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STUDIES OF SUSTAINABLE STRATEGIES TO CONTROL PHYTOPATHOGEN AGENTS

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CHAPTER 1

1. INTRODUCTION

Biological control, nontoxic for plants and a nontargeted microorganisms, is an eco-friendly approach with the aim to decrease the pesticide accumulation in the environment and in the food, to regulate the activity of natural predators and, in general, to increase the microbial diversity in the managed systems. This process is less evident but more stable and long-lasting if compared to physical and chemical control methods (Sewell et al. 1985; Gawai 2018).

Historically the term biological control meant the suppression of pests using native or specially introduced antagonist organisms (Smith 2003). Over time the term biocontrol of phytopathogens has been enriched with new extensions of meanings, which demonstrate the complexity of this concept. The intent to control phytopathogenic organisms, using methods different from those concerning the dispersion of synthetic products, allowed to develop innumerable biocompatible approaches and methodologies. The biological control can be divided into abiotic or biotic control strategies.

This thesis work highlights the importance of eco-sustainable systems to control phytopathogens based on screening strategies of organisms capable to conferring hypovirulence to their host (Figure 1.1). The great spread of mycoviruses within the fungal host is allowing to deepen the knowledge on the host-virus relationship and therefore to identify viral strains and methodologies to cope phytopathogenic fungi.

On the other hand, strategies based on botanical pesticides (Figure 1.1) were studied in chapter 3. In particular, a rapid method of screening of substances contained in a bio-formulate was developed in order to investigate the potential of specific drug delivery systems for the control of phytoplasma causing flavescence dorée disease.

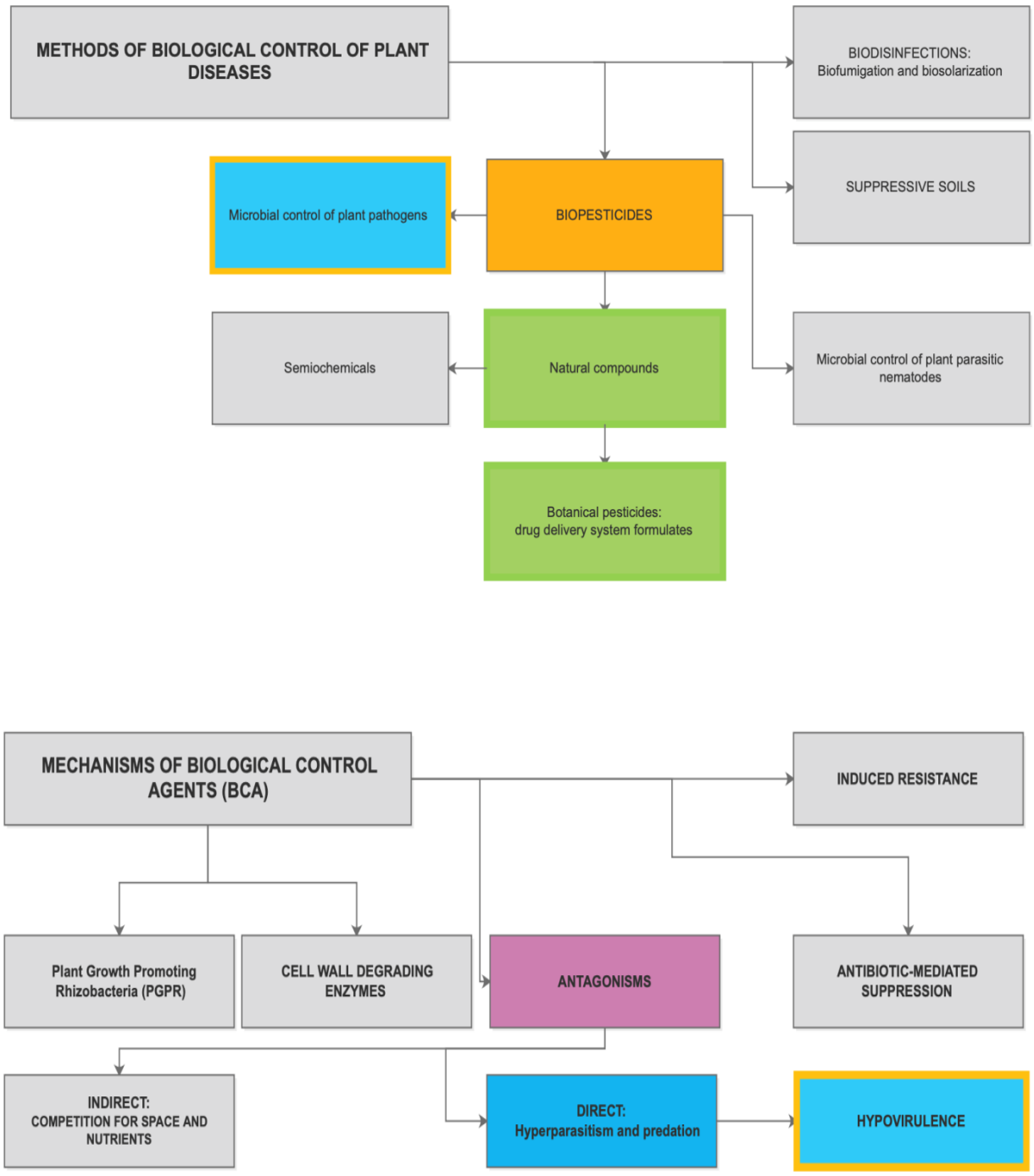

Figure 1.1 Schematic representation of biocontrol methods and the mechanisms used by biological control agents (BCA) to suppress phytopathogenic organisms. This map represents the guide to the thesis work. The theme in depth in chapter 2 was highlighted in blue, while the topic concerning chapter 3 was shown in green.

1.1 METHODS OF BIOLOGICAL CONTROL OF PLANT

1.1.1. Suppressive soils

The suppressive soils interest areas characterized by a non-pathogenic microbial flora which plays a role in competition and antagonism against phytopathogenic microorganisms.

Suppressive soils can be identified as areas in which the pathogen is present without causing serious damages to crops and over time the disease incidence decrease even if the pathogen persists in the soil (Sewell et al. 1985) suggesting that plants become moderately tolerant to pathogens.

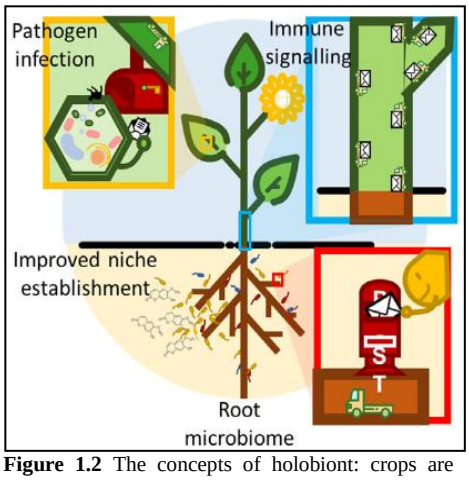

Figure 1.2 The concepts of holobiont: crops are functional units made up of plants and their microflora, which grows together contextually in various environments (Thomashow et.al, 2019).

Suppressive soils therefore represent a valid solution to the problem of soil "intoxication" and environmental infertility. The mechanisms by which this occurs are only partly known:

- Antagonism: bacteria that produce antibiotics e close to the root surface,
- Induced systemic resistance: a mechanism that closely resembles the induction of immune response in animals,
- Competition: microorganisms able to strongly compete with others for nutrients and for the colonization of ecological niches. These microorganisms colonize the surface of the root preventing access to other microorganisms, including pathogens,
- Predation and parasitism: in predation microorganisms kill the host to survive, during a parasitic relationship the host continues to live providing the parasite with nutrition,
- Production of siderophores: molecules that capture iron and make it unavailable to other microorganisms, which therefore cannot develop.

Some microorganisms can act also against insects, nematodes or weeds. The physical and chemical characteristics of the soil (Figure 1.2) can directly or indirectly influence the suppressing action versus plant diseases influencing the microbial activity but it has been shown that the functional holobiont established between the plant and its associated microbiome is directly associated with the action of soil suppression (Thomashow et al. 2019).

Fungal genera such as *Penicillium* spp., *Aspergillus* spp., *Ampelomyces* spp., *Candida* spp., *Coniothyrium* spp., *Gliocladium* spp., *Sporidesmium* spp. and *Trichoderma* spp. are known for their suppressive action against phytopathogenic fungi such as *Rhizoctonia solani*, *Phytophthora* spp., *Pythium ultimum, Rhizopus oryzae,* and *Sclerotium rolfsii*. One of the most versatile fungus acting in the suppression of diseases caused by phytopathogenic agents is *Trichoderma* spp., able to efficiently invade the rhizosphere producing antibiotics and capable to produce enzymes that degrade the fungal wall. This fungal genus also competes for nutrients with other organisms in the same area of growth producing auxin-like molecules that stimulate plant grow and, consequently, building a perfect holobiont system with its host (Howell 2002; Khan et al. 2004; Vinale et al. 2008).

Moreover, members of some bacterial genera such as *Agrobacterium, Arthrobacter, Azospirillium, Azotobacter, Bacillus, Enterobacter, Klebsiella, Pseudomonas, Serratia* and *Streptomyces* added to the soil can significantly reduce the incidence of diseases due to their antagonistic action (Nega, 2014; Gawai 2018).

The filamentous soil bacteria belonging to the genus *Streptomycetes*, can effectively colonizing plants from roots to leaves, exhibiting a direct or indirect action to defend the colonized host. Indeed, their ability to synthesize volatile organic compounds and antibiotics, combined with their endophytic nature, indicates these microorganisms as the perfect models of biocontrol agents (Vurukonda et al. 2018; Petkovic et al. 2017). Such antagonists, expressed by antibiotics and lytic enzymes production, by competition for food or by direct parasitism, prevent the pathogen to reach populations level able to cause severe disease (A. Hadwiger 2005).

1.1.2. Soil biodisinfection: biofumigation and biosolarization

Nowadays it is urgent to find alternatives to compounds of synthetic origin, such as methyl bromide, for soil disinfection. In this scenario the bio-disinfection is seriously taken into consideration as alternative (Palmero et al. 2011). In particular, two methods are used, the biofumigation, where the organic soil amendments, used during soil disinfection, are hydrolysed to chemical compounds with herbicidal and biocidal potential, and the biosolarization that exploits the solar radiation to generate high temperatures in the soil to obtain the sterilizing effect (Singh 2006).

Kirkegaard and collaborators have been the firsts to use the term "biofumigation" referring to the process involving the hydrolysis of compounds released from the return to the soil and subsequent maceration of the plant residues (Kirkegaard et al., 1993). In particular through the biofumigation the suppression of soil borne pests and pathogens occurs by biocidal compounds released when plants member of the family *Brassicaceae* such as cabbages (*Brassica oleracea capitata*), broccoli (*Brassica olearceae var italica*), rape (*Brassica napus*), cauliflower (*Brassica oleracea var botrytis*), chou mollier (*Brassica oleracea*), kale (*Brassica oleracea var acephala*), turnip (*Brassica campestris var rapa*), mustard (*Brassica juncea*) and radish (*Raphanus sativus*) are finely grinded and accumulate in the soil. The hydrolysis of macerated tissues produces sulfur compounds called glucosinolates, which are degraded in the presence of the enzyme myrosinase, releasing chemical substances with an effective biocidal action against fungi, nematodes bacteria, but also weeds (Bones and Rossiter 1996, 2006; Fahey et al. 1997). Total glucosinolate concentration generally decrease with the onset of flowering and reach the lowest contents at maturity, whereas spring-sown plants usually had greater concentration than those sown in autumn (Dutta et al. 2019; Matthiessen and Kirkegaard 2006).

Cyanogenic plants such as sorghum (*Sorghu bicolor (L.) Moench*) and Sudangrass (*Sorghum bicolor subsp. Sudanense (P.) Stapf*) also contain cyanogenic glucoside p-hydroxy- (S) - mandelonitrile-β-D-glucoside (dhurrin) which is a precursor for the activation of a secondary synthesis that produces hydrogen cyanide that has been proved to act as biofumigant (De Nicola et al. 2011). Plant families such as *Caricaceae, Moringaceae*, *Salvadoraceae* and *Tropaeolaceae* also exhibit a biofumigant action when harvested and prepared in the season in which they contain the higher concentration of glucosinolates at tissue level (Van Dam et al. 2009).

Therefore, biofumigation is one of the most used biological control methods and is considered as a possibility for reducing or even eliminating the use of pesticides.

Furthermore, despite this agronomic practice modifies the entire rhizosphere and consequently the holobiont system, it is not capable of repressing the growth of soil-borne organisms such as *Trichoderma* spp. which has been shown to be tolerant to compounds released by glucosinolate hydrolysis (Galletti et al. 2008).

