was used to perform all computational analysis. Most processing for the reads was done in DADA2 v 1.16.0 (Callahan et al., 2016), including further quality control sequencing filtering, dereplication, chimera identification, merging paired-end reads, and construction of ASV (Amplicon Sequence Variant) tables. Taxonomy identification was assigned using the Unite database v 10.5.2021 for fungal taxa. Phyloseq v 1.36.0 (McMurdie and Holmes 2013) and ggplot2 v3.3.5 packages (Wickham 2009) were used for much of the graphical and statistical analyses of the data. After removing poor quality reads and unassigned taxa, the fungal dataset totaled 173 samples (of the possible 324= 2 pruning types x 6 vines x 27 samples per vine), including 85 for Arcure- [Arm20 = 20 samples; Arm50 = 18 samples; Arm80 = 19 samples; Trunk20 = 12 samples; Trunk50= 8 samples;

Trunk80= 8 samples] and 88 for Poussard- [Arm20 = 15 samples; Arm50= 15 samples; Arm80= 21 samples; Trunk20= 12 samples; Trunk 50= 14 samples; Trunk80 = 11 samples] pruned vines. Unidentified microbes at the kingdom level were removed. After filtering the total ASVs were 1267. Alpha diversity was measured by observed taxa within the communities. Poisson generalized linear modeling with ad hoc Tukey was used to verify statistical differences among groups. Family bar charts were constructed by aggregating taxa at the family level. Samples were also constructed by tissue compartments and transformed to relative abundance. Bray–Curtis dissimilarity was used to calculate the compositional dissimilarities between samples. These dissimilarities were visualized with NMDS (Non-metric Multi-Dimenstional Scaling) plots using the Vegan package v 2.5-7. The Adonis test was run to determine the statistical significance of beta diversity.

Results

The two vineyard blocks were 45 years old in the first year of the survey and showed a high incidence of grapevine trunk diseases that was worsening each year of the survey (Table 1). Our results highlighted how the two training systems affected disease incidence and severity. Arcure-pruned vines displayed a statistically higher percentage of vines symptomatic for GTD in comparison to Poussard-pruned vines for all three years on record ($P< 0.0001$). ImageJ analysis

of the ratio of the necrotic area to that of the total wood area of the vine indicated a high percentage of wood decay in asymptomatic vines (~80%) regardless of the training system.

Our results indicated that pruning methods affected microbial diversity richness (Fig.1) and community composition (Fig.2). Alpha diversity plots showed a greater observed microbial diversity in trunks of Arcure- vines vs. Poussard-pruned vines [Poisson generalized linear model with Tukey; P<0.001], with trend indicating greater diversity near the head of the trunk. In arms, microbial diversity difference between the two pruning types was only noticed in section close to the trunk (arm 20) with Arcure displaying higher taxa richness. Pruning practice type also affected fungal community composition in both arm and trunk (Adonis test P< 0.001).

Table 1.1 Vine training (Arcure vs. Poussard) affects grapevine trunk disease incidence and severity.

Vineyard Training System	**Survey Year**	**Percent Asymptomatic Vines**	**Percent Symptomatic Vines**			
			Vines with Esca Foliar Symptoms[3]	Partially Unproductive Vines[4]	Unproductive Vines[5]	**Total**
Arcure[1]	2018	**42.5**	1.6	14.7	41.3	**57.5**
	2019	**40.1**	1.2	17.8	40.9	**59.9**
	2020	**38.7**	2.9	16.2	42.1	**61.3**
Poussard[2]	2018	**70.4**	0.7	14.9	14	**29.6**
	2019	**66.9**	0.4	18.6	14	**33.1**
	2020	**65.9**	1.7	17.9	14.5	**34.1**

[1]511 total vines; [2]692 total vines; [3]No other foliar symptoms recorded (e.g., Eutypa); [4]re-trained vines, one arm missing, one dead-arm; [5]dead or missing.

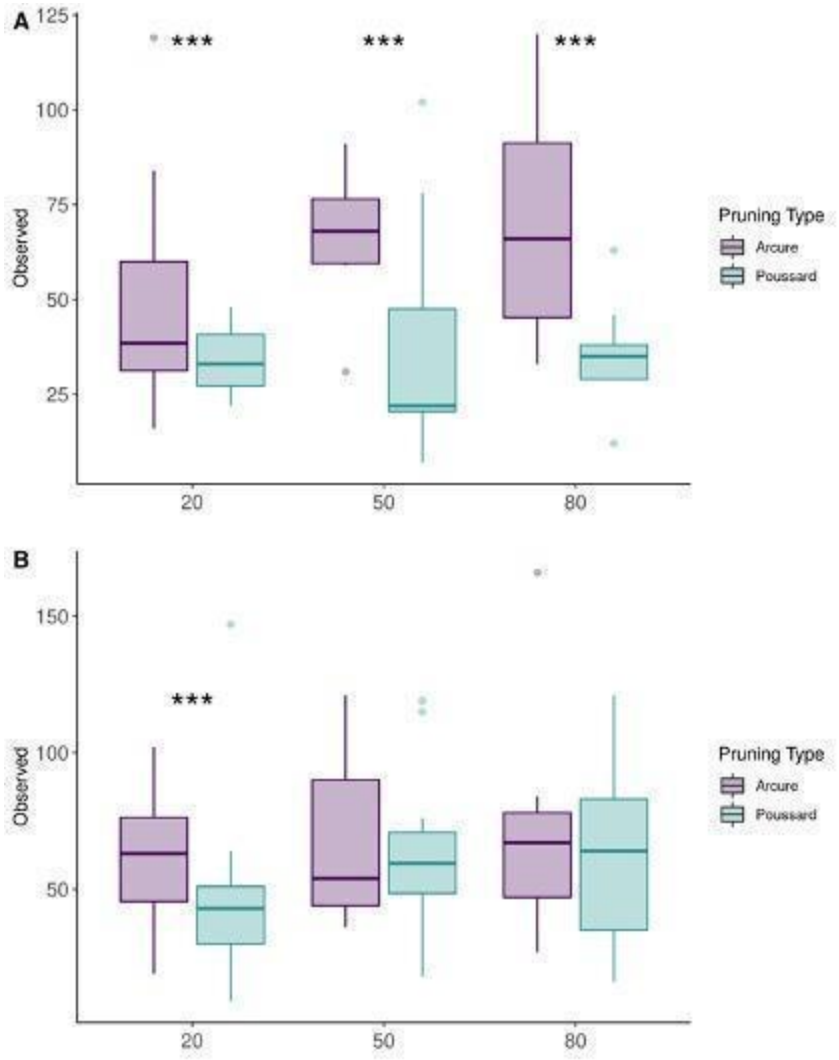

Figure 1.1 Alpha diversity plots indicating that microbial richness is affected by pruning practice. Bar plots represent observed diversity at the location on the vine (20%, 50% and 80%) on trunk (A) and arm (B). Statistical significance is indicated for $P < 0.001$ (***) based on Poisson generalized linear model with a pairwise Tukey test. Arcure: Arm20 = 20 samples; Arm50 = 18 samples; Arm80 = 19 samples; Trunk20 = 12 samples; Trunk50= 8 samples; Trunk80= 8 samples. Poussard: Arm20 = 15 samples; Arm50= 15 samples; Arm80= 21 samples; Trunk20= 12 samples; Trunk 50= 14 samples; Trunk80 = 11 samples

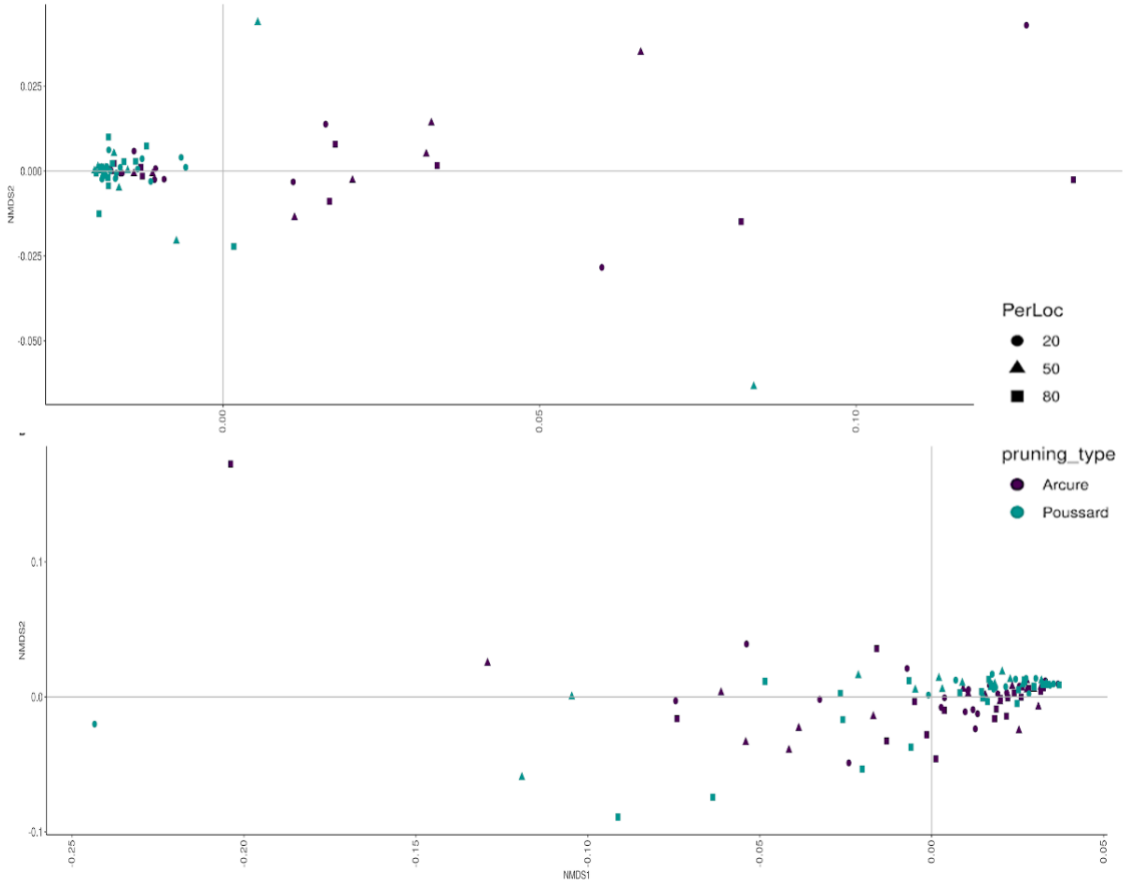

Figure 1.2 Bray Curtis beta diversity plots indicating that fungal beta diversity is significantly affected by pruning practice. Each dot represents the fungal community composition of a single vine. Points are colored by each pruning type (Arcure vs. Poussard) and shaped by the sampling location on the vine (20%, 50% and 80%) on trunk (A) and arm (B). Significant statistical P and R^2 values were measured by Adonis permutational multivariate analysis of variance for trunk (P=0.001 R^2=0.15) and arm (P=0.001 R^2=0.0865). Arcure: Arm = 57 samples; Trunk = 28 samples. Poussard: Arm = 51 samples; Trunk = 37 samples.

Microbial isolations from the 324 data points across the 12 grapevines (27 datapoints per grapevine) indicated that percent recovery was the highest for fungi belonging to the families Botryosphaeriaceae, Phaeomoniellaceae and

Togniniaceae with 42.9%, 44.8% and 31.5% respectively. The fourth most recovered pathogenic group belonged to the family Nectriaceae (13%) but incidence of other pathogenic groups including Diatrypaceae (1.2%) and Diaporthaceae (0.6%) was very low in comparison. *Fomitiporia* was only isolated from one trunk sample and one arm sample of Arcure-pruned vines. Arcure-pruned vines displayed significantly higher incidence of esca-causing fungi (*Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*) in both arm and trunk in comparison to Poussard-pruned vines (Fig.3). Similarly, Botryosphaeriaceae percent recovery was also significantly higher in trunk of Arcure- vs Poussard-pruned vines whereas the opposite was significantly measured in arms.

Percent relative abundance supported isolation data with the three main fungal families representing 81.6% in arms and 77.8% in trunks of all taxonomic groups in Arcure-pruned vines in comparison to 74.4% in arms and 71% in trunks of Poussard-pruned vines (See Supplemental Figure 3A). Of those, Phaeomoniellaceae was the most dominant family with over 60% abundance with both pruning methods, albeit higher for Arcure- vs. Poussard-pruned vines (See Supplemental Figure 3B), followed by Togniniaceae (~12%), Herpotrichiellaceae (~4%) and Botryosphaeriaceae (~2%).