The non-chemical approach of soil solarization has been used since 1940, even before the spread of the first synthetic pesticides (Newhall, 1955). Indeed, soil solarization is a hydrothermal process that occurs when solar energy is forced to persist in the ground due to the coverage of the ground with polyethylene sheet, chosen on the basis of its thickness, color, material quality and wavelength transmission capacity (Golzardi et al. 2014). The success of soil solarization is strictly dependent on physical and chemical factors between the temperature of the sun, exposure time to solar radiation, soil structure, but, above all, soil moisture (Al-Karaghouli and Al-Kayssi 2001). The temperature reached in this system is between 35-60° C, but to be effective it is important that the soil contains sufficiently moisture to a depth of 60-75 cm. For this reason, usually, it should be considered an irrigation phase before solarization (Stapleton and DeVay, 1986)

From the earliest experimental use, it was shown that in 14 days of coverage with transparent polyethylene film it was possible to obtain a reduction ranging from 94 to 100% of diseases caused by soil pathogens such as *Fusarium oxysporum* f. sp. *vasinfectum* and *Verticillium dahliae* (DeVay, 1991). As previous discussed, organic soil amendments can be used within the bio-fumigation approach but, when they are subjected to solarization, the process is called bio-solarization (Marín-Guirao et al. 2016). The solarization could be an approach suitable for reducing the use of pesticides in the field but it requires from 4 to 8 weeks to reach its full potential therefore, in areas where the period with the maximum solar irradiation is minimal, it is not possible to practice this form of biosterilization (Stapleton et al. 2016). Soil phytopathogenic infections can be very persistent and especially the intensive agriculture can cause the proliferation of various soil pathogenic organisms and consequently the accumulation of synthetic compounds, used to counter them, in the waters that seep into the soil. The different bio-disinfection techniques have an important eco-friendly role. The package for integrated management of soil pests therefore includes practices that lead to the reduction of chemical agents as much as possible.

1.1.3 Biopesticides

The approach discussed in this section concerns the role of biopesticides in controlling diseases caused by plant pathogens. Biopesticides are crop protection agents with biological origin produced by microorganisms or obtained from natural sources. They can be divided on the basis of active ingredients which include microorganisms, natural compounds (biochemicals substances) or semiochemicals (Chandler et al. 2011; Nega, 2014).

1.1.3.1 Microbial biopesticides

The microorganisms used in biological control (Biological Control Agents, BCA) are bacteria, fungi, oomycetes, viruses and protozoa which, generally, are able to effectively control phytopathogenic microorganisms and insects (OECD, 2009). The modes of action of the microorganisms exploited as biocontrol agents are different and not easily distinguishable but a classification can be hypothesized according to their ability to interact with environment that defines a potentially target plant. In general, the microorganisms that occupy the same ecological niche (antagonists) and the microorganisms that perform a direct action on the target, are well classified separately. Based on this subdivision, some characteristic examples of biological control have been highlighted. One of the most striking case of studies and applications concerns the entomopathogenic bacterium *Bacillus thuringiensis*. The biocide characteristic of this bacterium consists in the ability to secrete an endotoxin capable of inducing the lysis of the gut cells when it is ingested by a susceptible insect (Gill 1992). Other examples of the use of bacteria for the biological control of crops is the use of the *Agrobacterium radiobacter* K84 strain to control the crown gall disease caused by *Agrobacterium tumefaciens*. Moreover, *Bacillus subtilis*, *Pseudomonas fluorescens* and *Pseudomonas aurefaciens* are exploited for their ability to counteract diseases such as damping-off caused by *Rhizoctonia solani* and soft rots caused by *Erwinia carotovora* (Choudhary and Johri 2009; Haas and Défago 2005; Berg 2009).

The efficacy of entomopathogenic fungi play an active role as insecticides as there are at least 170 products based on fungi such as *Beauveria bassiana* or *Metarhizium anisopliae*. The latter is effectively used in Brazil against spittlebugs, locusts in Africa and grasshopper in Australia (Lomer et al. 2001; Li et al. 2010; Faria and Wraight 2007). Virocontrol, based on entomopathogenic viruses, is used in the USA and Europe against codling moth on apples, exploiting some viral strains belonging to the family *Baculoviridae*. These types of viruses are

particularly suitable for biological control as they only target invertebrates, are particularly virulent and not sensitive to environmental factors, as they are enclosed in a protein shell. The *Cydia pomonella GranuloVirus* (CpGV), once ingested by the codling moth larvae, reaches the intestine where, thanks to the alkaline pH, the protective protein matrix of the viral particle is dissolved. Then, the virus can pass through the intestinal wall, spreading throughout the body of larvae interrupting the activities of the vital organs. The larvae die within 5 days after virus ingestion, taking a flaccid, whitish appearance (Arthurs et al. 2005).

The cooperation between microorganisms has a resolutive role in the suppression of a phytopathology caused by pathogenic agents, In particular, in the case of diseases affecting the nutrient transport system a direct action against the pathogenic organism would be more effective with a preventive action. For this reason, the biological control approach of fusarium wilt and verticillium wilt, through the synergistic action of non-pathogenic *Fusarium oxysporum* Fo47 combined with the actinomycete *S. spectabilis* QLP12, was evaluated. The effectiveness of the two cooperating organisms derives from the competition exerted by the Fo47 strain and by the modification of the chemical-physical conditions of the soil in favor of the holobiontic relationship between plant and actinomyces (Zhang et al. 2018). Microbial antagonists such as the yeast *Aureobasidium pullulans* L1 and L8 strains are also used as control agents of post-harvest diseases resulting in up to 95% suppression of diseases caused by *Monilia laxa* on peach fruits. The extreme ubiquity and versatility to survive in different climatic conditions makes L1 and L8 strains a concrete example of biocontrol agents with an efficient antagonist activity (Di Francesco et al. 2018). More examples are shown by different species belonging to the genus *Trichoderma* used against phytopathogenic fungi, exploiting their ability to secrete biocide molecules such as antibiotics and enzymes able to degrade the cell wall of the pathogen. Those fungi can also compete for nutrients accessible in the soil such as carbon, nitrogen and other elements, but they can also produce compounds similar to auxins resulting in the stimulation of plant growth (Vinale et al. 2008). The specie *Trichoderma harzianum* has long been used for the control of *Rhizoctonia*, *Pythium*, *Fusarium* and other soil-borne pathogens by effective antagonism (Harman 2006).

Other BCA with antagonistic action against other phytopathogenic fungi are *Ampelomyces quisqualis*, *Verticillium lecanii*, *Nectra inventa* and *Gonatobotys simplex*. Another interesting example is the control of *Heterobasidium annosum*, the causal agent of root rot and wood caries, through *Phlebiopsis gigantea*. This is a subject of particular interest considering that the spores of

this fungus can be easily transmitted from one conifer to another as they can persist even in the oil of the chainsaws used for forest interventions (Kiss et al. 2004; Pratt et al. 1999). Moreover, a serious problem coming from the soil that afflicts crops are nematodes, phytoparasitic organisms with order lengths of 0.4-2 mm and diameters between 10 and 35 μm. Many phytoparasitic nematodes live in the soil attacking the hypogeal organs of plants (roots, rhizomes) but there are also species that feed on the epigeal parts (stems, leaves, flower buds) getting their nourishment exclusively from the host and causing not specific symptoms often difficult to diagnose, being common to those of other diseases The nematophagus fungus *Verticillium chlamydosporium* is able to infect nematode females and eggs but also to produce chymoelastase-like proteins that hydrolyse host proteins *in situ* (Segers et al. 1994; Kerry 2000). Even fungi such as *Arthrobotrys oligospora* and *Arthrobotrys dactyloides* can reduce the infection caused by nematodes using a specific hyphal net (Nordbring-Hertz 2004).

1.1.3.2 Natural compounds

The natural compounds used to control plant pathogens are called biopesticides. As described above there are microbial agents such as viruses, bacteria or fungi that can suppress other phytopathogenic organisms with various strategies but also compounds with vegetal origin (botanical pesticides), pheromones that inhibit the coupling of insects and secondary metabolites deriving from microorganisms (antibiotics) have to be considered as essential part of the biopesticide concept.

1.1.3.2.1 Semiochemicals

Semiochemicals are chemical compounds that regulate the interactions between living organisms. Semiochemicals are often able to induce behavioral modifications, but sometimes also alterations of physiological or, more rarely, anatomical nature. A characteristic of semiochemicals is to be active in their operation at low concentrations. Semiochemicals are generally classified into two main groups: pheromones and allelochemicals. Pheromones act at intraspecific level regulating communication within species while, on the other hand, allelochemicals have interspecific effect and mediate the communication between different species or higher groups up to the level of different kingdoms such as plants and animals. The most used semiochemical systems for plant protection are pheromones for insects monitoring and control by lure-and-kill approach and pairing interruption (Reddy et al. 2009; El-Sayed et al. 2009).

1.1.3.2.2 Botanical pesticides

Botanical pesticides derive from the extraction and purification of substances that are necessary for the metabolism or the defense of plants against plant pathogens. Therefore, essential oils, pyrethrum produced by *Chrysanthemum cinerariaefolium*, rotenone, neem oil, turnip seeds oil, quassia extract, nicotine, repellents such as citronella are just some examples of compounds used since centuries for the protection of plants from insects. Some compounds such as laminarine, fennel oil, lecithine are used as fungicides or herbicides (e.g. pine oil), germination inhibitors (e.g. caravay seed oil) and adjuvants such as spreader-sticker (e.g. pine oil) (Isman 2006; Isman and Machial 2006; Silvério et al. 2009). Essential oils (EOs) are synthesized through secondary metabolic pathways of plants and generally play important roles in direct and indirect plant defenses against herbivores and pathogens. Essential oils are often generated by the plant during the reproduction phase in order to attract pollinating insects, thus dispersing the seeds. EOs are synthesized and accumulated in appropriate organs such as trichomes, secretory cavities and resiniferous ducts (Regnault-Roger et al. 2012). Essential oils can be divided into terpenoids (monoterpenes, sesquiterpenes) and phenylpropanoids. Moreover, EOs are also of great industrial interest, especially for the preparation and formulation of biocompatible pesticides for the control of insects, bacteria, fungi and other microorganisms with different way of action (Burt 2004; Zabka et al. 2014).

Botanical pesticide applications have been made available on a large scale, demonstrating low toxicity for the environment and for the operators, permitting to define this approach as eco-chemical and biorational in plant protection systems (Dubey et al. 2010).

1.1.3.2.3 Bio-mimetic drug delivery systems

In recent years, new technologies allowed the generation of bio-mimetic drug delivery systems to enhance the efficiency of botanical pesticides. The idea behind the origin of the formulation is the same used in the medical field: carriers, also called drug carriers, are used to improve bio-availability, prevent premature degradation and enhance the uptake of a specific compound (Chen et al. 2016; Battiston et al. 2018). Based on this assumption, it was presumed to obtain similar results also when applied to plant defense. In particular, hydroxyapatite (HA) is the most widely used and one of the 11 known calcium orthophosphates with a Ca/P molar ratio between 0.5 and 2.0. HA is used in the medical field as a drugs and genes delivery agent but also as a filler for biocomposites. HA powder can be synthesized through many different methods including solid state and wet chemical methods, hydrothermal processes, mechanochemical techniques, pH shock waves, microwave processing, hydrothermal microemulsion or nanoemulsion techniques. An ion-covalent structural model of the apatite family shows a structure that can accept both cationic and anionic substituents, and for this reason, it is possible to substitute this mineral with many elements or molecules. This substitution induces a change in the crystallinity, without generating significant changes in the hexagonal apatitic system. The easy atomic doping or substitution in apatite has made available this mineral for a wide range of compositions (Šupová, 2015). In specific, HA used in the formulation with copper and zinc forms Cu-HA and Zn-HA complexes that were tested by Kim et al. (1998) for antimicrobial activity confirming the carrier capability of the HA.

1.2.MECHANISMS OF BIOLOGICAL CONTROL AGENTS

1.2.1 Antagonism: Hyperparasitism (direct antagonism) and Competition (indirect antagonism)

Biopestides act by different types of mechanisms, often difficult to group, such as antagonism which is the way of action of the micro-organisms responsible for biocontrol. In particular, direct antagonism is defined when the control agent become in contact with the pathogen or when it is extremely selective against it. An example is the strategy of hyperparasitism obtained employing obligate bacterial pathogens, hypoviruses, facultative parasites or predators to suppress phytopathogenic agents. Hyperparasitism also involves the protrusion of the BCA towards the phytopathogen, the consecutive wrapping and finally the killing after secretions of enzymes that prevent phytopathogen growing (Pal and Gardner, 2006). This phenomenon is largely exploited by *Trichoderma spp.* such as *T. harzianum* which is used to effectively control *Rhizoctonia solani*, thanks to its capability to act as a mycoparasitic fungus (Altomare et al. 1999).

The successful activity of a mycoparasite strictly depends on its ability to synthesize active enzymes, such as chitinases and β-1, 3 glucanases, often highly specific even against a single strain of the phytopathogen. Due to this specificity, mutations of the corresponding genes can results in a translation of a not functional enzyme with the consequent loss of the ability to control the pathogen (Harman et al. 1994; Gupta et al. 1995).

Several mycoparasites can also colonize the same pathogen such as in the case of *Acremonium altenatum, Acrodontium crateriforme, Ampelomyces quisqualis* and *Gliocladium virens*, fungi that have been studied for their capability to communally parasitize the causal agent of powdery mildew (Kiss 2003). Unlike hyperparasitism, some BCAs secrete lytic enzymes only in limited nutrient conditions, indeed, in experimental conditions in which fresh bark is made available, predators prefer this source of nutrients instead phytopathogens. In *Trichoderma* spp. the enzymes that degrade lignin are produced only when no other sources of nutrition are available allowing the direct attack of the phytopathogen *Rizoctonia solani* (Benhamou and Chet 1997).

Furthermore, also viruses can be used as control agents of phytopathogens if able to induce hypovirulent status on their hosts. Mycoviruses, for example, are viruses that parasitize fungi and many of the published research focused on fungi, and on their mycoviruses, with a considerable

economic importance, such as yeasts, cultivated mushrooms and pathogens of plants and animals. The main mycoviral model in the context of plant pathology is *Cryphonectria hypovirus 1* (CHV1), which has been successfully used as a biocontrol agent against the fungal pathogen *Cryphonectria parasitica*, responsible of the chestnut cancer (Nuss 2005; Turina and Rostagno 2007). The mycovirus-based biocontrol system will be deeply explored in Chapter 2.

Microorganisms that control pathogens through competition, strictly depends on the nutrients contained in the soil on which the plants are grown (Pal and Gardner, 2006). Therefore, the competition mechanism is considered an indirect relationship between the pathogen and its BCA when the biological control is based on the physical occupation of the site and on the depletion of nutrients (Lorito et al. 1994) as biocontrol agents, inside rhizosphere and phyllosphere, are much more efficient in absorbing nutrients than pathogens (Nelson 1990). This property is exploited by *Erwinia caratovora* as the siderophores of this protobacterium chelate Fe (II) and the membrane binds the receptor proteins, binding and adsorbing the siderophore-Fe complex. In this way the pathogen fails to have the necessary nutrients and, consequently, the infection decreases (Kn et al. 2012). This is also the case of a fast and efficient catabolism of nutrients inside the spermosphere by *Enterobacter cloacae* that contributes to the control of *Pythium ultimum* on seedlings (Van Dijk and Nelson 2000).

1.2.2 Antibiosis-antibiotics

Antibiotics are in most cases secondary metabolites produced by bacteria (2,900 different antibiotics), fungi (4,900) and actynomycetes (8,700) killing other microorganisms even at low concentrations (Bérdy 2005). In general, BCAs can produce three different types of antimicrobials: non-polar/volatile, polar/non-volatile and water-soluble. A single BCA can produce several compounds with antimicrobial activity, and this increases the chances of colonization of a site and makes the biocontrol agent more effective against plant pathogens. The most important characteristic of antibiotics is the extreme selectivity towards the target, in this way it is possible to preserve the other non-pathogenic micro-organisms not damaging crops (Islam et al. 2005; Thomashow et al. 2008). The strain of *Bacillus cereus* UW85 can produce several antibiotics such as zwittermycines and kanosamines giving an obvious advantage to suppress damping-off disease of alfalfa caused by *Phytophthora medicaginis* (Milner et al. 1996). Even genetically modified

Pseudomonas putida WCS358r can produce 2,4-diacetylphloroglucinol (DAPG) and phenazine-1-carboxylic acid (PCA), suppressing the diseases caused by different filamentous fungi that afflict wheat crops (Glandorf et al. 2001). Other examples of BCA with marked selectivity towards plant diseases are *Pseudomonas fluorescens* F113 capable to produce DAPG against *Pythium spp*. and *Agrobacterium radiobacter* that produces agrocin 84, against *Agrobacterium tumefaciens* (Kerr and Tate 1984). Moreover, *Bacillus subtilis* QST713 produces iturin A active against *Botrytis cinerea* and *R. solani*, while *B. subtilis* BBG100 produces mycosubtilin against *Pythium aphanidermatum* (Leclère et al. 2005; Paulitz and Bélanger 2001). *B. amyloliquefaciens* FZB42 produces bacillomycin and fengycin against *Fusarium oxysporum* (Koumoutsi et al. 2004). For *Trichoderma spp.* and closely related *Clonostachys* (former Gliocladium), 6-PAP, gliovirin, gliotoxin, viridin and many other compounds with antimicrobial activity have been investigated (Ghorbanpour et al. 2018; Köhl et al. 2019).

1.2.3. Secretion of Lytic Enzymes

Lytic enzymes are among the many varieties of compounds that microbial organisms release to interfere with the growth and activities of other microorganisms.

The target of these molecules is the hydrolysis of polymers (such as chitin, proteins, cellulose) as well as the interference with the biosynthesis of DNA. The cosmopolitan bacterium *Serratia marcescens* exploits the mediated action of the chitinase to control the pathogen *Sclerotium rolfsii*. Serratia marcescens has genes that code for different chitinases, so they have been used in multiple biocontrol experiments. In some cases, only one strain of this gram-negative bacterium is able to control *Botrytis* spp, *Rhizoctonia solani* and *Fusarium oxysporum* (Ordentlich 1988; Haran et al. 1996; Wang et al. 2013). Myxobacteria are also known to produce a large quantity of lytic enzymes which results in an antifungal action preserving the plant from diseases caused by fungal phytopathogenic agents such as *Phythium ultimum*, *Rhizoctonia spp*. and *Phytophthora capsici* (Bull et al. 2002). Additionally, *Lysobacter enzymogenes* C3 strain can produce lytic enzymes that degrade the cell walls of fungi and oomycetes, in particular beta-1,3-glucanase. Multiple *in vitro* tests demonstrated the viability of this organism in controlling *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet (Palumbo et al. 2005). Several strains of *Pseudomonas fluorescens* can produce antibiotics and siderophores but the *Pseudomonas fluorescens* CHA0 strain is also able to secrete cyanidric acid (HCN). This compound is extremely effective in

suppressing micro-organisms at the root level, as in the case of *Thielaviopsis basicola* causal agent of the black tobacco rot (Ramette et al. 2006).

1.2.4. Plant Growth Promotion Rhizobacteria (PGPR)

The bioinoculation by Plant Growth Promotion Rhizobacteria (PGPR) has led to the development of commercial products that are different each other mainly due to the way of action. In general, PGPR are classified in three main classes: biofertilizers, that improve nutrient acquisition; biostimulants, producing phytohormones and bioprotectors that suppress plant diseases (Ben Saad et al. 2016).

Bioformulates based on members of the genera *Azospirillium, Bacillus*, *Paenibacillus, Streptomyce*s, *Pseudomonas, Burkholderia* and *Agrobacterium* are already on the market not limited to the induction of systemic resistance but are also able to produce siderophores and antibiotics. Members of the genus *Azospirillium* and *Pseudomonas siderophores* can store nitrogen and iron and increase the plant absorption of these nutrients while *Bacillus spp.,* and other kinds of microorganisms, produce phytormones that can regulate root extension, increasing the surface available for nutrient absorption (Kang et al. 2019).

Additionally, the AN-27 strain of *Aspergillus niger* has been reported to produce growth-promoting compounds, 2- carboxy-methyl-3-hexyl-maleic anhydride and 2 methylen-3-hexyl-butanedioic acid (hexyl itaconic acid) directly involved in stimulating the growth of roots and shoot conferring more vigor to the plant. Finally, *Tricoderma viride* used since the seed coating increases considerably shoot, root and nodules in broad beans (Mondai et al. 2000; Woo et al. 2006).

1.2.5. Induced Systemic Host Resistance

Plants respond to innumerable chemical stimuli produced by the surrounding environment including the contact with BCAs, resulting in the production of biochemical signals designed to activate defenses and resistance mechanisms against infections caused by pathogens. The plant can mainly respond through three different ways: the hypersensitive response (HR), the Acquired Local Resistance (LAR) or the Acquired or Induced Systemic Resistance (SAR or ISR). SAR is generated through the release of salicylic acid which activates the expression of pathogenesis-related (PR) proteins and other enzymes. In ISR it is the jasmonic acid and/or the ethylene which mediates the cascade of signals following the contact with rhizobacteria. Salicylic acid and non-expressor of pathogenesis-related genes1 (NPR1) are key players in SAR as in the case of *Trichoderma harzianum* that after inoculation on to roots or on to leaves of grapes provides control of diseases caused by *Botrytis cineria* on leaves far from the site of application of the bio control agent (Deshmukh et al. 2006).

Plasticity adaptation of *Tricoderma spp*. makes this fungus very exploited also as an inducer of resistance due to the vast production of heterogeneous compounds.

A part of substances produced are proteins with enzymatic activities such as xylanase, cellulase and swollenin. Furthermore, a substantial increase in PR proteins was observed when the tricoderma endochitinase is released. The activation of defense mechanisms in plants after compound release is not limited to the production of PR proteins. Indeed, *T. virens* secretes a small hydrophobin-like protein (SM1) responsible for the induction of terpenoid phytoalexin biosynthesis and peroxidase activity in cotton plant (Martinez et al. 2001; Druege et al. 2007).

The knowledge regarding the number and the mechanism of compounds and chemical elicitors of SAR and ISR is increasing constantly.

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CHAPTER 2: '' FAST DISSECTION OF VIROME INFECTING *FUSARIUM SPP*. BY NGS APPROACHES''

2.1 INTRODUCTION

Mycoviruses are viruses that infect fungi and they can be found within the host as elements like viral particles (VLPs) but also as not encapsidated ribonucleic acid (RNA). Most of the known mycoviruses have dsRNA genomes packaged in isometric particles, but recently a growing part of known fungal viruses has been found to have filamentous or bacilliform shaped forms and an increasing number of positive- or negative-strand ssRNA and ssDNA viruses have been isolated and characterized (Pearson et al. 2009; Yu et al. 2013; Ghabrial et al. 2015). The majority of mycoviruses have quite simple genomes that usually consist in two genes, one encodes for the capsid protein and the other encodes for the replicase (some only have one gene for RNA replicase). While viruses of plants have long been recognized as important components of plant bio systems, mycoviruses have been largely ignored and their roles in fungi are pretty much unknown. Indeed, the number of mycoviral genomes fully characterized is small if compared to that of plant and animal viruses. Then using the term mycovirus, it is possible to separate viruses which are found in fungal cells, excluding the viruses that use fungi as a vector that have no the ability to replicate in the fungi (Rochon et al. 2004). Mycoviruses require a living cell to replicate, just as any other virus particle but unlike these aspects mycoviruses do not seem to have a movement protein, which is essential for the life cycle of animal and plant viruses. Furthermore, mycoviruses cannot be transmitted by extracellular routes, even if it was demonstrated the extracellularly transmission of Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1) (Yu et al., 2013), but they are transmitted intercellularly only through cell division, sporulation and cell fusion. Interesting, mycoviruses can be agents inducing hypervirulence behavior to their hosts. These modifications of the fungal status are now attracting a lot of attention, since it has been realized that they can serve as biocontrol weapon (Márquez et al. 2007; Dawe and Nuss 2013).

2.1.1 Origin of mycoviruses

The first record of a virus infecting a fungus has been reported more than 50 years ago: Sinden and Hausen described dieback disease on commercial mushrooms *Agaricus bisporus* found in a cultivation chamber of the company La France brothers (Sinden and Hausen 1950). A few years later 1962, Hollings isolated and transmitted the disease with particles, this event is considered to be the point of emergence in mycovirology. The origin of mycoviruses can be described by two different hypotheses. Firstly, the ancient co-evolution hypotheses, which suggest that these viruses and their fungi hosts emerged from the same ancestor and they evolved in parallel. Differently, the plant virus hypothesis implies that mycoviruses originate from plant viruses and regards mainly the mycoviruses found in pathogenic fungi (Ghabrial 1998). As mentioned above mycoviruses can cause hypovirulence, hypervirulence or they can have neutral activity in the hosts. In some cases, mycoviruses can interfere or enhance the production of secondary metabolites in fungi. This is the case of yeast on which a research performed at beginning of the 80nth century reported some strains with 'killer toxins', which leaded to the discovery of viruses associated with the presence of toxins in their host (Bevan et al. 1973). Later on, literature reports that toxins are not only found in yeast but also in several other fungi (Schmitt and Neuhausen 1994; Park et al. 1996).

Although the mycoviruses are widely distributed in nature, they lack of a transmission vector. Mycoviruses rely 'strictly' on horizontal and vertical transmission, which occurs in such efficiency that mechanisms of surviving out of the cells are not present (Ghabrial and Suzuki 2009). This statement has been partially correct since recently SsHADV-1 has proven to be an exception (Yu, 2013). The high rate of mutations within the mycoviral genomes brings substantial differences even among the strains of the same virus (Domingo and Holland 1997), while plant viruses are in a way to becoming more stabilized if compared to mycoviruses (García-Arenal et al. 2001). As described by numerous authors, this capacity is a highlighted advantage of the mycoviruses (García-Arenal et al. 2001; Schneider and Roossinck 2001).