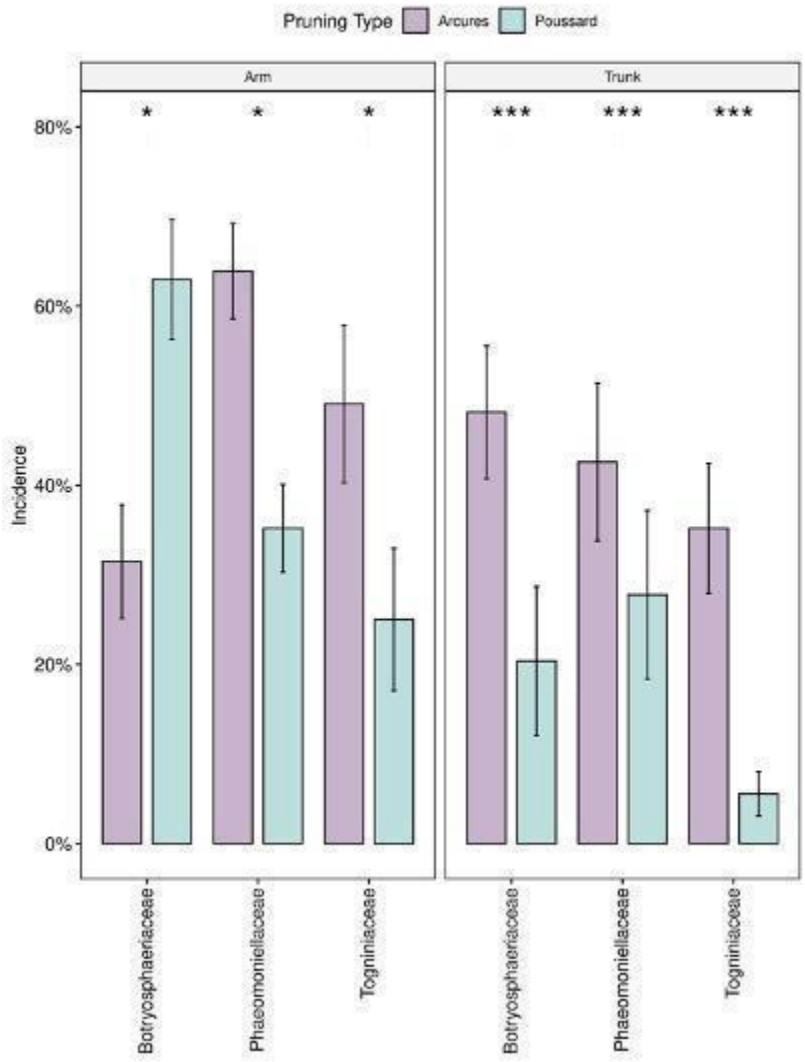

Figure 1.3 Statistical difference in percent recovery for Phaeomoniellaceae, Togniniaceae, and Botryosphaeriaceae fungi between Arcure- and Poussard-pruned vines in both arm (left panel; 6 grapevine replicates with 2 arms per vine and 9 data point per arm; n= 108) and trunk (right panel; 6 grapevine replicates with 1 trunk per vine and 9 data point per trunk; n= 54). Standard errors are shown on the bar graph and statistical P values are indicated with asterisks (* P< 0.5; *** P< 0.001).

Discussion

This study was designed to gain further knowledge on how pruning practices affects the incidence and severity of GTD. In addition, we evaluated in what capacity pruning strategies spatially affected the composition and diversity of the mycobiome in asymptomatic grapevine and its pathobiome profile. The incidence of GTD-foliar symptoms in the surveyed vineyards was overall low for all three years, even though vines showed extensive wood decay, regardless of the pruning strategy. Leaf stripe symptoms were indicative of esca disease, which was confirmed with culture-dependent and independent diagnosis. Esca has been identified as the major threat to vineyards across Mediterranean climates (Guerin-Dubrana et al., 2019; Lecomte et al., 2018).

Community composition analysis from non-symptomatic vines indicated that GTD-pathogens dominated the wood mycobiome, supporting previous data (Geiger et al., 2022). Fungi in the Phaeomoniellaceae (i.e., *Phaeomoniella chlamydospora*) and Togniniaceae (*Phaeoacremonium aleophilum*) were the overwhelmingly dominant members of the wood mycobiome and pathobiome with 60% and 12% in relative abundance, respectively. Profiling of the wood microbiome affected by GTD and esca using high throughput sequencing methods revealed that *Phaeomoniella chlamydospora* is a dominant member in many viticulture areas (Del Frari et al., 2019; Geiger et al., 2022; Kraus et al., 2022; Morales-Cruz et al., 2018; Niem et al., 2020; Vanga et al., 2022). However,

Phaeoacremonium aleophilum was not always the second most prevalent pathogen reported in esca-affected vineyards, as *Fomitiporia mediterranea* was often detected. The GTAA primers that were used in our study do not capture Basidiomycota fungi and are only specific to the Ascomycota phylum (Morales-Cruz et al., 2018). Nonetheless, the presence of white rot and *Fomitiporia* was very low in our vineyard sites based on wood observation and isolation results, even though grapevines were old with presence of heartwood. Sequencing data indicated that fungi in the Herpotrichiellaceae were the third most abundant taxonomic group but suspected grapevine pathogens within that family (*Phialophora, Exophiala*) were not reisolated from grapevines perhaps due to their slow growing nature or that other non-pathogenic represented this group. Botryosphaeriaceae (*Neofusicoccum*, *Diplodia*) were the fourth most abundant pathogenic fungi identified, although with a disparity between the low relative abundance and the high recovery rates from wood samples because of the fast-growing ability of these fungi in culture. Fungal species within this family cause bot canker in a broad host range and have been associated with esca disease in several studies (Bruez et al., 2014; Geiger et al., 2022; Kraus et al., 2022; Lecomte et al., 2018). Several other pathogenic fungi belonging to the Diatrypaeae (*Eutypa*), Diaporthaceae (*Diaporthe*) and Nectriaceae (*Fusarium*) were also identified but at low incidence and abundance and, as such, only appeared to play

a marginal role in the decline of the vineyard surveyed. Overall, these results support that the esca pathobiome showed similar profile to previous report and that its assembly in the Cognac region was likely driven by regional factors that include biogeography and cultivar (Bekris et al., 2022).

Efficient management of GTD in vineyards is done essentially by early adoption of preventative measures (Gispert et al., 2020; Kaplan et al., 2016). Post-pruning fungicide treatments is viewed as the most effective practice mainly because the causal agents are airborne with free water and infect vines through wounds (Rolshausen et al., 2010). Adjusting the timing of pruning during dry weather conditions when disease inoculum is low and/or when the period of susceptibility of wounds is narrowed under warmer temperature, is also recommended (Martinez-Diz et al., 2020; Munkvold and Marois 1995). However, those strategies are not always practical because those weather conditions are not always met at pruning time or in sync with the availability of the field labor.

Vine training and pruning practices have also been investigated as a way to manage GTD. Evidence suggests that severe pruning with high numbers of cuts and large wound size, increased GTD incidence and severity (Gu et al., 2005; Lecomte et al., 2018). Although, according to Henderson et al., (2021), the severity of pruning is better defined by the total surface area of pruning cuts per vine, which encounters both the number and size of wounds per vine. Incidence esca disease

(number of symptomatic vines) and severity (extent of wood decay) were clearly reduced after commercial vineyards in Germany and France were converted from an intensive pruning training system such as vertical spur position VSP to a minimal pruning strategy (Kraus et al., 2022; Kraus et al., 2019; Travadon et al., 2016). However, outcomes were only significant when adopting these practices occurred early in the life of the vineyards (Kraus et al., 2022). Our results supported those findings and showed a decrease in vine symptomology by 45% and vine mortality by 75% in Poussard-pruned grapevines because of the significant reduction of *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* incidence from both trunk and arms tissues, and Botryosphaeriace incidence in trunks. Interestingly, Botryosphaeriaceae infections were only lower in the arm Arcure-pruned vines, perhaps highlighting contrast in diseases etiology whereby frequent pruning in Arcure vines removed latent Bot infection while increasing entry points for esca pathogens. However, pruning methods did not affect the internal wood decay with all asymptomatic vines showing about 80% of necrosis in trunks and arms and perhaps differences in the extend of wood decay between pruning practices would be better observed in younger vines. The Guyot-Poussard pruning has been described to minimize the interruption of sap flow feeding foliage, whereas Guyot-Arcures pruning induces interruption of sap routes which causes xylem vessel occlusion and loss of physiological function of the host vasculature,

thereby stressing vines and supporting esca-pathogen colonization (Lecomte et al., 2018). Together, these results suggest that pruning methods that minimize the wound surface area per vine and preserve the integrity of continuous sap routes minimize the risks of GTD.

Our results also indicated that the pruning strategy affected fungal community diversity and composition of asymptomatic vines. Two studies from France (Travadon et al., 2016) and Germany (Kraus et al., 2022)compared minimal to spur pruning strategies in two cultivars and yielded inconsistent outcomes. Significant effects of pruning strategies on fungal community diversity and composition were inconsistent, and only found on one of the two cultivars (Syrah) from France. It was suggested that fungal abundance and diversity are driven by both cultivar susceptibility to wood-infecting fungi and the severity of pruning (Travadon et al., 2016). However, in both studies, all the vineyards were converted from spur pruning to minimal pruning after several years, which certainly confounded microbial composition analyses. Assemblage of the core endophytic microbiome in the perennial wood of grapevine is driven by several factors including aboveground wound colonization (Deyett and Rolshausen 2020; Martinez-Diz et al., 2020). Pruning methods have clearly shown to influence the

GTD pathobiome and disease outcomes as previously discussed. One would expect that it also influences the entire mycobiome as suggested by our data, although additional experiments should validate these findings.

In conclusion, our data support the current knowledge that severe pruning increasing the risks of GTD pathogen infections and shortening of vine longevity and vineyard productivity. Additional comparative studies between intensive and minimal pruning should be pursued overtime, starting at the establishment of the vineyard, to better understand the long-term effect of vine training systems on wood endophytic microbiome assembly dynamics and pathobiome profiles. Moreover, attention should be paid not only to the severity of the pruning with respect to the surface wound area per vine but also the quality of the pruning and how it impacts xylem integrity and sap flow routes. This gain in knowledge will improve recommendations to growers for practical ways to manage GTD in a cost-effective manner.

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Chapter 2

Targeted Delivery of Functionalized Nanoparticles to *Botrytis cinerea* in Grapevines

Introduction

Propelled by climate change, food security is a growing global threat, and within recent years, fruit and vegetable production has been compromised by extreme weather patterns (Branca et al., 2013; Gregory et al., 2005; Hasegawa et al., 2021). Environmental crises challenge the world's fruit production, with yields of grapes and other soft fruit at risk of decreasing by almost a third (Alae-Carew et al., 2020). Globally grapes are an economically important commodity and the highest-value fruit crop grown in the U.S., supplying fresh, dried, and processed fruit for table, wine, and juice production (Choi et al., 2015; Williams et al., 2018). However, a major impediment to grape production is the impact caused by punitive fungal pathogens. The pathogen Botrytis *Cinerea* is responsible for severe losses in economically important crops, including grapes, causing annual losses worldwide that surpass $10 billion (Roca-Couso et al., 2021).

The demand for more effective tools, technologies, methods, and processes that provide larger yields and higher quality, while reducing time, money and harming the environment is critical for agricultural productivity (McNamara, 2009). While pesticides boost crop yield and quality, they also traumatize the environment and human health (White, 1988; Zacharia, 2011). Pesticide overuse and indiscriminate application are particularly concerning

because only a small percent of the millions of metric tons of these agrochemicals reach the intended biological target (Schreinemachers et al., 2020); (Huang et al., 2020); (Bustamante et al., 2008); (van der Werf, 1996). This makes agricultural practices one of the greatest pressures on the planet's natural resources resulting in groundwater, soil, and air pollution, deforestation, and increased greenhouse gas emissions (Johnson et al., 2007); (Giannadaki et al., 2018). In addition, agrochemicals build up in the environment and can enter the food chain through biomagnification, causing harm to life (Cucurachi et al., 2019); (Kyei-Baffour et al., 1993). The key to addressing these inefficiencies is the ability to precisely deliver the agrochemicals to their intended targets in plants, where they will be most effective.

Over the past decade, nanotechnologies have emerged as tools to meet agricultural demands and provide opportunities for much-needed advancements in the agrochemical industry (Hofmann et al., 2020). Evolving agrochemical solutions include nano-fertilizers, antioxidative nano-enzymes, nano-pesticides, synthetic hormones, and nano-fungicides (Saritha et al., 2022). Other related technologies include nano-biosensors, seed nanopriming, genetic engineering, and nanobiofortification (Saritha et al., 2022). However, in plants, nano-agrochemicals and the interaction between nanoparticles (NPs) and fungi are often researched in the context of characterizing the antifungal properties of nanoparticles (Su et al., 2019).

Alluding data shows that delivery of NPs to fungi is practical but lacks proper technology and application. Quantum dots have been used to demonstrate the direct uptake of organic nitrogen in fungi (Whiteside et al., 2019). Poly(lactic-co-glycolic) acid (PLGA)-based non-toxic NPs are considered to hold considerable potential as drug carriers and were tested in *Vitis vinifera* cell cultures and grapevine-pathogenic fungi, *B. cinerea*, *A. niger*, and *A. carbonarius* mycelia (Cao et al., 2016; Grune et al., 2021). Their results suggest that PLGA NPs could be used to deliver antifungal compounds within fungal cells (Chronopoulou et al., 2019). Combined, these types of research efforts demonstrate gaps and the potential of nanotechnologies to mitigate the impacts of fungal pathogens.

One of the most dangerous pre- and post-harvest pathogens in fresh fruits and vegetables is *Botrytris cinerea*, a phytopathogenic fungus that causes Botrytis blight, Botrytis Bunch Rot, and other diseases (Zhang et al., 2014). It has a broad host range with a wide range of symptoms. The most notable symptom caused by *B. cinerea* infection is the observable growth of gray mold on the surface of both leaves and fruit. Over the past 50 years, chemical controls in the form of fungicides have been utilized to minimize the losses caused by *B. cinerea*. However, after repeated use of fungicides, *B. cinerea* has been shown to develop resistance to the market's most promising fungicides (Fernández-Ortuño et al., 2015; Leroux et al., 2002; Myresiotis et al., 2007; Shao et al., 2021)**.**

The tunability of the physical and chemical properties of nanoparticles makes nanobiotechnology possible (Rastogi et al., 2019; Sabir et al., 2014). In

addition, some engineered nanomaterials can enable a more efficient delivery of chemical and biomolecular cargoes in plants (Cunningham et al., 2018; Li et al., 2021). Properties such as the charge and size of nanomaterials can influence their delivery efficiency to plant cells and organelles, including stomata guard cells and chloroplasts (Gan et al., 2005; Hu et al., 2020; Vargas-Hernandez et al., 2020; Wu et al., 2022). Recently, Santana et al., have demonstrated that engineered nanomaterials can be led by a guiding peptide that targets chemical cargoes to plant organelles (Santana et al., 2020, 2022). Plant biorecognition approaches to target nanomaterials to plant-specific cells and organelles have been recently exploited for delivery to plant stomata, trichomes, and chloroplasts (Santana et al., 2020; Spielman-Sun et al., 2020). These advances are meaningful and pave the pathway for nanobiotechnology to better serve agricultural demands of disease mitigation. Optimizing the delivery of pesticides to crops, will address the inefficiencies of current agrochemical delivery.

The use of mechanical sprayers is one of the most popular ways to apply pesticides, especially in traditional agriculture (Das et al., 2015). Unfortunately, only 1% of pesticides sprayed reach the pathogen/pest (Das et al., 2015). Consequently, strategies that allow for more precise control over biomolecule or agrochemical delivery to pathogens in plants is needed. Because pesticides can be absorbed by plants through leaves, nanotechnologies must then be able to successfully enter the plant, though the leaves and enter spaces that pathogens occupy, (i.e., phloem, mesophyll) (Husted et al., 2023). Endocytosis has been

comprehensively reported both *in vivo* and *in vitro* and appears to be the primary mode of action for NP in leaf uptake (Husted et al., 2023; Liu et al., 2009; Torney et al., 2007).

Most recently, unpublished research conducted by Jeon et al. (2022), targeted the delivery of suc-β-GdCDs (sucrose coated-β-cyclodextrin CDs) to the plant phloem, a vascular tissue that transports systemically sugars and signaling molecules. It was hypothesized that the chemical affinity of sucrose molecules to sugar membrane transporters on the phloem cells enhances the uptake of sucrose-coated β-CDs. Results show that the distribution of fluorescent chemical cargoes into the leaf vascular tissue was significantly improved by the suc-β-CDs and made possible targeted root nanoparticle delivery, with roughly 70% of phloem-loaded nanoparticles reaching these belowground root organs. Coating sugars (i.e., sucrose) as a biorecognition onto CDs that carry β-cyclodextrin as a molecular basket, is novel. Although sucrose has been reported in the development of novel nanoparticles, it is often used as a step within synthesis and not as a biorecognition molecule to interact with fungi (Ghouri et al., 2023; Jimenez-Falcao et al., 2021). Fungi-specific interactions using nanomaterials carrying biorecognition molecules such as sucrose or glucose have not been studied (Sharma et al., 2017).

In this research, we aim to enable a pathway towards precision application of pesticides. To do this, we synthesize, and test functionalized beta-cyclodextrin carbon dots coated with sucrose, as a system that can efficiently target *B. cinerea*

structures in the leaves of young grapevines. We test the system *in vitro* using different spore counts of *B. cinerea* expressing GFP , and *in vivo* using detached leaf assays. We assess targeted delivery by imaging the fluorescence of suc-β-GdCDs in fungi and measure delivery efficiency via colocalization. Taking advantage of sucrose as a long transport molecule, and sucrose transport pathways, we hypothesize that *B.cinerea* will preferentially uptake suc-β-GdCDs, over β-GdCDs.

Emerging uses of nanobiotechnologies have the potential to bring innovative manufacturing techniques and goods for various industries, from agriculture to medicine to defense. Although significant funding has been given to nanotechnology research by government organizations, public and private research centers, and universities to realize the full potential of the field, the acceptability of nanotechnology and the tangible outcomes of its application across a range of application sectors is not sufficiently addressed (Brossard et al., 2009; Roco et al., 2005; Salerno et al., 2008).

Materials and Methods

Grapevine Plant Growth in Growth Chambers

Grapevines cultivar Sauvignon Blanc were grown in growth chambers under white LED lights of 30% Blue light, 35% Green light, 30% Red light, and 5% FR. To optimize grapevine growth within optimal growth conditions for *Botrytis cinerea*, the growth chamber settings were set at 21 ± 1°C during the day and 18± 1 °C during the night, with 12-hour light for each period and 90% relative humidity. Plants were grown in 1 gal nursery pots filled with UCR soil.

Fungal Growth Conditions

Botrytis cinerea isolate B05.10 (Schumacher, 2012) labeled with and without GFP was grown and cultured on potato dextrose agar (PDA) medium (Diego-Nava et al., 2023) containing Plant Preservative Mixture™,. *B. cinerea* conidia were taken from a glycerol stock; 20uL of glycerol stock solution was dropped on the PDA media, and a cell spreader was used to disperse the *Botrytis* spores. The Petri dishes containing *B. cinerea* glycerol stock solution were sealed using parafilm and stored in an incubator at 24^{o}C for 10-14 days. Plates were not stacked to ensure equal distribution of light.

Bioassays

A healthy 10-day-old culture of either wildtype or GFP-labeled *B. cinerea* was used for spore suspension. In a laminar flow hood, a sterile blunt-ended spatula was used to scrape 2mL of mycelium and conidiophores containing conidia. These structures were transferred to an Eppendorf tube, and 1% SMB media was added and shaken to produce a spore suspension. For leaf inoculations, a concentration of 10^6 spores/mL was used. For plate bio-assays, the initial spore concentration used was 100 spores/uL and then diluted to obtain 10 - 50 spores per uL per experimental run. pore suspension concentration calculated with a hemocytometer under a light microscope at 20x magnification.

Young healthy leaves were selected for spore inoculation And 5mL of a spore suspension was gently pipetted on the adaxial surface of the undetached grapevine leaf on both sides of the main midrib and allowed to grow for a 96h growth period at 25^{o}C. For the detached leaf assay, young healthy leaves were carefully detached from the mainstem at the node. Before inoculation, leaves were sterilized in the biohood by spraying 70% ethanol and allowed to sit for 30 seconds. Then they were sterilized using 1% bleach and allowed to sit for 1 minute. The detached leaves were pat dry with Kimtech wipes and immediately placed on a PDA Petri dish with the petiole embedded in the culture medium. Following spore inoculation, the Petri dishes containing the detached leaf and spore solution were sealed with micropore tape and allowed to grow at 25^{o}C for 96h (Figure 3.2.A-C).

Nanoparticle Solution and Inoculation

The working stock for the nanoparticle (NP) with Silwet was made up of GdCDs and Suc-β-GdCD at a concentration of 0.1mg/mL and varying concentrations of Silwet surfactant at 0.1%, 0.2%, and 0.5%. The NP stock solution was made by combining 20µL of NP stock solution with a concentration of 0.1mg/mL and 80µL of 100 mM TES buffer (pH=6). A 1.5µL NP working solution was inoculated to the abaxial surface of healthy grapevine leaves and infected grapevine leaves or to *B. cinerea* spores. Figure (3.2.A-C).

Confocal Fluorescent Microscopy of NPs in Grapevine Leaves

Both leaf and *in vitro* bioassays were imaged using the Zeiss inverted confocal microscope after a 3h incubation with the respective nanoparticles and Silwet concentrations. Microscope mounting slides were used to mount leaf samples and were prepared by placing a 2 cm wide circle of mounting gel that was pressed to 2 mm in thickness. A 15 mm cork borer was used to create a crater in the center of the mounting gel, where the circular leaf sample was placed for imaging. A 5 mm cork borer was used to excise a circular leaf sample near the NP inoculation site and placed on the mounting slide inside the crater created with the mounting gel with the abaxial side facing up. A 200 uL of Perfluorodecalin was pipetted on the leaf sample and covered with a cover slip. The Zeiss confocal microscope settings were as follows: 20x, 40xW, and 40xOil objectives were used; 355nm laser excitation for NPs at 1.5 intensity and 180 pinhole size; 633nm laser excitation for chlorophyll at 1.5 intensity and 180

pinhole size; 355nm laser excitation for GFP at 2.0 intensity and 120 pinhole size; z-stack section thickness = 2.0µm. The DAPI channel was used for NP detection with the detection range set at 410nm-570nm; the EGFP channel was set at 500-570nm for GFP-*Botrytis cinerea* detection; and chlorophyll was set at 610-750nm for chlorophyll autofluorescence. All images obtained were processed and analyzed using FIJI software.

Imaging GFP-Botrytis Spore Slides

An 18-well µ-slide designed for inverted fluorescent microscopy was used to grow 10, 50, and 100 GFP-*Botrytis cinerea* spores in 30µL well. Two growth periods, 48h and 72h, were tested to identify the optimal growth time for healthy fungal growth. After the optimal conditions for spore germination were established, 10µL of GdCD, SucGdCD, or Suc-*β*-GdCD with a concentration of 0.1mg/mL in 0.2% Silwet solution was placed in each well and incubated for 3h. After allotted growth periods, spores were imaged using a Zeiss Inverted confocal microscope using the EGFP channel set at a 500-570nm detection range (Figure 2.1.).

Statistical analysis

To obtain and evaluate colocalization measurements obtained between treatments we used the JACoP colocalization analysis tool within Image J software Fiji version 2.14.0 was utilized for statistical analysis (Schindelin et al.,

2012). The FIJI software conducts a 1:1 pixel spatial comparison between two different channels. In our datasets, channel 1 corresponds to nanoparticle fluorescence (410-470 nm) and Channel 2 corresponds to the GFP- B.cinerea fluorescence (500-570 nm) signal. FIJI compares the pixel intensities of GFP fluorescence and nanoparticle fluorescence channels. Assigning in this manner allows us to understand how much the NPs are colocalizing with GFP- The statistical data provided by FIJI were statistical measures of correlation indicating the strength of correlation between the two channels. Pearson's, Costes, and Mander's correlation coefficients were compared statistically using Graphpad (Version 10.0.2) (Motulski, 2023). For Mander's coefficient, Coste's automatic pixel threshold was utilized to obtain a Mander's coefficient using an auto threshold. The most effective statistical tool to compare the Mander's and Pearson's correlation coefficients was the unpaired, two-sample Student t-test which determines if two independent samples, in this case the correlation coefficients, are statistically different (Tomaso et al., 2022).

To run the statistical analyses, the individual Mander's or Pearson's correlation coefficients were an unpaired, two-sample t-test was selected as the statistical tool. The data was assumed to follow a Gaussian distribution indicating that the plotted data of both samples followed a normal and randomized distribution with similar standard deviations (Tomaso et al., 2022). This specific

test is a non-directional test aimed at only determining if the two-sample means are statistically different; the confidence interval was determined to contain out true correlation coefficient value 95% of statistical runs.

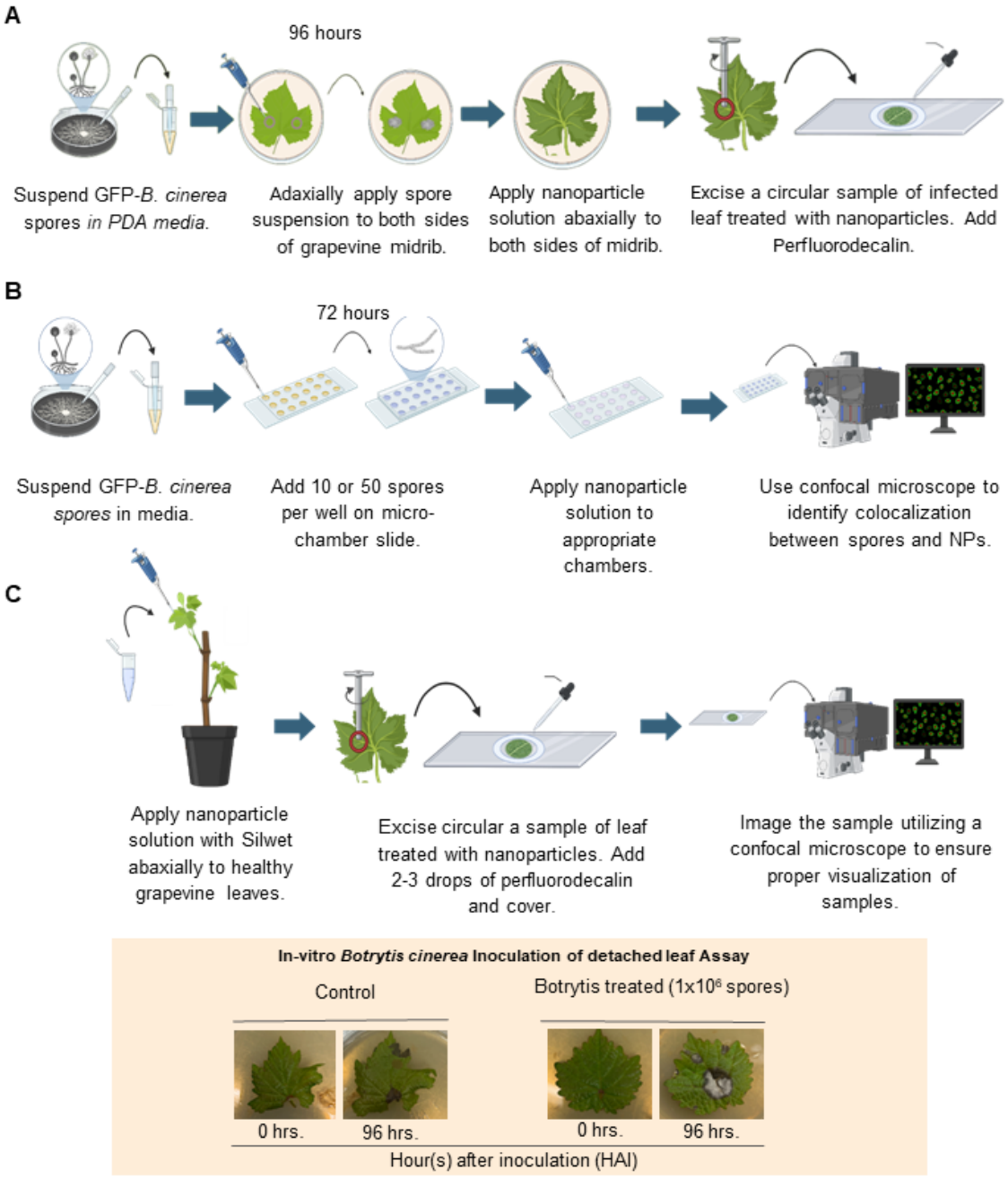

Figure 2.1 A-C, Workflow for *in vitro* and *in vivo* experiments (detached leave and whole plant assays). D, Demonstration of Botrytis-infected leaves for *in vivo* test. Inoculated leaves were incubated for 96 h until Botrytis fungal growth on the leaf surface was observed.