Mycoviruses are strictly transmitted through intracellular means and horizontal transmission is restricted among closely related fungi members, suggesting that the relation between the two organism is ancient (Buck, 1998). Varga *et al.* (2003), theorizes a coevolution between mycoviruses and hosts, which are exploited for their biosynthetic machinery. For example, *Helminthosporium victoriae virus* 190S uses enzymes found in its host for the capsid protein

(Huang and Ghabrial 1996). On the other hand, the possibility that the virus originated from plant viruses has been considered. Varga et al. (2003), also suggest the possibility that the fungus acted as a vector and therefore transformed the virus into mycovirus, although it is more possible an acquisition of mycoviruses directly from the plant. Supporting this evidence *Cryphonectria parasitica* hypovirulence- associated RNA viruses sequences revealed correlation with several plant viruses from genus *Potyvirus* (Fauquet et al. 2005). Studies over RNA depended RNA polymerase from *Fusarium graminearum dsRNA* has found analogue among *Cryphonectria parasitica hypovirus* (CHV) 1-3 and *Barley yellow mosaic virus* (Chu et al. 2002; Linder-Basso et al. 2005). Up to this date not enough evidence are collected to reject one of the two hypotheses. Furthermore, both are needed to fully explain the origin. In any case nature gave remarkable examples, which gives the possibility that both can be correct and randomly aligned after millions of years. Up to this date most mycoviruses reported happen to be dsRNA and they can be found in the families of *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Reoviridae*, *Quadriviridae* and *Megabirnaviridae*. ssRNA mycoviruses families are *Alphaflexiviridae*, *Barnaviridae*, *Gammaflexiviridae*, *Hypoviridae*, *Narnaviridae*, *Endornaviridae* and a negative ssRNA family *Mymonaviridae*. There are also new proposed family of Alternaviridae, Fusariviridae and Fusagraviridae (Hisano et al. 2018; Wang et al. 2016) (International Committee on Taxonomy of Viruses (ICTV), 2018)

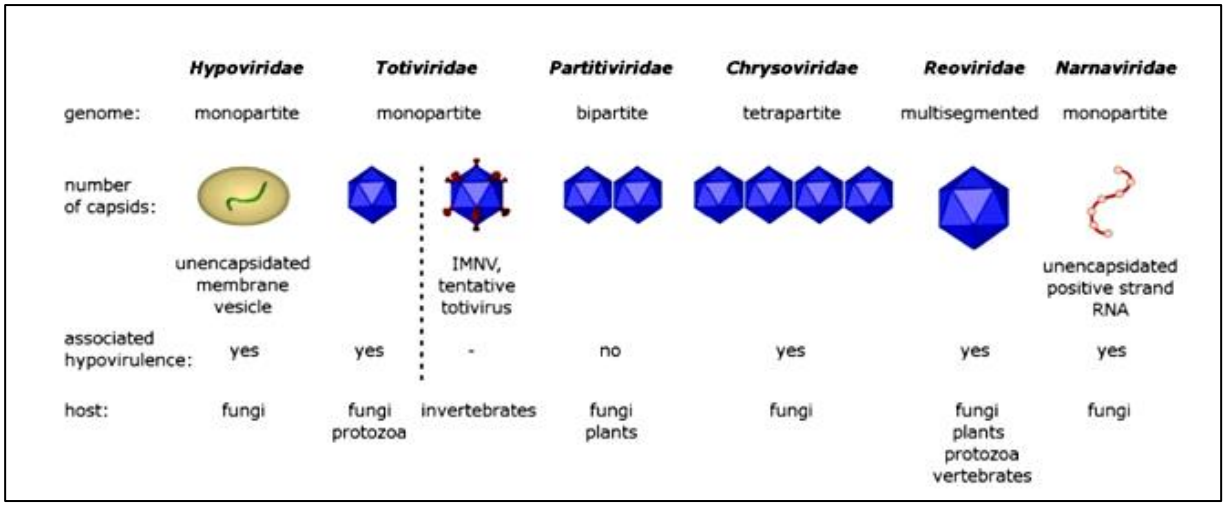


Figure 2.1 Characteristics and distribution of the most studied families of mycoviruses found in the hosts (Nuss, 2005; Van de Sande et al., 2010)

2.1.2 Distribution and transmission of mycoviruses in the hosts

The presence of a mycovirus in fungi is not a rare occasion but a very frequent situation. Profiles of nucleic acid can be found in a high diversity even among the same species. As literature reports, isolates of the same species in the same orchards or in the same trees can have different dsRNA profiles, which represent the mycoviral genomes (Tsai et al. 2004). A better representation of the diversity of profiles among fungal populations is given to Figure 2.1. Among mycoviruses, as in general among viruses, the presence of more than one viral species in the same host is frequent; co-existence of two or more mycoviruses can also be mutualistic. An example of this interrelation is described on *Agaricus bisporus*, which reported a high incidence ~60% of *Mushroom bacilliform Virus* (MBV) when *La France isometric Virus* (LIV) was present but in the absence of LIV the MBV was found only at 5%. This draws a direct relation between the two viruses (Romaine and Schlagnhaufer 1995).

Naturally transmission, in mycoviruses exists in two forms. The vertically transmission is the transmission through spores, in both sexual and asexual reproduction; where asexual sporulation refers to creation of spores from modified hyphal (Buck, 1998). The mycoviruses transmission efficiency in the sexual spores form can reach up to 100% as reported for *Fusarium graminearum* (Chu et al. 2004), but generally efficiency varies between different combinations of virus-hosts as well as the types of sporulation (sexual or asexual) (Buck, 1998). Fungal species which undergo sporulation more often show less success in the transmission (Varga et al., 2003). Finally, regarding vertical transmission no repeated frequencies are recorded. The second type of transmission is called horizontal and it describes the transmission of the virus through fungal anastomoses (Suzaki et al. 2005; Xie et al. 2006).

Anastomosis is the process of vegetative hyphal fusion for most filamentous fungi that can take place between two individual fungi. When this happen cell materials can be transferred from one member to another and vice versa. However, anastomosis does not happen between random fungal members since these members must belong to the same vegetative compatibility group (VCG). As far as ascomycete fungi matter, VCG is controlled by the loci *vic* and *het*, vegetative incompatibility and heterokaryon incompatibility respectively (Figure 2.2)(Saupe 2000; Glass and Dementhon 2006). VCGs act as a mechanism to regulate horizontal transmission between

individuals; they can create an obstacle even among members of the same species as demonstrated on CHV-1 (Milgroom and Cortesi 2004). In the case of incompatibility, cells undergo a self-destruction process. Some asymmetrical transmissions lead to the assumptions that compatibility reactions are regulated from both the fungus and the virus (Biella et al. 2002). This may give a possible explanation on experimental results demonstrating transmission of mycoviruses from *Fusarium poae* to black *Aspergillus* species through protoplast fusion (van Diepeningen et al. 2000). However, compatibility of different species was predicted since a virus of a known host has been recorded on another fungus, as for CHV 1 (Ikeda et al. 2005). Horizontal and vertical transmission regarding mycoviruses is powerful enough to maintain virus continuity at sufficient levels so they can survive in absence of any extracellular transmission mechanisms (Pearson et al. 2009). Means of transmission have drawn a massive attention in mycoviruses research in the past years as it is a crucial part in turning the virus in a bioweapon for agriculture. In the plant-fungi-virus interactions the effect of a virus can range from causing hypovirulence, cryptic existence or hypervirulence. Unfortunately, not all mycoviruses discovered have a detailed report about their influence on the host, this is because of various obstacles such as when phenotypic observations are correlated with a co-infection by 2 or more viruses. A notable example of co-infectivity is *Botrytis virus X* (BVX) and *Botrytis cinerea virus F* (BCVF) in *Botritys cinerea* (Howitt et al. 2006).

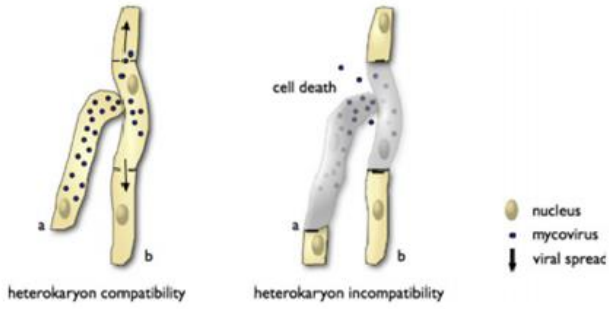


Figure 2.2 Transfer of mycoviruses between fungal hyphae is dependent on the compatibility of the donor (a) and acceptor (b) hyphae upon contact. (Source : Van De Sande et al. 2010).

Among the range of influence, the term cryptic instead of no influence was used is the more appropriate one, since these viral presence has no phenotypic influence. Regardless to directly phenotypic observations, it makes sense to assume that all viruses have even the slightly impact

on their hosts (Pearson et al., 2009). It has been demonstrated on *Aspergillus* spp. strains that sporulation, growth rate and competitive ability is affected at minimum statistically significant level (van Diepeningen et al. 2006). Environmental conditions have a direct impact on the infectivity of the virus, in some conditions it can be cryptic and in other can cause virulence (Pearson et al., 2009). The majority of mycoviruses, unfortunately, are cryptic. This is not a surprise, since a virus is an organism having the need to survive and replicate in the hosts. Hence, causing hypovirulence on their hosts is not considered as a wise strategy for the virus (McCabe et al. 1999). This phenomenon can be described with the term symbiosis; which is defined as two or more different entities coexisting (Bary 2019). Hypovirulence is a state of reduced fitness of the host. Model of hypovirulence behavior is the case of *Cryphonectria parasitica* when infected with viruses of the genus *Hypovirus*. The phenotypic hypovirulence induced in this situation is referred to reduced sporulation, growth rate and competitive ability (Nuss 2005). Aside to these influenced parameters, laccase activity can be decreased, and invasiveness of the fungus can be restricted; as it was described for a dsRNA from a work of Castro *et al.* (2003). The diversity of mycoviruses strategies are related to different mycovirus groups (Xie et al., 2006). An idea of induced hypovirulence behavior related to the fungal host and the presence of dsRNA was easily rejected from studies like the one from Van Diepeningen (2006). This study examined 668 strains of *Aspergillus* spp. and found one dsRNA-infected strain without the ability to sporulate, no other strain when infected by the same dsRNA showed a phenotypic hypovirulence. In contrast to hypovirulence, there is hypervirulence; which is the exact opposite. This group of mycoviruses deserves an equal attention as the hypovirulence since they can be agents of an extra problem for many agricultural systems. For example, the presence of BVX in some strains was demonstrated, *in vitro*, to improve its host's fitness by increasing the growing speed (Tan et al. 2007). Another example showing positive results for horticultural purposes is *Curvularia Thermal tolerance virus* (CThTV). This mycovirus was found to increase the thermal resistance of the host fungi *Curvularia protuberate*, but also of the plant that host this fungus, *Dechanthelium lanuginosum* (Márquez et al. 2007). And lastly a dangerous hypervirulence symbiosis is the promotion of killer phenotypes in the yeast and *Ustilago* spp. (Wickner 1996).

2.1.3 Vegetative Incompatibility

The natural host range of mycoviruses is likely to be restricted to the same or closely related vegetative compatibility groups that allow lateral transmission. This phenomenon is governed genetically and controlled by a series of gene loci known as *vic* (vegetative incompatibility) or *het* (heterokaryon incompatibility). The existence of compatibility groups creates a natural barrier to horizontal transmission of mycoviruses, making this gene-connected relation a complication for using hypovirulent mycoviruses as biological control agents. When a virus-infected strain contacts a virus-free strain from a not compatible group, an incompatibility response occurs, often leading to programmed cell death (PCD), which limits the transmission of mycoviruses. Interspecies transmission has been reported between members of the same fungal genus including *Cryphonectria, Sclerotinia,* and *Ophiostoma* spp. that share the same habitats, but it is rare that this would happen in nature. Recent researches have demonstrated that the seven *vic* genes associated with five of six *vic* loci in *C. parasitica* contribute to incompatibility and affect virus transmission. For instance, the prototype mycoreovirus (mycoreovirus 1) can replicate and induce phenotypic alterations in different vegetative compatibility groups of the chestnut blight fungus *C. parasitica* (Zhang et al. 2014).

2.1.4 Concept of hypovirulence

Strains of plant pathogenic fungi differ in their capability to induce various severity of disease symptoms or degree of virulence on their host. Strains of a pathogen may be classified according to their virulence (Table 1), ranging from avirulent to virulent strains, and, in between, low virulent strains which are considered hypovirulent. These groups of viruses can reduce the ability of the pathogenic fungus to cause a disease. This phenomenon is very attractive due to the importance of fungal diseases in agriculture and the limited strategies that are available to control them. Hypovirulent fungal strains are a promising biocontrol agent for management of fungal disease by attenuating virulence of a phytopathogenic fungi. This strategy consists in transmitting copies of mycoviral nucleic acid from hypovirulent to virulent strains so that the virulent ones becomes hypovirulent.