Results

A multi-step synthesis was used to functionalize nanoparticles. Sucrose molecules were coated on β-CD (suc-β-CD) surface by strong binding between boronic acid groups and carbohydrates (i.e., sucrose) containing syn-periplanar hydroxyl groups (Santana et al., 2020). We functionalized the CD with β-cyclodextrin molecular baskets to act as targeted nanocarriers for the delivery of agrochemicals in plants (Figure 2.2.). Transmission electron microscopy (TEM) images showed an average size for suc-β-CD of approximately 9.1 ± 2.8 nm (unpublished data). The suc-β-CD were doped with gadolinium (suc-β-GdCD) for X-ray fluorescence mapping analysis. Gadolinium is incorporated into the core structure of the CDs via coordination bonds during the carbon dot synthesis (Wang et al., 2022; Yu et al., 2016).

The hydrodynamic diameter of CD and GdCD was measured before and after functionalization with sucrose or β-cyclodextrin. The core CD and GdCD DLS sizes were 3.6 ± 1.5 nm and 7.5 ± 4.1 nm, respectively. After functionalization with sucrose or β-cyclodextrin, the sizes of suc-β-CD and suc-β-GdCD increased to a similar level of 20.3 ± 3.6 nm and 17.4 ± 5.7 nm, respectively (in 10 mM TES, pH 7.4). The sucQD are highly negatively charged with ζ potential magnitudes of -57.1 ± 2.5 mV and -45.9 ± 7.4 mV (10 mM TES, at pH 7.0), respectively. The core CD and GdCD also have negatively charged zeta potentials of -28.9 ± 7.7 mV and -12.2 ± 1.9 mV, respectively. Coating the CD or GdCD with sucrose and β-cyclodextrins slightly reduces the zeta potential of suc-β-CD and suc-β-GdCD to -

26.1 ± 4.1 mV and -9.9 ± 2.4 mV. The DLS size of nanomaterials in this study is in the range (<40 nm) (Avellan et al., 2019) reported to allow internalization through leaf biosurface barriers, including the plant cell wall, plasma, and chloroplast membranes. The ζ potential for all nanoparticles except for GdCD and suc-β-GdCD is within the range expected to facilitate uptake through plant lipid membranes (>20 mV) (Lew et al., 2018). Unpublished work shows the maximum fluorescence emission peak for sucQD, and suc-β-GdCD was 590, and 420 nm, respectively. Although sucQD absorption peaked at 575 nm, and the absorption spectrum of suc-β-CD showed the broadening of CD absorption in the UV and visible range due to the introduction of both sucrose molecules and β-cyclodextrins. The suc-β-GdCD also showed an absorption peak at 355 nm (Xu et al., 2018; Yu et al., 2016) and a broad absorption in the range of 300 to 450 nm after functionalization.

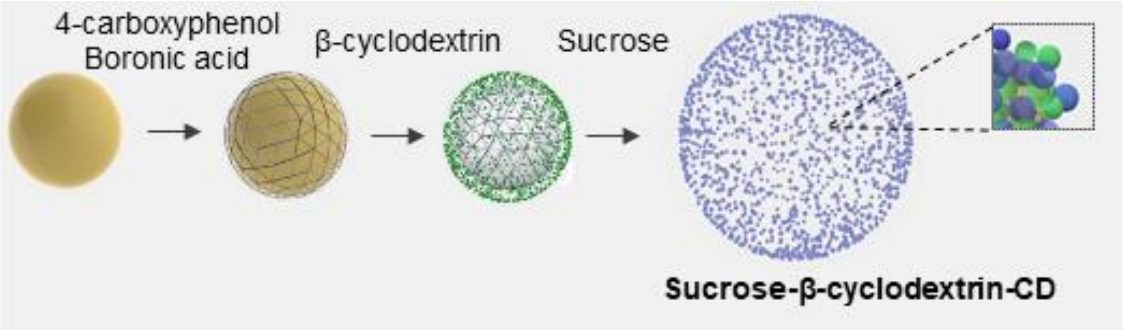

Figure 2.2 Simplified schematic of Suc-β-GdCD nanoparticle synthesis.

We established *in vitro* and *in planta* bioassays to image the fluorescence of β-GdCDs and suc-β-GdCDs nanoparticles in fungi and measure delivery efficiency via colocalization. Confocal imaging captured spore germination and hyphal colonization of plant cells by the GFP-labeled *B. cinerea* in both artificial medium *in vitro* and *in situ* with the detached leaf assay (Figure 2.3.A,B).

In vitro growth of 10 GFP- *B.cinerea* spores after a 72-hour growth period treated with either 0.1mg/mL GdCDs or Suc-β-GdCDs in 0.2% Silwet is shown in Figure 2.4 and 2.5. The GFP signal of the fungal pathogen is demonstrated in green within 500-570 nm and GdCDs or Suc-β-GdCDs signal in red within 410-470nm nm. The images highlight the presence of GFP-labeled *B. cinerea* spores (Figure 2.4.A) and hyphae (Figure 2.5.A). However, detection of nanoparticles in hyphae or spores was rarely measured (Figure 2.4.A). This would be visible with a yellow hue signal indicating a colocalization event between fungal structures and nanoparticles.

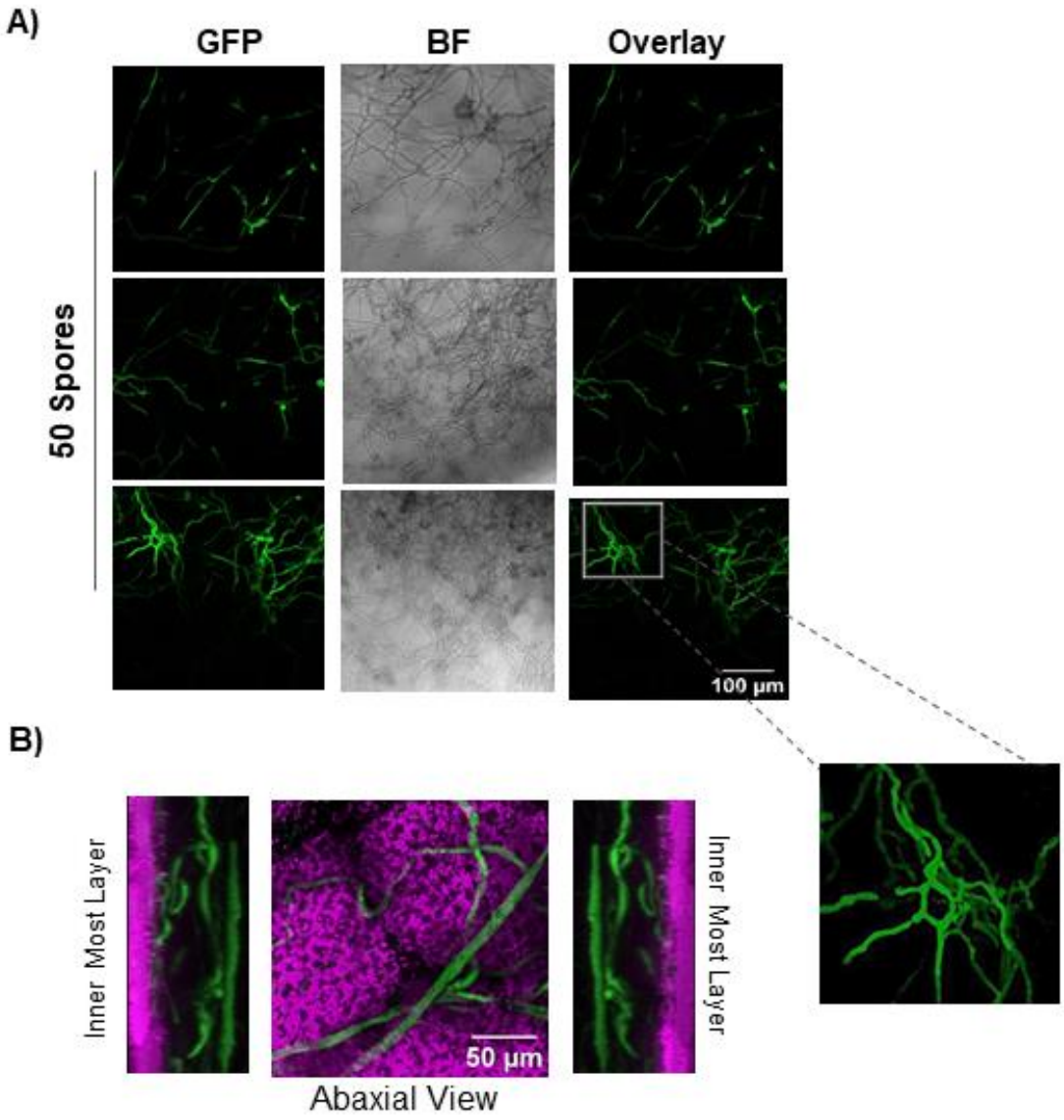

Figure 2.3 Confocal Images of healthy GFP-Botrytis both *in vitro* and *in vivo* (leaf shown in purple). A) Confocal images of healthy *B. cinerea* hyphal structures (shown in green) and detection of GFP fluorescence, displaying GFP, brightfield (BF) and composite (overlay) Channels. B) Orthogonal views from confocal images (z-stack) of GFP-Botrytis growth (shown in green) on infected grapevine leaf (in purple) following a 72 hour growth period.

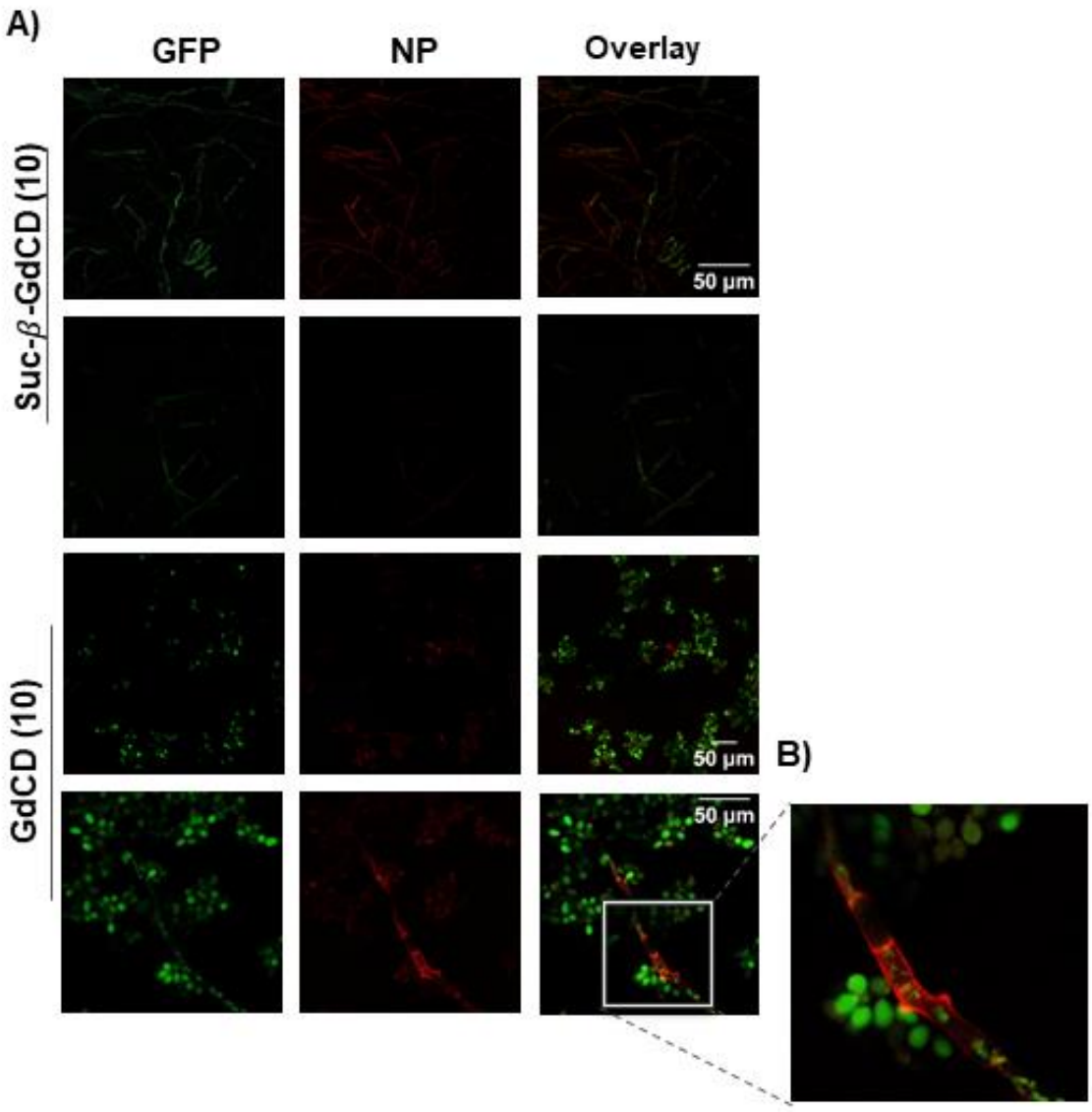

Figure 2.4 Targeted delivery of GdCDs and Suc-β-GdCDs to GFP-Botrytis spores (10). A) Confocal images of *B. cinerea* hyphal structures indicating colocalization with either GdCDs (shown in red) and Suc-β-GdCDs (shown in red) in *in vitro* spore (shown in green) inoculation. B) Highlighted region (gray square) within the *in vitro* sample demonstrates that GFP fluorescence (in green) is expressed in fungal spores (shown in green); outline of hyphae structure, coated in NPs, shown in red.