Table 1. List of mycoviruses able to cause hypovirulence. Source (García-Pedrajas et al. 2019)

Mycovirus acronym/source fungus	Phenotypic effects on the fungal host[a]	References[b]
Family *Hypoviridae*: unencapsidated and monosegmented (+) ssRNA genome		
CHV1[c]/*Cryphonectria parasitica*	Strain CHV1/EP713: strong hypovirulence,[d] reduced growth and pigmentation, drastically reduced conidiation Strain CHV1/Euro7: moderate hypovirulence,[d] reduced conidiation and pigmentation Strain CHV1/EP721: moderate hypovirulence,[d] reduced conidiation and pigmentation	Hillman and Suzuki 2004; Lin et al. 2007
CHV2/*Cryphonectria parasitica*	Strain CHV2/NB58: strong hypovirulence,[e] marked debilitating effect on the fungus, slow growth, isolates brown in color, thin mycelium, reduced conidiation	Hillman and Suzuki 2004; Smart et al. 1999
CHV3/*Cryphonectria parasitica*	Strain CHV3/GH2: strong hypovirulence,[e] colonies often exhibit lobed margins, normal sporulation, orange pigment production	Hillman and Suzuki 2004; Smart et al. 1999
SsHV1/*Sclerotinia sclerotiorum*	Strong hypovirulence,[e] abnormal colony morphology, enhanced pigmentation; phenotypic alterations are only observed in isolates coinfected with an SsHV1-associated sat-like RNA	Xie et al. 2011
SsHV2/*Sclerotinia sclerotiorum*	Strain SsHV2/5472: hypovirulence[e] Strain SsHV2/SX247: hypovirulence,[f] reduced growth, abnormal colony morphology Strain SsHV2L (potential recombinant of SsHV2 and an unknown hypovirus): hyporivulence,[d] delayed maturation of sclerotia	Hu et al. 2014; Khalifa and Pearson 2014; Marzano et al. 2015
FgHV2/*Fusarium graminearum*	Hypovirulence,[e] reduced growth and conidiation, reduced DON production	Li et al. 2015
AsHV1[c]/*Alternaria alternata*	Hypovirulence,[e,f] reduced growth	Li et al. 2019
Family *Alphaflexiviridae*: encapsidated and monosegmented (+) ssRNA genome		
SsDRV[g]/*Sclerotinia sclerotiorum*	Hypovirulence,[e,f] reduced growth, abnormal colony morphology	Xie et al. 2006
Family *Botybirnaviridae*: encapsidated and bisegmented dsRNA genome		
SsBRV2/*Sclerotinia sclerotiorum*	Hypovirulence,[h] reduced growth, absence of sclerotia	Ran et al. 2016
BpRV1/*Botrytis porri*	Hypovirulence,[h] reduced growth	Wu et al. 2012
BmBRV1-BdEW220 (strain of Bipolaris maydis botybirnavirus 1)/*Botryosphaeria dothidea*	Hypovirulence,[e,f] reduced growth, abnormal colony morphology	Zhai et al. 2019
Family *Chrysoviridae*: encapsidated and multisegmented dsRNA genome		
FgV-ch9/*Fusarium graminearum*	Hypovirulence,[h] reduced growth and conidiation, reduced perithecia formation	Darissa et al. 2012
BdCV1/*Botryosphaeria dothidea*	Hypovirulence,[e,f] reduced growth	Wang et al. 2014
FodV1/*Fusarium oxysporum* f. sp. *dianthi*	Hypovirulence,[f] reduced growth and conidiation	Lemus-Minor et al. 2018, 2019; Torres-Trenas et al. 2019

Mycovirus acronym/source fungus	Phenotypic effects on the fungal host[a]	References[b]
MoCV1-A/*Magnaporthe oryzae*	Hypovirulence,[f] infection promotes host race conversion	Aihara et al. 2018
Family *Endornaviridae*: unencapsidated and monosegmented (+) ssRNA genome		
HmEV1-670/*Helicobasidium mompa*	Hypovirulence,[e,f] alterations in growth not reported	Ikeda et al. 2003; Osaki et al. 2006
RsEV1/*Rhizoctonia solani*	Hypovirulence,[f] thin mycelium, small sclerotia, dark pigmentation	Zheng et al. 2019
SmEV1/*Sclerotinia minor*	Hypovirulence,[f] reduced growth and sclerotial production	Yang et al. 2018
Family *Megabirnaviridae*: encapsidated and multisegmented dsRNA genome		
RnMBV1[c]/*Rosellinia necatrix*	Strong hypovirulence,[h] reduced growth. A variant with genome reorganization lost the ability to induce hypovirulence	Chiba et al. 2009; Kanematsu et al. 2014
RnMBV2/*Rosellinia necatrix*	Hypovirulence,[h] reduce growth and irregular colony morphology, when coinfected with partitivirus RnPV1	Sasaki et al. 2016
Family *Narnaviridae*: unencapsidated and monosegmented (+) ssRNA genome		
BcMV1/*Botrytis cinerea*	Hypovirulence,[e,f] reduced growth, greatly reduced conidial production, absence of sclerotia	Wu et al. 2007; 2010
SsMV1/*Sclerotinia sclerotiorum*	Hypovirulence,[f] reduced growth, reduced sclerotia production	Xu et al. 2015
Family *Partitiviridae*: encapsidated and bisegmented dsRNA genome		
SsPV1[c]/*Sclerotinia sclerotiorum*	Hypovirulence,[f,h] reduced growth, reduced sclerotia production, cytoplasm leaks at hyphal tips	Xiao et al. 2014
RsPV2/*Rhizoctonia solani*	Hypovirulence,[h] reduced growth	Zheng et al. 2014
HetPV13-an1/*Heterobasidion annosum* complex	Hypovirulence (reduces the ability of *H. parviporum* to colonize wood),[e,f] reduced growth, increased hyphal branching	Vainio et al. 2018
BcPV2/*Botrytis cinerea*	Hypovirulence,[f] absence of conidia and sclerotia	Kamaruzzaman et al. 2019
Family *Reoviridae*: encapsidated and multisegmented dsRNA genome		
RnMYRV-3(W370)[c]/*Rosellinia necatrix*	Hypovirulence,[e,f,h] reduced growth, virus-transfected strains exhibit "mosaic" colonies due to areas with different growth rate	Kanematsu et al. 2004; Sasaki et al. 2007; Wei et al. 2003
Family *Totiviridae*: encapsidated and monosegmented dsRNA genome		
HmTV1-17/*Helicobasidium mompa*	Hypovirulence,[f] effects on growth not reported	Suzaki et al. 2005
HvV190S[c]/*Helminthosporium victoriae*	Hypovirulence,[h] reduced growth, abundant white aerial mycelium, sectored colonies	Xie et al. 2016
PdV1/*Penicillium digitatum*	Hypovirulence,[e,h] reduced growth	Niu et al. 2016
dsRNA and (+) ssRNA mycoviruses not assigned to a known family		
FgV1[c]/*Fusarium boothii* (formerly *F. graminearum* strain DK21)	Hypovirulence,[f] reduced growth, increased pigmentation, decreased production of trichothecene mycotoxins	Cho et al. 2013
BcRV1, monopartite dsRNA virus/ *Botrytis cinerea*	Hypovirulence,[f] reduced growth	Yu et al. 2015
BdRV1, dsRNA virus 5 segment genome/*Botryosphaeria dothidea*	Hypovirulence,[f] reduced growth, sectored colony edges, absence of picnidia	Zhai et al. 2016
Family *Mymonaviridae*: encapsidated and monosegmented (–) ssRNA genome		
SsNSRV-1/*Sclerotinia sclerotiorum*	Hypovirulence,[h] reduced growth, absence of sclerotia	Liu et al. 2014
DNA mycoviruses: encapsidated and monosegmented circular ssDNA genome		
SsHADV-1/*Sclerotinia sclerotiorum*	Hypovirulence,[i] reduced growth, small sclerotia, abnormal colony morphology	Yu et al. 2010

2.1.5 Viral effect on the host

Since the first discovery of their presence, mycoviruses have been found frequently and numerously among fungi, but, they do not seem to affect the host phenotypically (Ghabrial 1998) even though there are several well-documented cases where infection results in marked structural and/or physiological changes. Up to now only few strains of mycoviruses have shown hypovirulent capability, although their high presence in fungal cells, mycoviruses are symptomless, latent and persistent. Latency benefits the host for survival while persistence helps the virus to live in absence of extracellular modes of transmission. To ensure their retention, some mycoviruses have evolved to bestow selective advantage to their host (e.g. killer phenotypes in yeasts and smuts). Macroscopic symptoms caused by fungal viruses are a consequence of alterations in complex physiological processes that involve interactions between host and viral factors. Mycoviruses can change the phenotypes of their hosts providing some advantages or deleterious effects, but as said before ,they normally do not produce obvious symptoms on their host. Because of the lack of an infectivity assay and the frequent occurrence of mixed infections, there is a problem with assigning any fungal phenotypic change to a pecific mycovirus (Romaine and Schlagnhauffer, 1995; McCabe et al., 1999; Howitt et al., 2006). Some mycoviruses, however, cause dramatic changes in their hosts, including irregular growth, abnormal pigmentation and altered sexual reproduction. The most interesting effect is the reduced virulence (hypovirulence) of plant-pathogenic fungi.

2.2 MYCOVIRUSES OF SIGNIFICANCE AND CASE STUDIES

2.2.1 *Cryphonectria parasitica*-Chestnut blight

Chestnut Blight is the diseased caused by *Cryphonectria parasitica*. The disease has been reported on *Castanea dentata, C. sativa, C. mollissima, C. crenata* trees, important in providing chestnuts, wood and considered to have a high aesthetic role in people's life. This pathogen in the decade of 1960s caused almost total extinction of *C. dentata* in eastern USA (Hepting 1974; A. Hadwiger 2005).

2.2.2 Biology

C. parasitica is an ascomycetes fungus that is spreader throughout wind, rain, beetles (*Agrilus* spp.) and birds. The primary infection is established from wounds created by insects' activity. The fungus can be found near the cambium region within the bark of the tree. One of the characteristics of this fungus, that makes it dangerous, is the ability to survive as saprophyte in numerous other species beside its true host (Darpoux et al., 1975). Conidia are produced through pycnidia and spread by rain. Their size ranges from 2 to 3 μm, they have an almost straight shape and have variation of yellow colorings. The fungus can also produce ascospores through perithecia (Figure 2.3), found on the soil. Ascospores have a glass-like look, they are composed out of two cells, with a thinner layer near the septum and they size 10 x 4 μm. The mycelium can live on dried wood almost 10 months (Hepting, 1974) and the fungus does not interact with the fruit but only with the nutshell (Jaynes 1984).

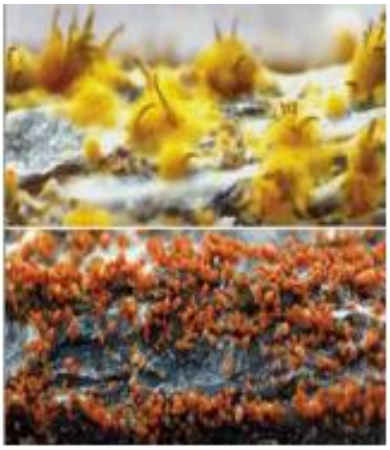


Figure 2.3 Mature perithecia (sexual fruiting bodies) with protruding ostiolar necks formed by the chestnut blight fungus *Cryphonectria parasitica* on chestnut tree twigs following mating of two strains of the opposite mating type (top panel). Disruption of the hypovirus-responsive *C. parasitica* gene encoding the Zn(II)2Cys6 transcription factor PRO1 results in female infertility, i.e., the inability to form perithecia when the mutant strain serves as the female partner of a sexual cross (bottom panel). Source: Eukaryotic Cell – American Society for Microbiology.

2.2.3 Symptoms

The fungus creates a canker at the bark of branches (Figure 2.4). This canker is creating a full circle around the branches causing leaves to wilt, but they do not drop. The growing speed of the canker can be so fast that the plant cannot restrict it with the formation of callus. By the time the canker is created, the tree develops adventitious shoots underneath the dead area (Darpoux et al., 1975).

Figure.2.4 On the left, cortical cancer on a young chestnut is reported. The tree tries to heal the infection, recognizable by the reddish color of the bark. The infection caused by *C. parasitica* compromises the phloem and tissue tissues, inducing in the tree the formation of epicormic twigs below the point of infection (Rigling and Prospero 2018).

2.2.4 The first successful use of mycoviruses

As mentioned before, in 1938 the fungus was introduced in Europe through Italy (Heiniger and Rigling 1994). Unlike eastern USA, in Europe the fungus was expressed differently. Chestnut trees in Italy were restricting the fungus and there were no devastating symptoms. A closer look to the fungus showed different pigmentation, less dangerous cankers and suppressed sporulation (Grente, 1965). In 1978 it was proposed to release the fungus infected with the virus by Grente, the idea has been proven successful. Some orchards still today use fungus infected with the virus as a biocontrol agent with success.

2.2.5 Compatibility

The use of mycoviruses has been successfully applied mainly in EU. This is because compatibility between individual strains of *C. parasitica* is controlled with specific alleles of the *vic* loci (Milgroom and Cortesi 1999). In the case that these alleles do not pair among two strains, anastomosis becomes programmed cell death (Biella et al. 2002; Glass and Kaneko 2003).

Literature reports that *in vitro* and *in vivo* comparisons of the transmission rates do not match, with *in vivo* experiments resulted more successful. The reasons for this are upon various parameters such as the contact time among the donor and the host target (Carbone et al. 2004). However, rates of transmission can be predicted from the available alleles; each one has a proportional influence (Cortesi et al. 2001). A case study has demonstrated the non-respective rate of transmission between *in vivo* and *in vitro* experiments: numerous individual members of *C. parasitica* belonging to separate VCGs has been collected and subjected to comparison (Figure 2.5). Transmission trials showed 14% success *in vitro* and 44% success *in vivo* (Brusini and Robin 2013). These results verify the assumptions made in the past from several authors that the ability of mycoviruses to be transmitted in natural ecosystems is underrated (Hogan and Griffin 2002; Carbone et al. 2004; Milgroom and Cortesi 2004; Robin et al. 2010). However, recently specific primers have been designed to address the compatibility without the need of *in vitro* or *in vivo* trials (Short et al. 2015). All these reasons explain the successful applications of hypovirulent strain in the EU but not in the USA where, only in Connecticut and West Virginia more than 90 VCGs are recorded (Anagnostakis 1987; Milgroom et al. 1991). In contrast, all European regions report less than 35 VCGs members distributed in different countries (Anagnostakis 1986).