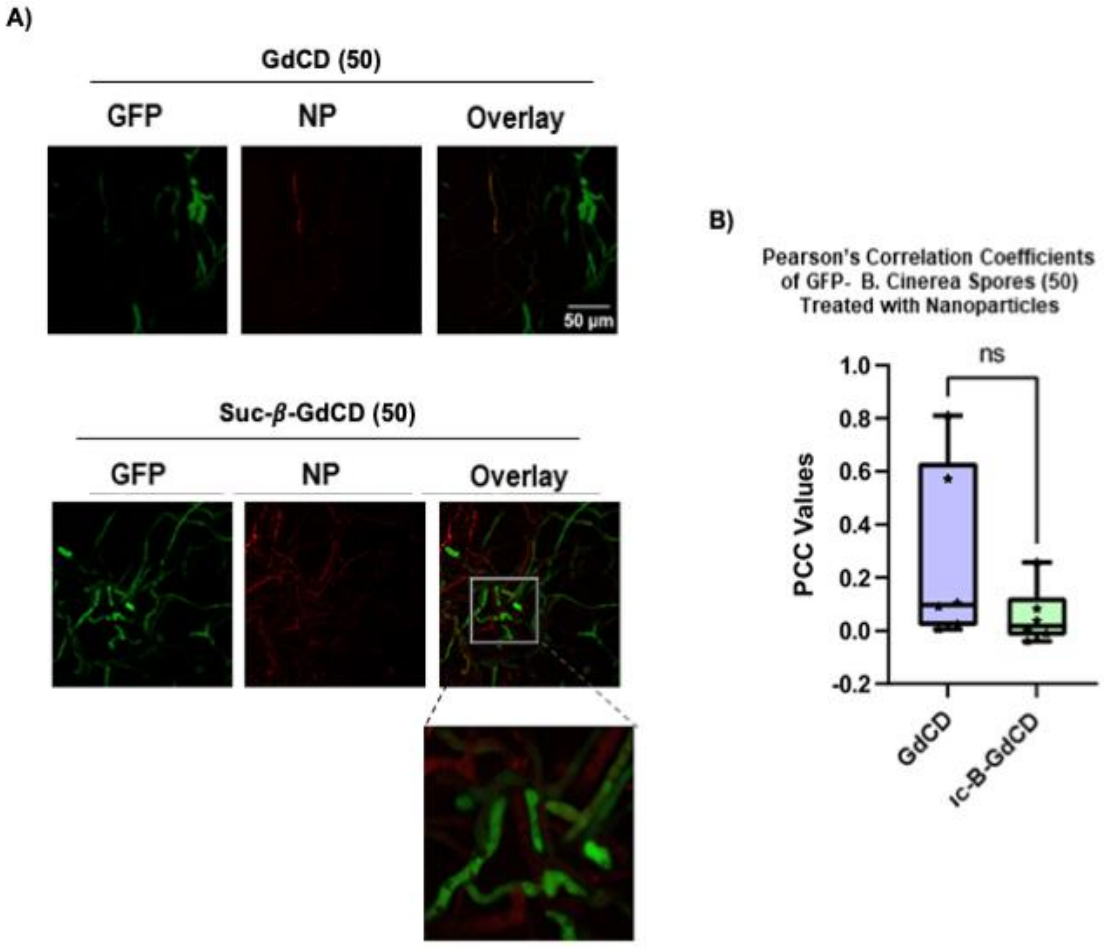

Figure 2.5 Targeted delivery of GdCDs and Suc-β-GdCDs to GFP-Botrytis spores (50). A) Confocal images of B. cinerea hyphal structures (shown in green) indicating colocalization with either GdCDs (shown in red) and Suc-β-GdCDs (shown in red) in in vitro spore (50) inoculation. B) Quantitative in vitro colocalization analysis of Nanoparticles with GFP fluorescence after growth of 50 GFP-Botrytis spores. PCC statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, p = 0.1726, n = 6).

To evaluate whether *in vitro* colocalization measurements obtained between GdCDs and Suc-β-GdCDs, differ significantly from one another, we conducted two statistical tests commonly used in those assays (Dunn et al., 2011; McDonald et al., 2013); a Pearson correlation coefficients (PCC) and Mander's correlation coefficient (MCC) statistical analyses. MCC (Manders' Colocalization Coefficient) and PCC (Pearson's Correlation Coefficient) are widely employed metrics for assessing colocalization in microscopy and image analysis (citation) . MCC quantifies the portion of intensity from each channel that overlaps with the other, considering intensity thresholds and enabling separate coefficients for both channels. This metric is valuable for evaluating partial colocalization and can be less influenced by background noise. On the other hand, PCC measures the linear correlation between pixel intensities in both channels across the entire image. It indicates the degree and direction of a linear relationship between channels' intensity variations. While MCC is suited for gauging overlap between channel intensities, PCC is better suited for assessing the similarity and directionality of intensity variations. Researchers often utilize both metrics in tandem to comprehensively understand different aspects of colocalization phenomena.

A two-sample Student's t-test determined the PCC values for GFP-*B. Botrytis cinerea* spores treated with either GdCDs or Suc-β-GdCDs in 0.2% Silwet were not statistically significant (Figure 2.4.A). The PCC values for GdCD with GFP fluorescence increased to 0.4134 ± 0.234 (95% Confidence Interval, N=5, SD population 0.26644) from 0.45 ± 0.187 (95% Confidence Interval, N=6, SD

population 0.2341) for Suc-β-GdCD with GFP fluorescence. The two-sample Student's t-test used to determine the Mander's correlation coefficient (MCC) values for GFP-*B B. cinerea* spores treated with either GdCDs or Suc-β-GdCDs in 0.2% Silwet also indicated no significant statistical differences (Figure 2.4.B). The MCC values for GdCD with GFP fluorescence increased to 0.6748 ± 0.192 (95% Confidence Interval, N=10, SD population 0.31012) from 0.563 ± 0.149 (95% Confidence Interval, N=12, SD population 0.2625) for Suc-β-GdCD with GFP fluorescence. Comparing PCC values is a more reliable comparison than utilizing MCC values because MCC values are highly susceptible to influence from background signals and thus require threshold adjustments between replicates.

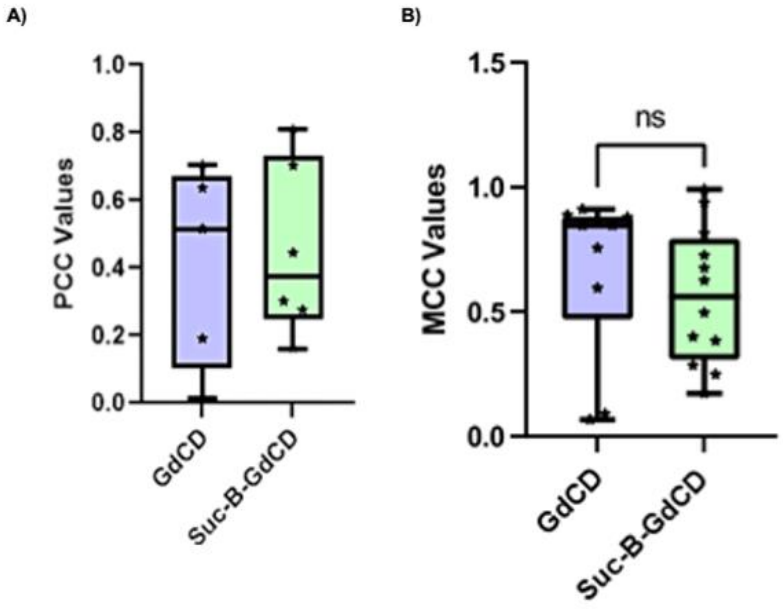

Figure 2.6 Quantitative *in vitro* colocalization analysis of NPs with GFP fluorescence. A) Quantitative *in vitro* colocalization analysis of NPs with GFP fluorescence after growth of 10 GFP-Botrytis spores. Statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, $p = 0.8313$, n = 5 - 6). B) Quantitative *in vitro* colocalization analysis of Nanoparticles with GFP fluorescence after growth of 10 GFP-Botrytis spores. Statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, $p = 0.3929$, n = 10 - 12).

Experiments were also carried out *in planta*. Following spore inoculation on leaves, the growth of GFP-*B. cinerea* was measured at 96-hour of incubation after treatment with either 0.1mg/mL GdCDs or Suc-*β*-GdCDs in 0.5% Silwet (Figure 2.7.A). A strong signal emitted within the GFP-labeled *B. cinerea* confirmed its presence on grapevine leaf. (e.g., stomata). Figure 2.7.B shows the orthogonal view of the healthy grapevine leaf infected by GFP-*B. cinerea* treated with GdCD.

In this image, the green shows tissue penetration by *B. cinerea,* and the red indicates the nanoparticle. The overlay image displays a yellow hue that demonstrates the colocalization between *B, cinerea,* and the GdCDs an Suc-β-GdCDs nanoparticles and suggest fungal uptake during the colonization of leaf tissues. Samples treated with GdCDs nanoparticles containing a higher Silwet centration (i.e., 0.5%) showed consistently a stronger GFP and NP signal making the colocalization yellow hue more distinguishable. Colocalization analysis of Nanoparticles and GFP fluorescence showed no statistical differences (Figure 2.7.B). The PCC values for GdCD with GFP fluorescence decreased to 0.7825 ± 0.0381 (95% Confidence Interval, N=5, SD population 0.0435) from 0.87583 ± 0.0357 (95% Confidence Interval, N=8, SD population 0.05145) for Suc-β-GdCD with GFP fluorescence.

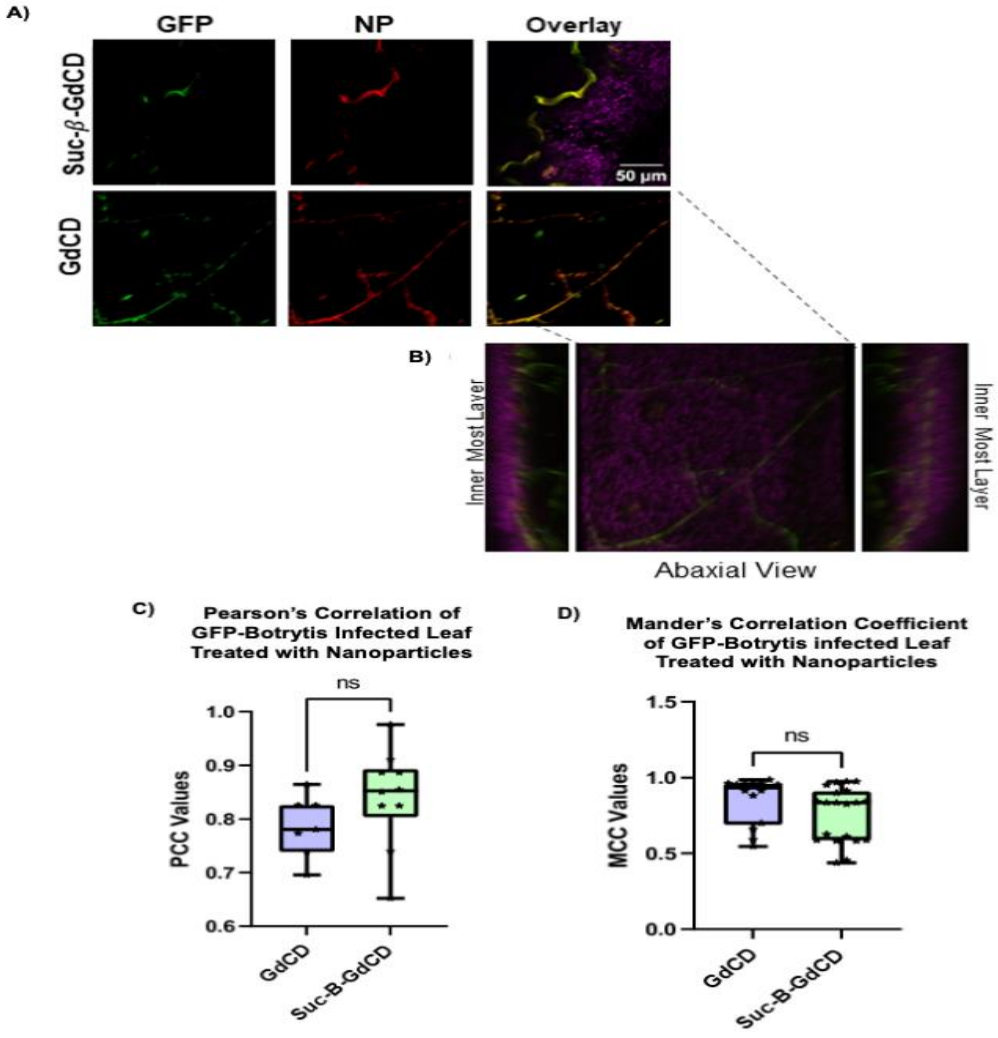

Figure 2.7 Targeted delivery of GdCDs and Suc-β-GdCDs to GFP-Botrytis infected grapevine leaves. A) Confocal images of B. cinerea hyphal structures (shown in green) indicating colocalization with either GdCDs (shown in red) or Suc-β-GdCDs (shown in red) in leaf (shown in purple). B) Orthogonal views from confocal images (z-stack) of GdCD colocalization with GFP-Botrytis on an infected leaf.C) Quantitative in vivo colocalization analysis of Nanoparticles with GFP fluorescence on GFP-Botrytis infected grapevine leaf. Statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, p = 0.1851, n = 7 - 10).D) Quantitative in vivo colocalization analysis of Nanoparticles with GFP fluorescence on GFP-Botrytis infected grapevine leaf. Statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, p = 0.1201, n = 14- 20).