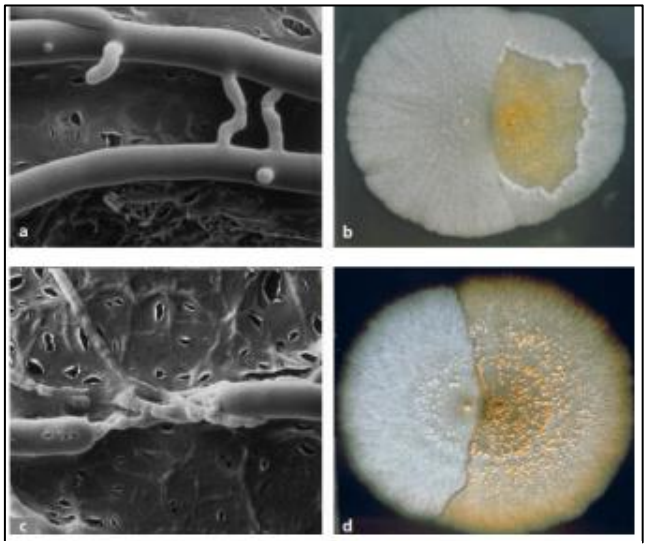


Figure 2.5 Virus-free *C. parasitica* cultures have an orange pigmentation and numerous asexual (picnidia) sporulations. Instead, cultures of virus-infected strains are whitish in color and no picnidia. In case of transmission of the virus through the formation of hyphal anastomosis (a) between the vegetatively compatible fungal strains, the infected strain acquires the morphological characteristics of a hypovirulent strain (b). In the case of vegetative incompatibility between the two fungal strains, the hyphae degenerate in the contact area (c), preventing the transmission of the virus (d). Source (Rigling et al., 2018)**.**

2.2.6 Viruses found in *Cryphonectria parasitica*

C. parasitica serves as a host for several mycoviruses, ranging from the families of *Hypoviridae, Reoviridae, Narnaviridae, Chrysoviridae, Partitiviridae* and more, that are currently unclassified (Hillman and Suzuki 2004; Liu et al. 2007). From the pool of mycoviruses reported as infectious for *C. parasitica*, CHV1, from the family *Hypoviridae,* is considered as the most important (Allemann et al. 1999). A wide variation of interactions with the host within its closely related members CHV2, CHV3 and CHV4 is reported and could have been expected (Chung Pei Hua et al. 1994; Enebak et al. 1994; Peever et al. 1998; Allemann et al. 1999) but a diversity of such effects has been also observed even among individual strains of CHV1. These effects are mainly concerning symptom expression and RNA accumulation (Lin et al. 2007). The noble strain of CHV1 is EP713, it is proven as the most effective for biological control because of its persistence, distribution and its ability to restrict sexual and asexual sporulation of the host (Zhang et al. 1998; Milgroom and Cortesi 2004). The distribution of EP713 occurred mainly with host fusion in the short distance and through spores carried from vertebrates at long distances (Heiniger and Rigling 1994). Detailed studies using microarray technologies over the use of CHV1-EP713 have been done by Allen and Nuss in 2004. These studies revealed that half of the gene expression influenced by this mycovirus was also influenced by mitochondrial mutations (*mit2* gene). Here the authors note that 68 of the 70 common influenced genes were correlated increased or decreased. This finding suggests that hypoviruses causes mitochondrial abnormalities. Reduced pigmentations and less asexual sporulation are hypothesized to be related to retaining the fungus in a juvenile state (McCabe and Van Alfen 2001). Evidence supporting this statement came in 2009 from Dawe and collaborators, when they used analytical methods of small biomass quantities to measure physiological changes. They captured the respiratory quotient and the ratio of CO_2 production related to O_2 consumption. Their results showed that strains infected with CHV1-EP713 did not change their metabolism with age as in the case of virus-free strains. Research over the changes of metabolism provided evidence for the alterations of the molecular compounds found in the fungal cell. A study listed 165 fungal compounds and reported that when host was infected with CHV1 the 33% of these compounds were changed. Additionally, the profiles of these compounds are corresponding to the hypovirulence behavior (Dawe et al. 2009). These findings can be used to address the question of why the virus is causing hypovirulence behavior to its host. Dawe and Nuss

in 2013 in their review speculate if these manipulations are enhanced for providing a suitable membrane surface material on which RdRp undertake its function.

2.2.7 *Botrytis cinerea* case

B. cinerea is a pathogen in the phylum *Ascomycota*, infecting several of the most important horticultural species (Figure 2.6). It is estimated that the worldwide losses due to this pathogen are estimated above 10 billion dollars. Its behavior is considered necrotrophic and it developed mechanisms to offset its hosts SAR responses. Following asexual sporulation, massive amounts of spores are released. When these spores land on the plant germinate forming appressorium. From there on, the fungus goes through the plant epidermis. To ensure the success of the penetration the fungus excretes cutinases, lipases and H_2O_2 (Elad et al. 2007). Until now, the control of this pathogen has been done with the use of chemical compounds. Side strategies such as removal of infected material and reduction of humidity has positive effects; but is not enough to replace pesticides. Like for all pathogens, horticulturists are called to address this issue therefore the *B. cinerea* control needs to be redesigned with modern standards requiring reduced input of industrial compounds. To fulfill this gap scientist are looking at the use of microorganisms. Mycoviruses are one of the options available for suppressing this pathogen (Pearson and Bailey 2013). In the past few decades, several authors published a number of interesting papers regarding viruses found in *Botrytis* spp. with the potential of practical application (Castro et al. 1999, 2003; Wu et al. 2007, 2010, 2012). Yet, reported hypovirulence is far from commercial use, since *B. cinerea* has more than 65 VCGs that create transmission problems (Beever and Weeds 2007).

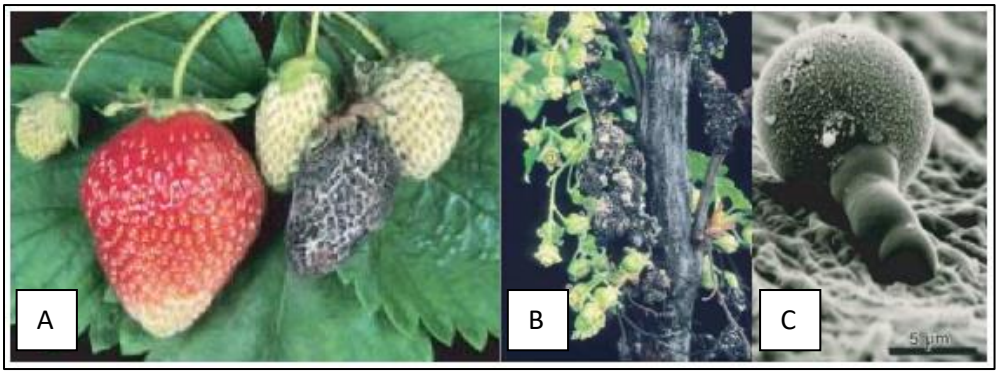


Figure 2.6. Redrawn from Williamson et al., 1995; 2007. Picture (A) shows B. cinerea infection on strawberries and and the effects on grapevine are also shown (B). In picture (C) a germinating conidia of *B. cinerea* was observed through scanning electron microscope (SEM).

2.2.8 Viruses found in *B. cinerea*

Many different dsRNAs were found in *B. cinerea*, however, only a small portion of them has been sequenced or even characterized as a virus. In particular Howitt and collaborators, from New Zealand, demonstrated the abundance of mycoviruses in *B. cinerea* by reporting 143 members with ds RNAs in 200 isolates, but from the pool of dsRNAs they studied only 5 of them (Howitt et al. 1995). Another research group detects dsRNAs that were associated with both 28 and 33 nm isometric particles (Castro et al., 1999, 2003). Then the first virus isolated from *B. cinerea* belonging to the *Mitovirus* has been characterized resulting a not encapsidated dsRNA (Wu et al., 2007, 2010). Elsewhere, two ssRNAs from the family *Flexiviridae* were reported. These viruses are *Botrytis virus F* (BVF) and *Botrytis virus X* (BVX) (Howitt et al., 2001, 2006), *Botrytis cinerea mitovirus 1* BcMV 1, *Botrytis cinerea mitovirus* 1-S BcMV 1-S and *Botrytis porri RNA virus* 1 BpRV 1 (Wu et al., 2007, 2010, 2012).

2.2.9 Hypovirulence on *Botrytis cinerea*

Castro and his colleagues (1999, 2003), reported *B. cinerea* isolates infected with a virus showing degeneration, reduced sporulation, less production of laccases and fitness compared with isolates without dsRNA. The affected strains produced unusual mitochondria. Generally, lab growth was reduced by 3 times, sporulation was restricted and no sclerotia were observed for 15 days (Wu et al., 2007, 2010). BVX and/or BVF were studied over the *B. cinerea* isolate B05-10 and reported important changes in some synthesis of macro-molecules. Less threonine and glycine amino acids were produced, while homocysteine, norvaline and methionine were increased (Boine 2012).

2.2.10 *Rosellinia necatrix* case

R. necatrix is an *Ascomycete* infecting numerous perennial species. The pathogen mainly develops on its hosts roots (Figure 2.7). Consequently, early infections are hard to diagnose through the above-ground plants phenotype. It is worldwide distributed, within all reference zones. A study over Japan of *R. nectatrix* infection found infectivity to 3.2% in apples and 6% in pears in all the country (Eguchi, 2010). The pathogens life cycle starts with asexual spores, conidia that are dispersed from a mass of conidiophores, called synnemata, which is not commonly found in commercial orchards since it is produced in extensive disease stage and needs to be protected from

light. In contrast of asexual spores, sexual spores, are produced under the protection of synnemata. Sexual reproduction creates perithecia containing ascospores in *R. necatrix* (Nakamura et al. 2000). Notably ascospores can be pathogenetic but conidia are believed to lack the pathogenic ability (Ikeda et al. 2004). Ascospores, because of their sexual propagation, create novel mycelium compatibility groups (MCGs). These MCGs are used to study *R. necatrix*, similarly to VCGs. However, Nakamura and colleagues in 2000 point out the farmers practice on removing infected roots in an early stage to avoid developing of perithecia and reduce the spreading of this pathogens in commercial fields. Besides, *R. necatrix* is known to be distributed in a field in patches (Pliego et al. 2012). MCGs of *R. necatrix* can have a great diversity globally but in orchards are usually limited. Massive amounts of fungicides have been sprayed with no expected results, breeding has failed and therefore available pathogen restriction strategies diminished. For this reason the idea of searching among mycoviruses for strains that cause hypovirulence was introduced (Matsumoto 1998).

Figure 2.7 *Rosellinia necatrix* induced to woolly root rot at the base of the poplar tree (source: https://agrobaseapp.com)

2.2.11 Important viruses reported in *Rosellinia necatrix*

Within the *R. necatrix* strains examined, a wide range of viruses has been recorded. Up to this date all these viruses belong to five families. Most of them commonly have a dsRNA genome; with a few exceptions of ssRNA. Most of mycoviruses researches in *R. necatrix* have been performed without favoring also DNA viruses detection then many researchers bringing the idea to reinvestigate strains already stored in the banks (Kondo et al. 2013). *Megabirnaviridae* family was created for Rosellinia nectrix megabirnavirus 1(RnMBV 1), the family name describes a bi-segmented virus for which the ability to cause hypovirulence to their host was demonstrated (Ikeda et al. 2005; Chiba et al. 2009). Although not yet assigned in the ICTV, literature reports also a second member of the *Megabirnaviridae* family, RnMBV 2 (Sasaki et al. 2015). In the family

Partitiviridae there are six members, *Rosellinia necatrix partitivirus* 1-6 (RnPV1-6), and it is assumed that more viruses will be reported soon as members of this family. One of the strains, RnPV1 – W8, was studied more extensively than the other since it shows interesting hypovirulence on its host (Sasaki et al. 2005). From *Reoviridaes* there is only one virus assigned, *Mycoreovirus* 3 (MyRV 3). A strain of this virus (MyRV 3-W370) was found as an agent of hypovirulence behavior of its host (Suzuki et al. 2004). A similar case with *Megabirnaviridae* is the family of *Quadriviridae*. This family was designed over the discovery of Rosellinia necatrix quadrivirus 1 (RnQV1). In this case studies over the effect on its host fitness showed no significant effects (Lin et al. 2012). A commonly found family among mycoviruses is *Totiviridae*. Up to this date in this family there is only one member, hosted by *R. necatrix*, assigned in the ICTV: *Rosellinia necatrix victovirus* 1 (RnVV 1) (Chiba et al. 2013). The viruses of this family regarding *R. necatrix* are not yet very well characterized.