A statistical comparison between PCCs of Nanoparticles with GFP fluorescence of *in vitro* vs. *in vivo* experimental replicates was performed to determine if nanomaterial uptake and colocalization differ significantly (Figure 2.8). An unpaired, two-sample Student's t-test determined that the PCC values for GdCD with GFP fluorescence in-planta were significantly higher than PCC values for GdCD with GFP fluorescence *in vitro.* An unpaired, two-sample Student's t-test was used again to determine that the PCC values for Suc-*β*-GdCD with GFP fluorescence *in-planta* were significantly higher than PCC values for GdCD with GFP fluorescence *in vitro* (Figure 2.8. A & B). Comparing PCC values is a more reliable comparison than utilizing MCC values because MCC values are highly susceptible to influence from background signals and thus require threshold adjustments between replicates.

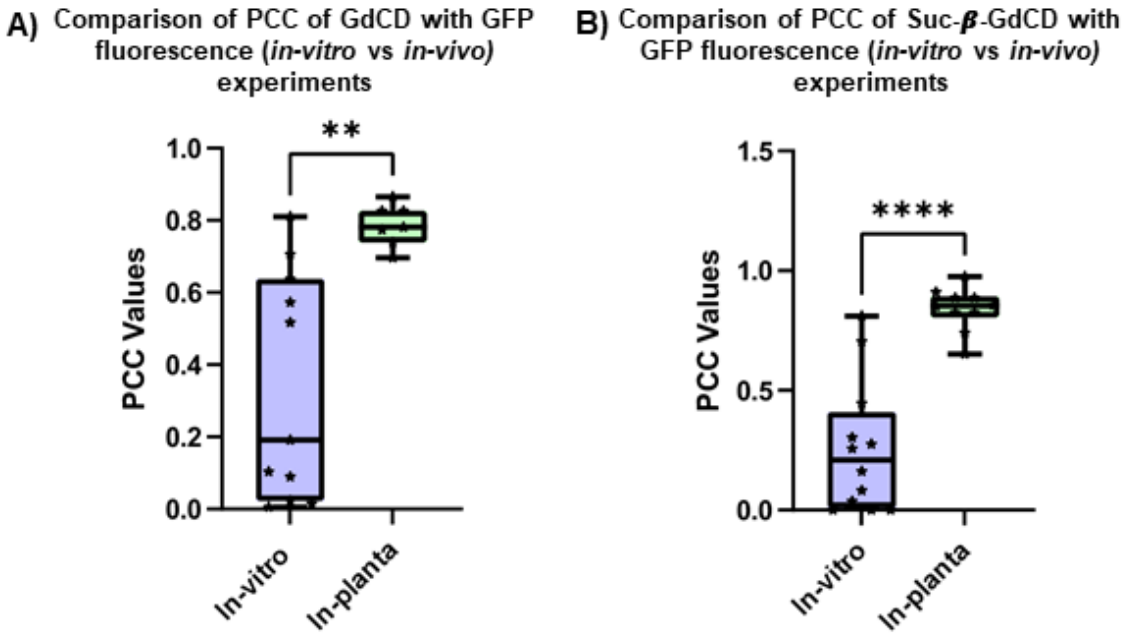

Figure 2.8 Comparing Pearson's correlation coefficients (PCC) between nanoparticle inoculation of GFP-Botrytis *in vitro* vs *in-planta*. A) Comparison of PCC of GdCD with GFP fluorescence between *in vitro* vs. *in vivo* experiments. Statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, $^{**}p = 0.0018$, n = 7-11). B) Comparison of PCC of Suc-β-GdCD with GFP fluorescence between *in vitro* vs. *in vivo* experiments. Statistical comparison was performed by unpaired, two-sampled, Student's t-test (two-tailed, $^{****}p < 0.001$, n = 10-12).

A positive correlation coefficient, which suggests a strong spatial relationship between the structures labeled by the two fluorescence channels, was observed across all three colocalization coefficients (Mander's, Pearson's, and Coste's) of *in vitro* inoculation of 10 GFP-*B. cinerea* with GdCD (Supplemental Table 2.1) and Suc-β-GdCD (Supplemental Table 2.2). *In vitro* inoculation of 50 GFP-*B. cinerea* with GdCD in 0.2% Silwet also shows positive correlation coefficient across all three colocalization coefficients (Supplemental Table 2.3).

However, a few Pearson's coefficients obtained are very small and close to 0, which may indicate that there is no colocalization between the GFP signal and the nanoparticle signal. A positive correlation coefficient was not observed across all three colocalization coefficients of *In vitro* trials of 50 GFP-*B. cinerea* with Suc-*β*-GdCD in 0.2% Silwet (Supplemental Table 2.4.). Within the Pearson coefficients, a few data points are negative, indicating a negative correlation between GFP and nanoparticle signals. Though they are negative, it is unlikely that these negative values indicate true anti-correlation but indicate no spatial overlap between both signals. Therefore, these negative values most likely indicate the absence of colocalization rather than anti-colocalization. *In vivo* inoculation of GFP- *B.cinerea* infected grapevine leaves with GdCD (Supplemental Table 2.5) and Suc-*β*-GdCD in 0.5% (Supplemental Table 2.6), show a positive correlation across all three colocalization coefficients.

Discussion

This research provides a framework to evaluate nanotechnology for the management of plant diseases. Nanotechnology is broadly defined as the manipulation of material at dimensions between 1 and 100 nm (Tripathi et al., 2017). To date, when nano-technologies have been explored as nano-pesticides, the NP is usually in the form of active ingredient(s), either manufactured nano-materials or metal oxide nanoparticles (e.g., silver and copper, zinc oxide, copper oxide, manganese dioxide, silicon dioxide, titanium dioxide) (Bratovcic et al., 2021;

Jogaiah et al., 2020). For example, molybdenum oxide nanoparticles inhibit the growth of fungal conidiophores and, in turn, can damage fungal structures (Raj et al., 2021). Copper oxide NPs, because of their metal properties, have antifungal activity against *Aspergillus flavus* , *Fusarium,* and *Phytophthora infestans*, among others (Dwivedi et al., 2016; Vanathi et al., 2016). However, within this research, we used manufactured carbon dots and functionalized them with β-cyclodextrin complexed to act as a molecular encapsulation, or "basket". This in itself is not novel, as numerous hydrophobic pesticides are transported on CDs, including α-, β-, and γ-CDs (Liu et al., 2022). In addition, β-cyclodextrins have been shown to extend the prevention and control time by increasing the stability of pesticides and preventing oxidation and decomposition, thus lowering the pesticide dosage (Liu et al., 2022). However, the novelty of our approach is that we leverage β-cyclodextrins attributes and further extend its utility by having it serve as a platform to deliver agrochemicals to fungal structures by coating it with sucrose bio-molecule. Innovative applications of nanoparticles have emerged, with sucrose-based nanoparticles showcasing their versatility in various fields, from protein detection to DNA sensing. Sucrose-modified gold nanoparticles (AuNPs) have been developed for sensitive protein detection (Shrivas et al., 2019, 2020) . These AuNPs are coated with sucrose and recognition ligands, enabling them to bind to target proteins selectively. This interaction induces optical changes that facilitate precise protein detection, offering potential applications in diagnostics. Sucrose-conjugated silica nanoparticles have found utility in DNA sensing (Ali et al., 2022).

By functionalizing these nanoparticles with DNA probes complementary to specific target sequences, they can detect the presence of target DNA through aggregation or other changes. The successful applications of sucrose-based nanoparticles for bio-recognition, such as protein detection and DNA sensing, underscore their potential as versatile tools in various biomedical and analytical domains. While nanoparticles have been explored for various purposes in agriculture, for example, nanosensors for detecting contaminants, sucrose-based nanoparticles for bio-recognition in agricultural contexts might need to be more widely documented (Riquelme et al., 2017).

We developed bioassays to capture targeted delivery of nanoparticles to *Botrytis cinerea* and quantify the efficacy of delivery of fully functionalized, sucrose-coated carbon dots with β-cyclodextrin molecular baskets (suc-β-CDs) compared to GdCD, which lacks the β-cyclodextrin molecular basket necessary for the delivery of targeted treatment. Considering preliminary data that examined suc-β-CDs and GdCD uptake in leaves, it was expected that both GdCDs and Suc-β-GdCD were capable of penetrating plant tissue and entering through the stomata. Because of *B.cinerea's* affinity for host-derived sucrose (Li et al., 2021), it was expected that both *in vivo* and *in vitro* treatment of *B. cinerea* with Suc-β-GdCD would result in a higher uptake by the pathogen compared to treatment with GdCD. Colocalization analysis was determined by the degree of interaction between the fungal structures containing the green fluorescent protein (GFP) gene and the nanoparticles. Application of GdCD and Suc-β-GdCD to healthy grapevine leaves

demonstrated that both nanoparticles are capable of penetrating plant tissue and entering plant structures. However, statistical analysis showed that *B. cinerea* uptake of nanoparticles Suc-β-GdCD was not significantly higher than GdCD as initially hypothesized. These results may suggest that plant and fungal uptake is identical between the formulations and that sucrose coating does not offer any advantage. Further experiments will need to improve delivery of nanoparticles to fungal structures to capture higher quality images of colocalization events.

In vitro inoculation of 10-50 spores coated with either GdCD or Suc-β-GdCD demonstrated that both nanoparticles are positively colocalized in GFP- *B. Cinerea* hyphae. Statistical analysis showed that *B. cinerea* uptake of nanoparticles was not significantly different between GdCD and Suc-β-GdCD. The standard deviation was high, contrary to *in vivo* results, which suggests that the large distribution makes these results unreliable and further experiments should be conducted to include more replicates. In addition, it is noteworthy that there was no marked uptake of NPs in fungal spores. To our knowledge, sucrose transporters in *B.cinerea* spores and fungi have not been documented. Spores are resting structures in the life cycle of fungi, they are not biologically active until they find a proper substrate with ideal conditions to germinate. We allowed a 48h growth period in microplates which may not be enough time for the Suc-β-GdCD(Lian et al., 2018; Wang et al., 2018). Manufacturer instructions recommend using Silwet® L-77 at a concentration range of 0.005-0.05% in 5% sucrose(Li et al., 2019) and several trials in tomato, cotton and maize have applied different concentrations for

nanoparticle delivery (Monroy-Borrego et al., 2022). Future experiments should optimize the Silwet working solution for nanoparticle delivery in grapevine leaf.

Findings from this work indicated that i*n vitro* experiments were essential for evaluating the interactions of sucrose-coated nanoparticles in a controlled environment. This has the potential for breakthrough applications in agriculture especially with the possibilities that systemic delivery of Suc-β-GdCD in plants would offer. Since sucrose is the end product of photosynthesis, and the main sugar translocated in the phloem (Greer, 2012) target for distant transport. Optimizing transport and delivery of nanoparticles in the vascular system or root of plants could support integrated pest and disease management programs. For example, *Candidatus* Liberibacter asiaticus is a phloem-dwelling bacterium that causes citrus Huanglongbing, the most serious disease to citrus globally (Bové, 2006). The pathogen impairs phloem sucrose movement and accumulation in roots due to sieve tube occlusion which weakens tree vigor and productivity (Etxeberria et al., 2009). The disease is managed by application of synthetic insecticides and antibiotics. Deploying nanoparticles technology for targeted delivery of active anti-CLas products *in situ* would offer alternative sustainable solutions to the problem.

The next frontier in agriculture is to research nanoparticles equipped with bio-recognition molecules as this is essential to bridge the gap between theoretical concepts and practical applications. One notable application involves the targeted delivery of pesticides using nanoparticles coated with peptides or proteins (Vega-

Vásquez et al., 2020). These engineered nanoparticles adhere specifically to receptors on pest surfaces, allowing for precise pesticide administration and reducing ecological impact. Additionally, by coupling nanoparticles with amino acids, researchers have facilitated nutrient absorption by plant roots, resulting in improved nutrient transportation, enhanced plant growth, and increased agricultural productivity (Hafez et al., 2021).

The efficacy of targeted delivery of nanoparticles to *Botrytis cinerea* in grapevine proves to be a promising model system that could be leveraged for screening efficacy of suc/glu-β-GdCD loaded with fungicides. Nanotechnologies will change the agricultural landscape when they become widely adopted in farming practices. However, they must receive consumer's acceptance and comply with human and environmental safety standards. Nanobiotechnologies related to pesticides and food, face challenges within regulatory frameworks (Paradise, 2019). Education of consumers, regulatory agencies and farm workers will be a huge component of the future of their success (Malik et al., 2023).

Conclusion

We set out to test a targeted delivery approach to fungi in plants using novel nanoparticles. Sucrose molecules were coated on the β-cyclodextrin CD to create suc-β-CD. Then the functionalized CD was abaxially delivered to *B.cinerea* -infected leaves. Overall, although there does not seem to be a preferential uptake with sucrose-coated NPs, the results of the targeted delivery, which shows the uptake of suc-β-CD in the presence of *B.cinerea* on its own, are suggestive and promising. We think the uptake in plant and fungi is being mediated and enters plant and fungal structures via plant and fungal sucrose transporters (Figure 2.9.). Current research by Jeon et al., aims to test the efficacy of this platform by loading the β-cyclodextrin molecular basket with an active ingredient. Thereafter, fully functionalized nanoparticles will be tested in a pathosystem to test the ability to limit fungal infection. The potential of the targeted delivery as a pest control method needs further development. Still, it should aim to test the ability to halt the survival and spread of *Botrytis cinerea* before widespread infection occurs. Further, for any nanotechnology seeking to enter the market, understanding how nanoparticles interact with biological systems and the suitability of testing procedures for determining the safety, efficacy, and quality of goods using nanomaterials are imperative, and the main objectives of the FDA's regulatory science research agenda on nanotechnology.

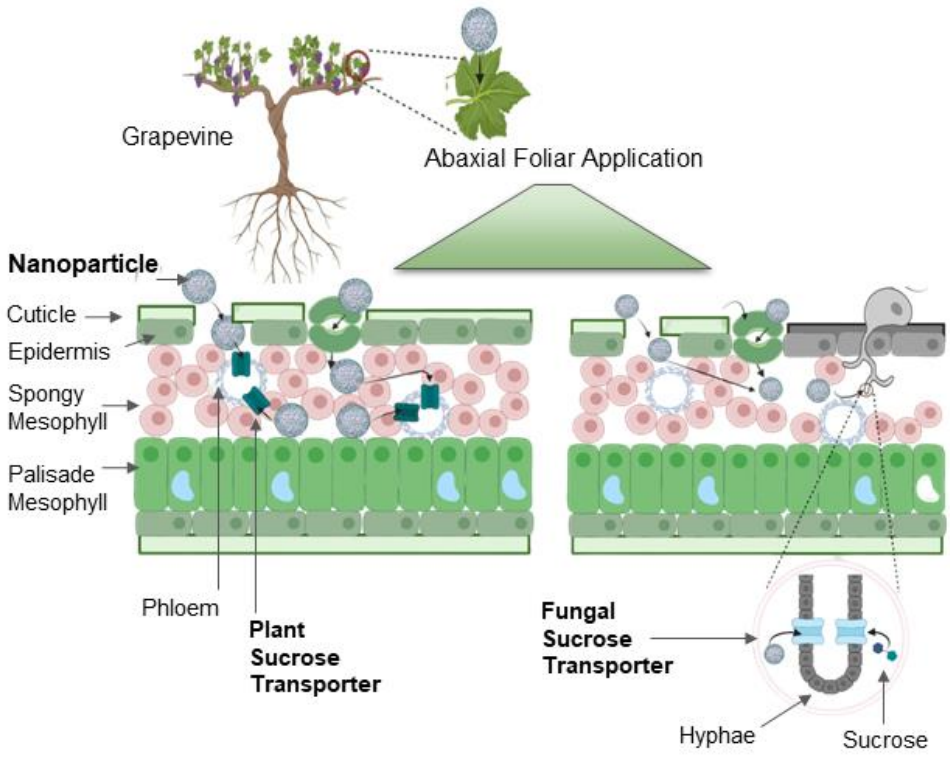

Figure 2.9 Schematic showing abaxial foliar application of suc-β-CD nanoparticles for targeted delivery to healthy plant tissue (left) and to B. cinerea infected plant leaf tissue (right). In healthy plant tissue the nanoparticles are uptaken by the phloem through the Plant Sucrose Transporter, while in the infected tissue the nanoparticles are uptaken by fungal cells through the Fungal Sucrose Transporter.

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Conclusion

Plant disease outbreaks represent serious threats to agricultural sustainability and the world's food supply chain. Applying pesticide is the backbone of growers farming practices to reduce disease threat. One goal of my work was to reduce chemical footprint in the environment by way of using traditional pruning practices and new nanotechnologies. Reducing chemical input is important because plant-based foods carry the heaviest burden of the global pesticide footprint, comprising 59% of the total pressure (Tang et al., 2022). Among these, the orchard fruits and grape sector is the primary contributor, responsible for 17% of the global footprint (Tang et al., 2022). Frequent and improper use of fungicides in grape production can also lead to chemical-resistance in fungal populations, diminishing the effectiveness of disease control measures (Harper et al., 2022). In grapes, several fungi have developed resistance to single-site fungicides (Hawkins et al., 2018; Massi et al., 2021). The work presented here is aimed at supporting Integrated Pest Disease Management (IPDM) strategies and reducing crop loss to diseases while preserving the longevity and diversity of an ecosystem (Pertot et al., 2017).

Within this thesis, I aim to better understand pathogenic interactions in grapevines to optimize management tools and strategies. In my first chapter, I achieved this goal by comparing two pruning types, Guyot Arcure (severe pruning) and Guyot Poussard (minimal pruning), used in the Cognac region, France. Our findings corroborated the hypothesis that severe pruning decreased vine longevity

and increased the likelihood of esca-pathogen infections caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*. Our data support that the composition of the endophytic mycobiome community is influenced by the type of pruning. Increased wounding provides gateways for microbial colonization and we clearly measured higher taxa richness in severely pruned wood. However, it also favored pathogen entrance. These results align with previous research and highlight the need for a trained and educated workforce. Best cultural practices are the backbone of IPDM programs and pruning is a key practice in viticulture. Optimizing vine training will not only influence berry quality and vine productivity but will also have a lasting impact on its health and longevity. Those practices are basic yet foundational to an integrated system with long-term profits.

Our results are specific to pruning practices in France. However, in the US, comparable practices known as spur and cane pruning are commonly practiced in viticulture. Spur pruning involves cutting back the previous season's growth to a short, permanent spur with a few buds, which encourages the development of new shoots and clusters of grapes. On the other hand, cane pruning involves selecting one or more long, flexible canes from the previous season's growth and removing the rest, allowing for greater flexibility in training the vine and potentially higher yields. The choice between these two pruning methods depends on factors such as grapevine variety, vineyard goals, and regional practices. Cane pruning would be the closest practice to minimal pruning. Whereas spur pruning might be

considered a more severe form of pruning. Exploring how these practices impact sap routes might be of lesser interest in vineyards that are mechanically pruned.

This thesis also aimed at optimizing current management strategies by testing a novel nanoparticle delivery method to the necrotrophic pathogen *B. cinerea* in grapevine leaf. This transformative approach would allow for efficient delivery of active ingredients remotely, and for off-site pathogens. This would have a broad economic impact because it will decrease the amount of pesticides used and its leaching in the environment. The research offers a platform to study target-specific new classes of nano-pesticide (Deka et al., 2021). The successful delivery of suc-β-GDCDs to fungal structure in grapevine, sets the pathway towards delivering functionalized nanoparticles with active fungicide ingredients, thus enabling precision application. However, continuous efforts should be made towards understanding the nanoparticles environmental footprint. The strategy to use β-cyclodextrins as a molecular basket has already been shown to load and deliver a wide range of chemicals, including pesticides and herbicides (Saha et al., 2016; Santana et al., 2020; Szejtli, 1998). The size and charge of nanoparticles coated in polymers have been reported to control their distribution in plant cells or organelles (Avellan et al., 2019; Hu et al., 2020). In this research, we tested sucrose as a biorecognition molecule, but little is known on how the properties of other sugars such as glucose influence the translocation and distribution of nanoparticles. Glucose is heavily involved in various phytohormone systems that directly impact plant development, metabolism, and senescence (Sami et al.,

2019; Singh et al., 2014). The translocation potential of β-cyclodextrins coated in glucose might hold better efficiency across plant systems. However, despite being the most biocompatible of all nanoparticles, CDs have been proven to inhibit plant growth and mass in toxicity studies (Li et al., 2020). Therefore, using glucose might hold the potential to counteract that inhibitive effect. The accumulation and decomposition of CDs within the plant system is still a subject of debate and is especially important for user acceptance, regulation, and sustainable/biodynamic guidelines. Further, distinct challenges impede the registration of carbon dot nanomaterials. Regulation submissions are hampered by the lack of specific and universal testing procedures and characterization methodologies designed for carbon dot nanomaterials, making it more challenging to demonstrate safety and efficacy (Great Britain. Department of Trade and Industry, 2005). In addition, the lack of international regulatory consensus, public perception, ethical and environmental concerns compound challenges. Addressing these requires collaboration between regulatory bodies, researchers, stakeholders, and policymakers for adapted frameworks, testing methods, and risk assessments specific to CD's.