2.2.12 Hypovirulence in *R. necatrix*

From the evidence gathered and reviewed regarding mycoviruses in *R. necatrix* the most interesting members is Rosellinia necatrix megabirnavirus 1. The study over the hypovirulence of this virus comes from the strain W779 and the isogenic cured strain W1015 (Figure 2.8). Transmission through anastomosis between the two strains was successful and reduction of host fitness has been recorded *in vitro* and on apple rootstocks (Chiba et al. 2009). The virus is built out of two dsRNA segments, approximately of 7 and 9 kbp, that are encapsidated separately in

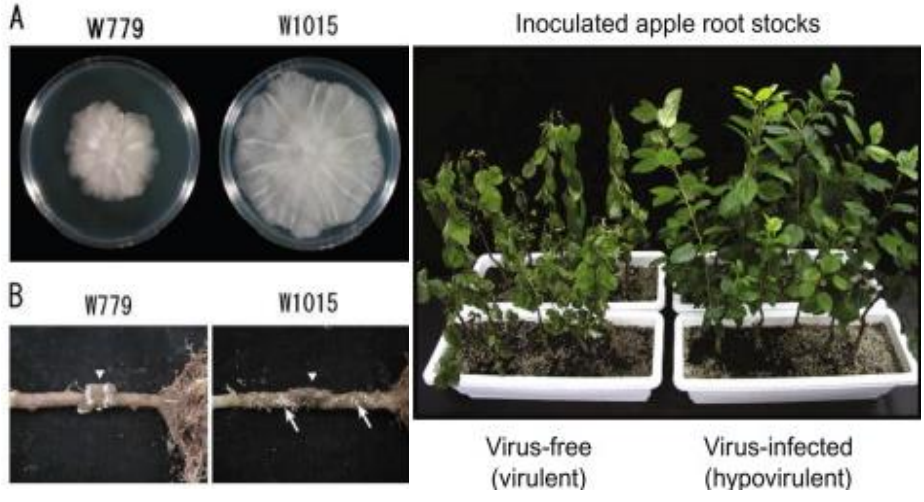


Figure 2.8 Mycelial growth of virus-carrying field strain W799 and Virus-free W1015. Colony morphology on PDA of both strains (W779 and W1015) after 5 days (A), (B) Mycelia growth on apple rootstock from the point of inoculation. The effect of the hypovirulent strain on the pathogenicity of *R.necatrix* inoculated on apple trees is shown in the picture to the right Source: (Chiba et al., 2009).

particles of 50nm in diameters. Each of the two dsRNAs has two ORFs (open read frame). In the dsRNA 1 the ORFs are responsible for the CP and the RdRp. In the case of the dsRNA 2 the ORFs have an unknown function (Salaipeth et al. 2014). Hypovirulence behavior was reported in a more recent study from the co-infection RnMBV 2 + RnPV 1 showing reduced mycelia growth in PDA Petri plates, as well as on apple fruits. The same study confirms also the ability of these two viruses to be transmitted to an isogenic strain that is virus-free (Sasaki et al. 2015).

2.2.13 *Fusarium* species

Fusarium spp. can be the causal agents of great economical loss in a wide range of hosts. Tomato, maize, wheat and barley are only few *Fusarium* hosts (Parry et al., 1995). The importance of these species is not concerning only economic issues, since it poses a public health danger from the production of mycotoxins (O'Donnell et al. 2018) such as fumonisins and trichothecenes that are linked to esophageal cancer and aleukia respectively (Adam et al. 2017).

2.2.14 Mycoviruses of *Fusarium* spp.

Methods for mycovirus screening on *Fusarium* spp. has been focused on dsRNA extraction, ignoring possible viruses with DNA genome. A population belonging to the genus *Fusarium* can have a diversity of dsRNA strains. For example, a study over *Fusarium solan*i reported 1 out of 34 strains containing dsRNA (Nogawa et al. 1993) or similarly, another study over *Fusarium proliferatum* reported 4 out of 100 positive strains (Heaton and Leslie 2004). In contrast *Fusarium poae* strains from wheat, originating from several regions of the world, showed all 55 isolates infected by dsRNAs and encapsidated virus like particles (Fekete et al. 1995). Interestingly, the screening of these isolates reveled different combinations of infections. Unfortunately, this study did not report any hypovirulence behavior but identified the Fusarium poae virus 1 (FpV1) (Compel et al. 1999). Hypovirulence behavior in Fusarium spp. was not reported frequently. However, there are some interesting members such as *Fusarium graminearium virus* 1 (FgV1) that are characterized as agents of hypovirulence (Chu et al. 2002). For example, an *in vivo* trial over mango plants suggests that a virus like particle can suppress *Fusarium moniliforme* (actually known as *F. proliferatum* and *F. verticillioides*) from creating abnormal shoots in plants. Based on genomic data and genetic analysis, *Fusarium spp* mycoviruses possibly belong to the families

of *Chrysoviridae*, *Hypoviridae*, *Partitiviridae* and *Totiviridae*. Some examples of viruses found in *Fusarium spp* are presented in the following Table 2.

Table.2. Examples of conferring mycoviruses-hypovirulence in *Fusarium* spp.

		Family	Segments		Reference
Fusarium graminearum virus 1	FgV1	Unassigned	1	6.6 kbp	Kwon et al., 2009
Fusarium graminearum virus 2	FgV2	Chrysoviridae	5	2.4-3.6 kbp	Chu et al., 2004
Fusarium graminearum virus 3	FgV3	Totiviridae	1	9 kbp	Chu et al., 2004
Fusarium graminearum virus 4	FgV4	Partitiviridae	2	2.3 & 1.7 kbp	Chu et al., 2004
Fusarium graminearum virus-China 9	FgV-ch9	Chrysoviridae	5	2.4-3.5 kbp	Darissa et al., 2011
Fusarium poae virus 1	FpV1	Partitiviridae	2	2.1-2.2 kbp	Compel et al., 1999
Fusarium oxysporum virus 1	FoV1	Chrysoviridae	3	0.6-2.5 kbp	Sharzehei et al., 2007
Fusarium solani virus 1	FsV1	Partitiviridae	2	1.4-1.6 kbp	Nogawa et al., 1996

2.2.15 Hypovirulence in *Fusarium* spp.

In many situations of dsRNA reported in *Fusarium* spp. there is no effect in the hosts fitness (Nogawa et al. 1996) but the strain DK21 of *Fusarium graminearum virus* 1, in contrast to the majority of mycoviruses, induced hypovirulence. Phenotypic alterations recorded through this strain were red pigmentation and restriction in mycelia growth (Chu et al. 2002). *Fusarium graminearum virus* 1-DK21 (FgV1-DK21) has been identified to reduce mycelia growth and sporulation as shown in the Figure 2.9, but also mycotoxins production was decreased to non-traceable levels. In the same paper a deeper study using 3'-tiling microarray revealed the ability of FgV1-DK21 to regulate the expression of genes of the fungal host in favor of the virus (Cho et al. 2012).

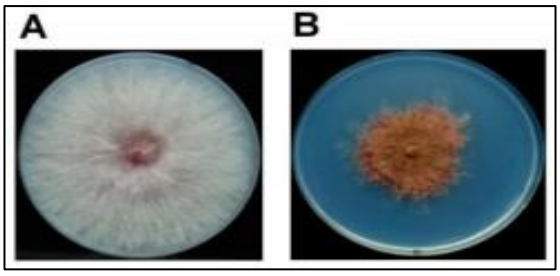


Figure 2.9 On the left (A) is a virus-free strain of *F. graminearum* while on the right (B) is an infected strain of *F. graminearum* with FgV1-DK2. Source: (Cho et al. 2012)

2.2.16 The case of *Fusarium circinatum*

Fusarium circinatum is the causal agent of pitch canker disease of pines (Figure 2.10)(Nirenberg and O'Donnell 1998; Wingfield et al. 2008). It has been reported in numerous countries all over the world: Mexico, USA, South America, Japan, Korea, Haiti, South Africa and southern Europe (Vainio et al. 2010). This invasive necrotrophic fungus in one of the most important pine pathogens, it can infect branches, stems, seeds, cones and roots causing pre- and post- damping off in seedlings and resinous bleeding cankers (Hepting and Roth 1946; Aegerter and Gordon 2006). *F. circinatum* spreads through contaminate materials (seeds, wood, seedling), soilborne spores or vectors (insects) (Bezos et al. 2015).

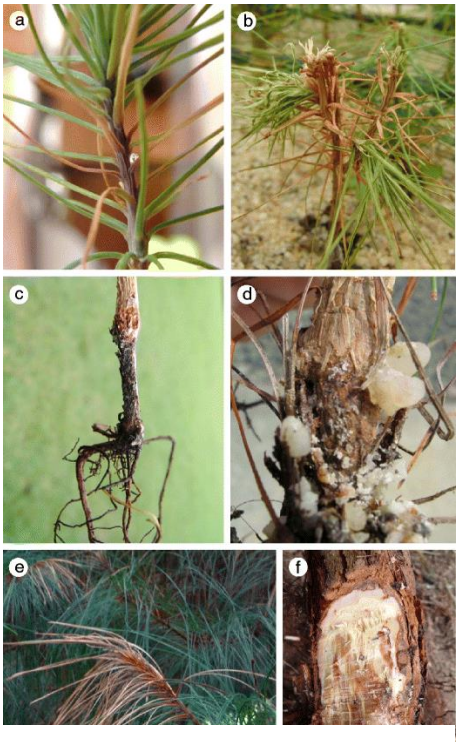


Figure 2.10 Symptoms associated with the pitch canker fungus: (a) Steam discoloration on a four month-old seedling; (b) Wilting and dieback symptoms; (c) Constricted root collar; (d) Resin bleeding from a canker; (e) Wilt branches on 11-years old pine; (f) Pitch-soaked, resinous wood, (f) Source: (Steenkamp *et al.*, 2012)

Viruses of *Fusarium* spp. have been isolated and classified into four mycoviruses families: *Chrysoviridae, Hypoviriade, Partitiviridae* and *Totiviridae* and unassigned *Fusarium graminearum virus 1* (FgV1), a dsRNA virus that showed promising hypovirulence characteristics by reducing mycelial growth and increased pigmentation (Chu *et al.*, 2002). Three species of the genus *Mitovirus*, belonging to the *Narnaviridae* family, have been detected in *F. circinatum* isolates: Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMv2-2 (Martínez-Álvarez et al. 2014). They were detected from a Spanish isolate of the fungus using CF11 cellulose

chromatography affinity, sequenced and classified within the genus *Mitoviruses.* Moreover, dsRNA elements were found in 14 out of the 135 isolates analyzed (Vainio et al. 2015), while most of the mitoviruses infection were not detected because they usually have ssRNA(+) genome (Park et al. 2006; Schoebel et al. 2014). The aim of the study promoted by Muñoz-Adalia and collaborates in 2016 was to analyze possible effects of *F. circinatum* mycoviruses FcMV1 and FcMV2-2 on laccase activity and pathogenicity of the fungus. Laccase extracellular enzymes are used by the fungus to degrade phenolic compounds such as lignin, mycoviral activity could decrease this metabolite production and decrease the virulence of the fungus (Rigling and Heiniger 1989; Castro *et al.*, 2003). Several *F. circinatum* isolates were obtained from two Spanish regions (Asturias and Cantabria) and laccase activity as well as growth of the fungus were compared between virus-free, single virus-infected and mixed infected (FcMV1 and FcMV2-2) isolates. Unfortunately, either *in vitro* or *in vivo* research did not produce results that qualified these mycoviruses as a valuable biological control treatment. In particular, no significant differences in pathogenicity for FcMV2-2 infected and virus-free isolates was detected while infection with FcMV1 increased the fungus pathogenicity leading a dieback process of the seedlings. Mycelial growth did not change if related to the presence or the absence of the mycovirus. In conclusion outcomes from this study exclude induction of hypovirulence by FcMV1 and FcMV2-2.

2.2.17 Fusarium head blight (FHB)

FHB is a disease concerning the main cereal crops such as *Triticum turgidum, T. aestivum, Hordeum vulgare, Avena sativa* and *Secale cereale*. This cereal disease is caused by diverse groups of pathogenic agents, which are distributed globally. All pathogenic agents are species undersigned in the genus *Fusarium*. The main species reported for this disease are *Fusarium graminearum, F. poae, F. avenaceum* and *F. culmorum* (Xu et al. 2008). In China have been reported seasons with nearly 7 million hectares of infected fields and losses reaching 1 million tons in severe years. In 1993, USA estimated 70 million tons of losses in barley. A possible pick of epidemic was reached between 1998 and 2002 with estimated losses of 2.8 billion dollars in the northern Great Plains and central USA (McMullen et al. 1997; Nganje et al. 2002; Leonard and Bushnell, 2003). Beside the economic issues from this disease, human and animal health threats also arise. These fungal species biosynthesize mycotoxins, secondary fungal metabolites, causing a wide range of toxic effects in humans and animals (Ostry 2008; Ferrigo et al. 2016). In general, products with mycotoxins accumulation in a severe level are withdrawn from market. Mycotoxins type and accumulation found from these species range according to the type species infecting the cereal crops. For example *F. graminearum* and *F. culmorum* are agents of accumulation of deoxinivalenol (DON, trichotechenes) and nivanelon (NIV) (Ferreira Geraldo et al. 2006). *F. avenaceum* in the other hand is known to accumulate moniliformin (MON) and despipeptides (Luz et al. 2017). *F. poae* can produce mycotoxins from the known group of trichotechenes but also toxins that do not belong to this group. Moreover, *F. poae* can produce trichotechenes such as Diacetoxyscirpenol (DAS), neosolaniol (NEOS), nivalenol (NIV) and fusarenonx (FX) (Thrane et al. 2004; Vogelgsang et al. 2008; Stenglein 2009). Toxins that do not belong to the trichothecenes group are arebeauvericin (BEA) and enniantins (ENNs) (Stępień and Chełkowski 2010). Some of these toxins (DAS and NEOS) have been linked to Alimentary toxic Aleukia (ATA) syndrome. This is a disease on humans and animals that can cause simple symptoms such as diarrhea to fatal shocklike syndromes (Rotter et al. 1996). Aside to the threads of human health and animals, these mycotoxins can decrease the quality of food and drinks, for example beer. FHB primary infections can originates from chlamidiospores, alternative hosts or saprophytic inoculum if was already in the fields soil, conidia or ascospores if it arrives from later stage infection or infected seeds (Parry et al. 1995). The dispersion of the inoculum along the field is through means like arthropod vectors, expansion through stem infection, wind and rain. Once the inoculums reached their destination it

is up to the weather if it will germinate or not. This complex of pathogens relies on wet weather (relative humidity 100%) and moderated hot weather (25°C). The available fungicides categorized by their mode of action have become more and more narrow. Commonly used in fields are pesticides that target mitochondria respiration inhibitors and demethylation inhibitors (Odds et al. 2003). These chemicals used over the time have developed fungal resistance strains, public health and ecological issues. Although we are aware of this situation the alternatives for FHB control is very limited, therefore application of fungicides is not reduced (Agrios, 2005; Fisher et al. 2012).