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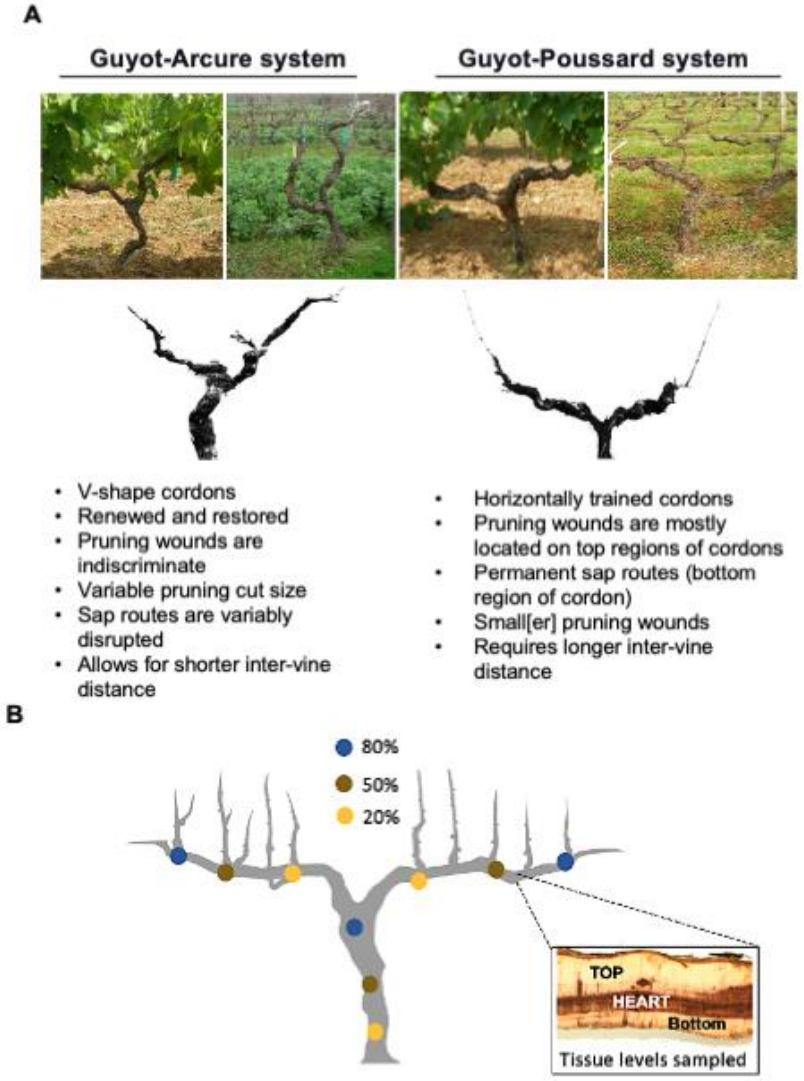
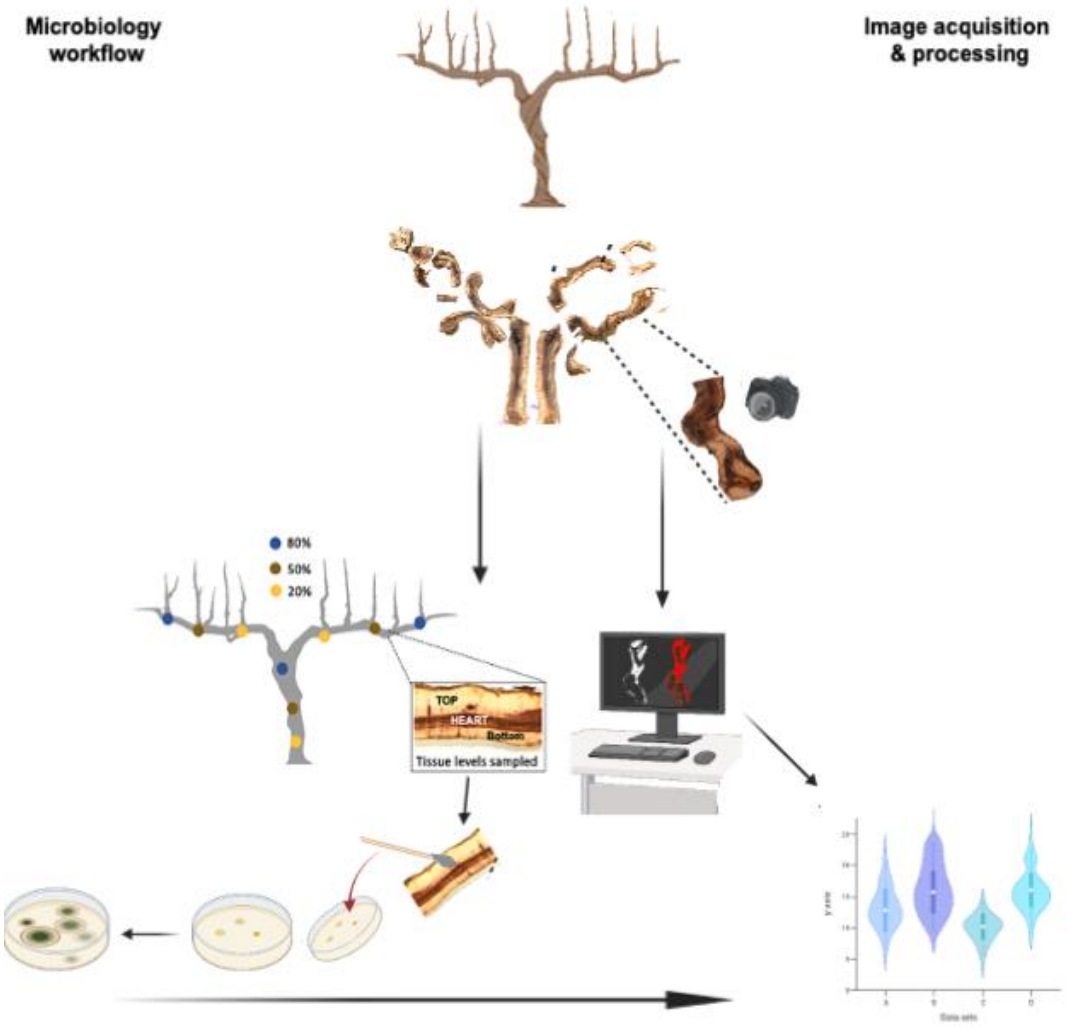
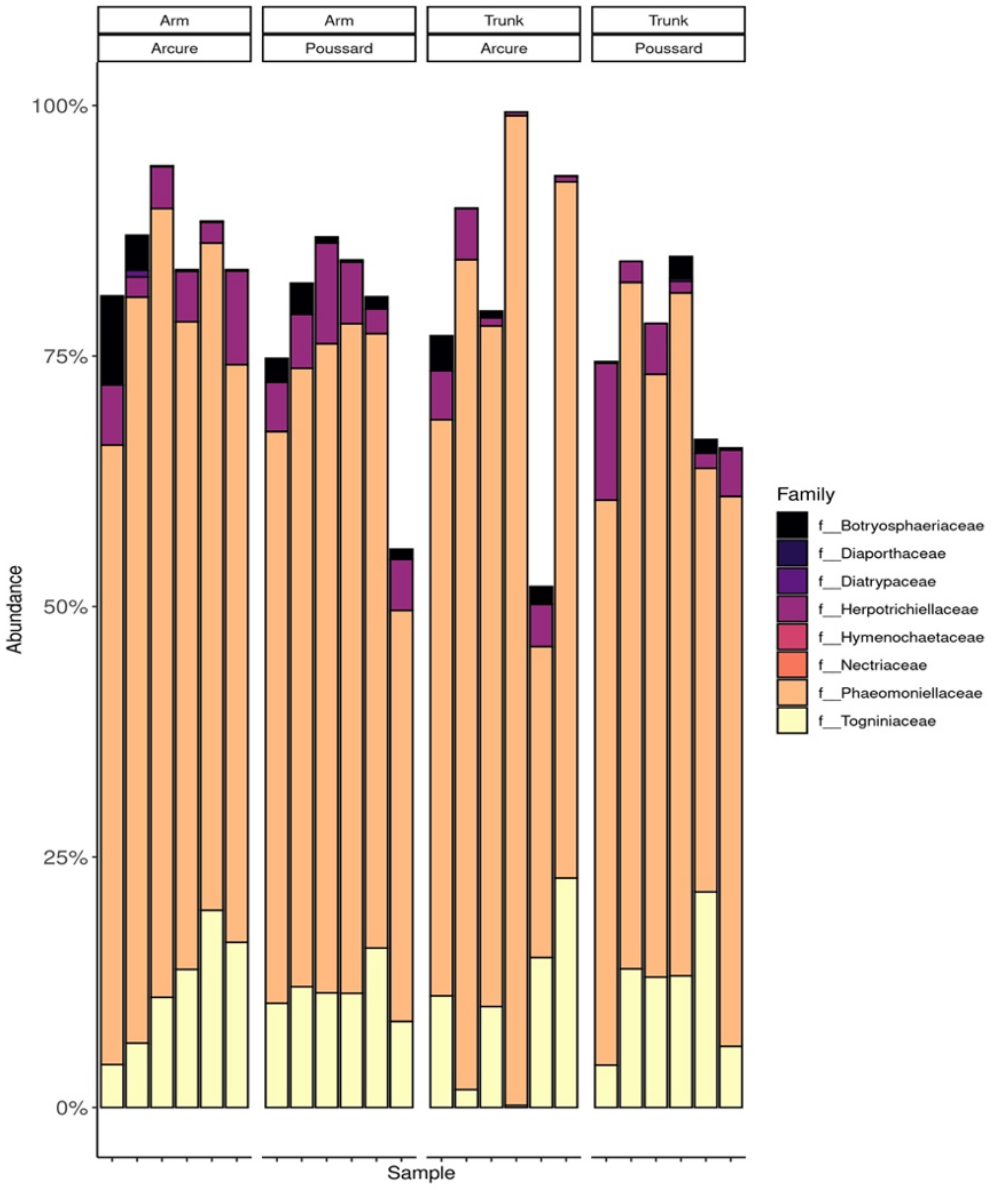
Appendix



Supplemental Figure 1.1 Illustrations and main features of the training systems surveyed in the Charente region in southwestern France, 'Guyot-Poussard' and 'Guyot-Arcure.' A) Guyot Arcure (left) and Guyot Poussard (Right); binary images of Guyot Arcure (left) and Guyot Poussard (Right). B) Location of sampling areas for the 12 vines.



Supplemental Figure 1.2 Workflow for image analysis and culture dependent and independent assays.



Supplemental Figure 1.3 Relative abundance of 8 fungal taxa shown at the family level, separated by tissue type, pruning type, and location of sample harvest, as measured through DNA metabarcoding.

Supplemental Table 2.1

Table 2.1. All data for experiments using GFP-Botrytis Spores (10) with GdCD in 0.2% Silwet *in vitro*. This includes 6 replicates where images were obtained using the Zeiss Inverted Fluorescent Microscope. Pearson's, Coste's, and Mander's values were obtained using the JACoP plugin on ImageJ Software. Channel 1 corresponds to nanoparticle fluorescence, and Channel 2 corresponds to GFP fluorescent signal.

Pearson's [a]	Coste's [b]	Channels		Mander's[c]	Adjusted Threshold[d]	Mander's w/ Thresholds[e]
				0.847	15	0.847
				0.912	18	0.846
		Channel 1	GdCD	0.377	58	0.353
				0.757	21	0.596
				0.091	72	0.067
0.638	1			0.888	73	0.879
0.517	1					
0.15	1					
0.706	1					
0.015	1			0.735	25	0.427
0.191	1			0.438	25	0.282
		Channel 2	GFP-Bot	0.131	67	0.126
				0.078	16	0.783
				0.007	16	0.002
				0.042	34	0.042

Pearson's correlation coefficient measures the linear covariance between two variables and values range from -1 to 1. A value of 0 indicates no correlation; a value of -1 indicates perfect anti-correlation; a value of 1 indicates perfect correlation.
b Coste's method for determining co-localization between two variables assumes that only a positive correlation is of interest. Coste's values range from 0 to 1, where 0 indicates no correlation and 1 indicates perfect co-localization.
c Mander's Correlation Coefficients are sensitive to pixel threshold values and background noise. The two values provided, M1(upper) and M2(lower), correspond to Channels 1 and 2, respectively. Mander's values quantify the fraction that one channel overlaps the other and provides a fraction; therefore, values range from 0 to 1.
d Since Mander's correlation coefficients are sensitive to pixel thresholds, an adjusted threshold ensures that pixels that surpass a predetermined threshold are accounted for while those that are below the threshold do not affect the Mander's Values. The Coste's auto threshold was used because it sets the threshold at the lowest value in which Pearson's correlation coefficients are positive since we are interested in the co-localization of NPs and GFP-Botrytis.
e Mander's Correlation Coefficients that have a Coste's-adjusted pixel threshold are reported to reassert the accuracy of our correlation coefficients. A Student's t-test is used to determine whether the Mander's Values significantly differ from the Mander's Values that use Coste's adjusted threshold pixel values.

Supplemental Table 2.2

Table 2.2. All data for experiments using GFP-Botrytis Spores (10) with Suc-β-GdCD in 0.2% Silwet *in vitro*. This includes 6 replicates where images were obtained using the Zeiss Inverted Fluorescent Microscope. Pearson's, Coste's, and Mander's values were obtained using the JACoP plugin on ImageJ Software. Channel 1 corresponds to nanoparticle fluorescence while Channel 2 corresponds to GFP fluorescent signal.

Pearson's	Coste's	Channels		Mander's	Adjusted Thresholds	Mander's w/ Thresholds
				0.816	12	0.727
				0.495	11	0.399
		Channel 1	Suc-β-GdCD	0.383	38	0.284
				0.676	28	0.625
				0.284	36	0.172
0.303	1			0.992	22	0.939
0.162	1					
0.445	1					
0.704	1					
0.276	1			0.181	25	0.09
0.81	1			0.135	25	0.048
		Channel 2	GFP-Bot	0.816	22	0.784
				0.982	24	0.83
				0.46	21	0.432
				0.695	15	0.658

Supplemental Table 2.3

Table 2.3. All data for experiments using GFP-Botrytis Spores (50) with GdCD in 0.2% Silwet *in vitro*. This includes 6 replicates where images were obtained using the Zeiss Inverted Fluorescent Microscope. Pearson's, Coste's, and Mander's values were obtained using the JACoP plugin on ImageJ Software. Channel 1 corresponds to nanoparticle fluorescence and Channel 2 corresponds to GFP fluorescent signal.

Pearson's	Coste's	Channels		Mander's	Adjusted Thresholds	Mander's w/ Thresholds
				0.759	24	0.612
				0.862	24	0.8
		Channel 1	GdCD	0.146	21	0.131
0.573	1			0.086	21	0.04
0.81	1			0.437	30	0.27
0.104	1			0.029	21	0.029
0.005	0.99					
0.09	1					
0.023	1					
				0.58	32	0.526
				0.883	26	0.777
		Channel 2	GFP-Bot	0.168	61	0.153
				0.021	19	0.017
				0.032	33	0.028
				0.066	38	0.066

Supplemental Table 2.4

Table 2.4. All data for experiments using GFP-Botrytis Spores (50) with Suc-β-GdCD in 0.2% Silwet *in vitro*. This includes 6 replicates where images were obtained using the Zeiss Inverted Fluorescent Microscope. Pearson's, Coste's, and Mander's values were obtained using the JACoP plugin on ImageJ Software. Channel 1 corresponds to nanoparticle fluorescence while Channel 2 corresponds to GFP fluorescent signal.

Pearson's	Coste's	Channels		Mander's	Adjusted Thresholds	Mander's w/ Thresholds
				0.389	32	0.249
				0.065	45	0.044
		Channel 1	Suc-β-GdCD	0.094	32	0.07
				0.326	33	0.192
				0.208	31	0.161
0.257	1			0.045	18	0.045
0	0.42					
-0.04	$3.45*10^{-21}$					
0.082	1					
0.037	1					
-0.011	$3.39*10^{-5}$			0.278	26	0.257
				0.018	39	0.015
		Channel 2	GFP-Bot	0.03	42	0.016
				0.079	40	0.061
				0.029		0.023
				0.013	20	0.013

Supplemental Table 2.5

Table 2.5. All data for experiments using detached Grapevine leaf infected with GFP-Botrytis Spore Suspension ([10^6spores/mL]) treated with GdCD in 0.5% Silwet. This includes 5 replicates where images were obtained using the Zeiss Inverted Fluorescent Microscope. Pearson's, Coste's, and Mander's values were obtained using the JACoP plugin on ImageJ Software. Channel 1 corresponds to nanoparticle fluorescence while Channel 2 corresponds to GFP fluorescent signal.

Pearson's	Coste's	Channels		Mander's	Adjusted Thresholds	Mander's w/ Thresholds
				0.662	5	0.7
				0.987	23	0.96
		Channel 1	GdCD	0.963	11	0.915
0.781	1			0.96	19	0.883
0.774	1			0.954	26	0.955
0.826	1					
0.696	1					
0.865	1					
				0.862	9	0.767
				0.651	37	0.564
		Channel 2	GFP-Bot	0.595	5	0.608
				0.697	16	0.449
				0.744	52	0.722

Supplemental Table 2.6

Table 2.6. All data for experiments using detached Grapevine leaf infected with GFP-Botrytis Spore Suspension ([10^6spores/mL]) treated with Suc-β-GdCD in 0.5% Silwet. This includes 5 replicates where images were obtained using the Zeiss Inverted Fluorescent Microscope. Pearson's, Coste's, and Mander's values were obtained using the JACoP plugin on ImageJ Software. Channel 1 corresponds to nanoparticle fluorescence while Channel 2 corresponds to GFP fluorescent signal.

Pearson's	Coste's	Channels		Mander's	Adjusted Thresholds	Mander's w/ Thresholds
				0.975	18	0.975
				0.901	46	0.914
		Channel 1	Suc-β-GdCD	0.628	23	0.613
				0.438	23	0.454
0.851	1			0.584	23	0.59
0.911	1			0.836	20	0.844
0.74	1			0.953	28	0.969
0.652	1			0.827	12	0.835
0.825	1					
0.887	1					
0.976	1					
0.855	1			0.721	22	0.719
				0.899	43	0.876
		Channel 2	GFP-Bot	0.958	39	0.947
				0.973	38	0.936
				0.999	29	0.999
				0.937	?	0.931
				0.999	21	0.968
				0.919	20	0.885