2.3 AIM OF THE STUDY

The purpose of this chapter was to develop a new approach capable of faster dissecting the virome of filamentous fungi belonging to the genus *Fusarium*. The semiconductor-based sequencer Ion Torrent™ and the nanopore-based sequencer MinION have been exploited to analyze DNA and RNA referable to viral genomes infecting fungal hosts. Some of these mycoviruses genera have been studied as inducers of hypovirulence in several phytopathogenic fungi, therefore future works will focus on the comparison of the morphology and physiology of the fungal strain infected and cured by the viruses identified and their possible use as a biocontrol agent.

2.4 MATERIALS AND METHODS

2.4.1 Fungal Strains and Culture Media

All the strains used to perform the experiments were selected from fungal collections, maintained at the Department of Agricultural Food and Sciences (University of Bologna) as reported in the Table 3. Strains were firstly grown on Petri plates containing potato dextrose agar medium (PDA) in presence of antibiotics Streptomycin 0.2 gr/L and Neomycin 0.1 gr/L. The fungus was then grown for 5 days in a dark chamber at 25 to 27°C (according to the strains) to maximize the production of mycelium. When the colonization of the PDA was completed the growth was stopped overnight at 4°C then, 1cm x 1cm of mycelium was moved from PDA plates to v8 vegetable juice liquid media and incubated by shaking in the same conditions previous described.

Table3. Fungal collections

Strains	Species	Host	Origin
B1017	*Fusairium culmorum*	Wheat caryopsis durum	Meriermi
B1030	*Fusairium culmorum*	Wheat caryopsis durum	Ussana
B1037	*Fusairium culmorum*	Wheat caryopsis durum	Ussana
B393	*Fusairium culmorum*	Wheat caryopsis durum	Ottawa
B394	*Fusairium culmorum*	Wheat caryopsis durum	Ottawa
B398	*Fusairium culmorum*	Wheat caryopsis durum	Serramana
B431	*Fusairium culmorum*	Wheat caryopsis durum	Sedini
B921	*Fusairium culmorum*	Wheat caryopsis durum	Ussana
B957	*Fusairium culmorum*	Wheat caryopsis durum	Ussana
F0242	*Fusarium poae*	Unknown	Unknown
F0419	*Fusarium poae*	Wheat spike	Ancona
F0448	*Fusarium poae*	Wheat caryopsis	Baricella
F0456	*Fusarium poae*	Wheat spike	Baricella
F0481	*Fusarium poae*	Wheat caryopsis	Baricella
F0504	*Fusarium poae*	Wheat spike	Baricella
F0541	*Fusarium poae*	Wheat spike	Idice
F0570	*Fusarium poae*	Wheat spike	Urbino
F0596	*Fusarium poae*	Wheat caryopsis	Grosseto
F0630	*Fusarium poae*	Wheat caryopsis	Taglio di Po-Rovigo
F0702	*Fusarium poae*	Wheat spike	Caselecchio
F0703	*Fusarium poae*	Wheat caryopsis	Bari
F1066	*Fusarium poae*	Wheat caryopsis	Umbria
F1067	*Fusarium poae*	Wheat caryopsis	Umbria
F1077	*Fusarium poae*	Wheat caryopsis	Umbria

F1080	*Fusarium poae*	Wheat caryopsis	Umbria
F1085	*Fusarium poae*	Wheat caryopsis	Umbria
F1088	*Fusarium poae*	Wheat caryopsis	Umbria
F1099	*Fusarium poae*	Wheat caryopsis	Cadriano
F1103	*Fusarium poae*	Wheat caryopsis	Cadriano
F1104	*Fusarium poae*	Wheat caryopsis	Cadriano
F1111	*Fusarium poae*	Wheat caryopsis	Verona
F1112	*Fusarium poae*	Wheat caryopsis	Verona
F1124	*Fusarium poae*	Wheat caryopsis	Palermo
F1136	*Fusarium poae*	Wheat caryopsis	Palermo
F1188	*Fusarium poae*	Barley dressed	Ferrara
F1189	*Fusarium poae*	Barley dressed	Ferrara
F1191	*Fusarium poae*	Barley dressed	Ferrara
F1193	*Fusarium poae*	Barley dressed	Ferrara
F1194	*Fusarium poae*	Barley dressed	Ferrara
F1196	*Fusarium poae*	Barley naked	Bologna
F1197	*Fusarium poae*	Barley naked	Bologna
F1199	*Fusarium poae*	Barley	Unknown
F1214	*Fusarium poae*	Orzo caryopsis	Unknown
F1217	*Fusarium poae*	Orzo caryopsis	Unknown
F1226	*Fusarium poae*	Orzo caryopsis	Unknown
F1278	*Fusarium poae*	Orzo caryopsis	Perugia
F1284	*Fusarium poae*	Wheat Durum caryopsis	Unknown
T1036	Fusarium acuminatum species complex	Wheat durum caryopsis	Unknown
T1093	fusarium tricinctum species complex	Wheat durum caryopsis	Umbria
T1281	fusarium tricinctum species complex	Barley caryopsis	Perugia
T1390	fusarium tricinctum species complex	Wheat durum caryopsis	Reggiolo
T1391	fusarium tricinctum species complex	Unknown	Unknown
T444	fusarium tricinctum species complex	Unknown	Unknown
T459	fusarium tricinctum species complex	Unknown	Unknown
T577	Fusarium tricinctum species complex	Wheat durum	Baricella

2.4.2 Growth rate of *Fusarium poae* strains

To assess growth rates, 5-mm-diameter agar disks from the margins of actively growing colony of 30 *F. poae* strains were transferred onto 9-cm-diameter Petri dishes containing 20 ml PDA and then incubated at 20°C. The diameter of *F. poae* colonies was measured at 24 and 48 hours post inoculation (hpi). The hyphal growth rate of the two strains was calculated as follows: growth rate (cm/d) = (48 hpi diam. - 24 hpi diam.)/2 (Zhang et al. 2009).

2.4.3 dsRNA extraction and purification

Up to 1gr of dried mycelium was ground by liquid nitrogen and immediately transferred in 5 ml of Extraction Buffer (EB) (0.2M NaCl; 0.1M Tris-HCl; 0.004M EDTA pH 8.0) then added with 5 ml of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, v/v/v). After shaking samples was centrifuged at 3,000 g for 15 min and the resulting aqueous phase was recovered. The whole dsRNAs present in the aqueous phase were isolated from the total nucleic acids through a modified version of Valverde et al., 1990 protocol, using Adavantec® C cellulose powder (300 mesh) and 1.5ml tubes instead glass columns (Figure 2.11). After purification a double digestion with DNAse and S1 Nuclease (Promega, USA) was performed. DsRNA segments were electrophoresed on a 1% agarose gel stained with ethidium bromide, using 1kb DNA Ladder (Promega, USA), to check quality and integrity of nucleic acids.

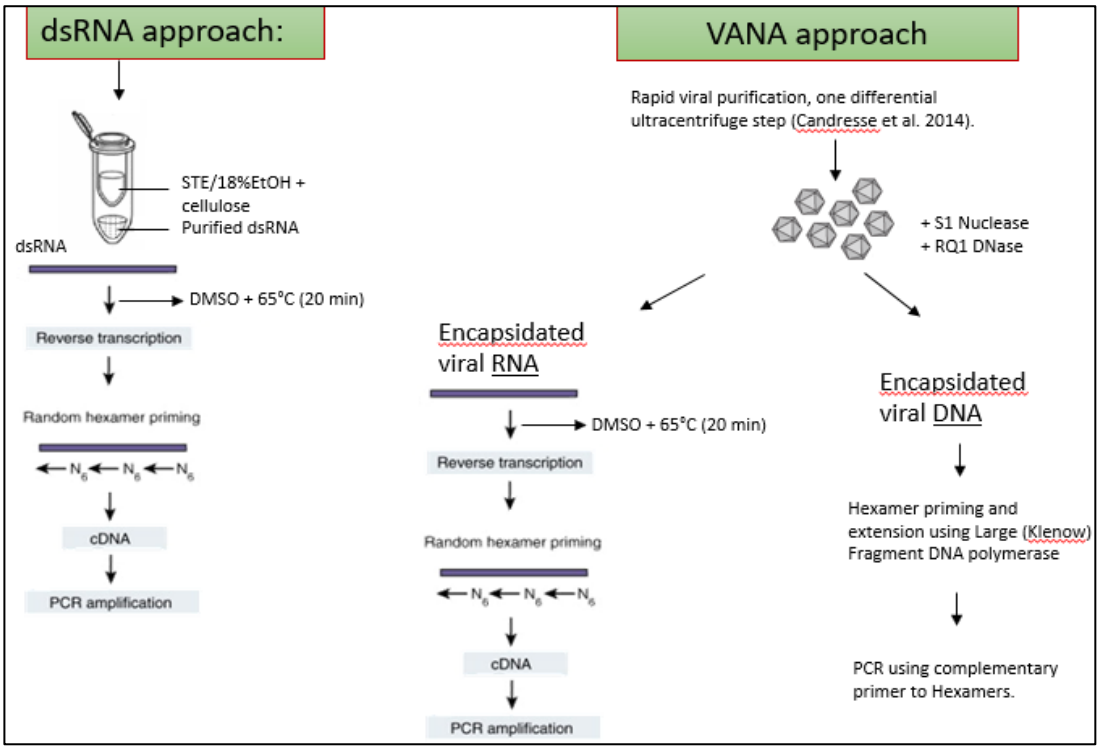


Figure 2.11 Methodologies for nucleic acids extraction and library preparation systems.

2.4.4 Virion-associated nucleic acids (VANA) isolation

Up to 1gr of dried mycelium has been blended and the fungal debris were trapped by a 0.45µm filter, large enough to allow the passage of viral particles. The filtered homogenate was further clarified by differential ultracentrifuges and the viral pellet obtained was resuspended overnight at 4°C in 200 µl of Hank's Balanced Salt Solution (HBSS). Subsequently a random library of both

RNA and DNA was prepared according to previously published protocol (Candresse et al. 2014) (Figure 2.11).

2.4.5 NGS random library preparation

2.4.5.1 Ion Torrent ™

The whole amount of precipitated dsRNA or VANA was resuspended in 10 ul of nuclease free water and 90 ul of Dimethyl sulfoxide (DMSO). The mixture was denatured for 20 min at 65 °C and then subjected to ethanol-based precipitation (Chiba et al. 2009). The ssRNAs obtained from the denaturation were resuspended directly in the volume of water necessary to perform the RT reaction by the ImProm-II RT system kit with 400 nM Universal primers-dN6 (5'-GCCGGAGCTCTGCAGAATTCNNNNNN-3') for cDNA synthesis (Froussard 1992). Then cDNA was PCI-cleaned and pellet was resuspended in 50µL of nuclease free water. GoTaq® DNA Polymerase (Promega, USA) was used for the RT-PCR reaction using 500 nM Universal Primer-Tag (5'-GCCGGAGCTCTGCAGAATTC-3') and up to 5ul of cDNA as template. Eventually viral DNA were subjected to a hexamer priming and extension using Large (Klenow) Fragment DNA polymerase (Promega, USA) before PCR setting as described by Froussard, 1993. The random amplicons obtained from both methods were used in the library preparation kit provided by the semiconductor-based sequencer Ion Torrent ™.

Ion Torrent sequencing was obtained starting from 200 ng of DNA amplicons using the Ion Torrent PGM sequencer (Thermo Fisher Scientific Inc.). Library preparation was generate following the manufacturer protocols including enzymatic shearing, end repair and adapter ligation using Ion Xpress Plus Fragment Library kit (Thermo Fisher Scientific Inc.). Samples were size-selected using the e-gel system (Invitrogen) and fragments corresponding to 200 bp were collected. The library was quantified by qPCR with the Ion Library Quantitation Kit (Thermo Fisher Scientific Inc.) using the ABI 7000 Real-Time PCR System (Applied Biosystems). The prepped samples were then amplified by emulsion PCR using PGM™ Hi-Q™ OT2 kit (Thermo Fisher Scientific Inc.) and sequenced with the Ion PGM™ Hi-Q™ Sequencing kit using one Ion 314 v2 chip (Thermo Fisher Scientific Inc.) (Ribani et al. 2018; Utzeri et al. 2019).

2.4.5.2 MinION: Nanopore DNA sequencer

Based on the accuracy derived from the Ion Torrent sequencing approach, the efficiency of the MinION sequencer was evaluated (Figure 2.12). MinION is a third-generation technology developed from studies on DNA translocation through artificial nanopores, in this way the sequencing is based on the conversion of the electrical signal of the nucleotides that pass through a pore of α-hemolysina (nanopore) covalently linked to the molecule of cycle dextrin, the nucleotide binding site (Pareek et al. 2011).

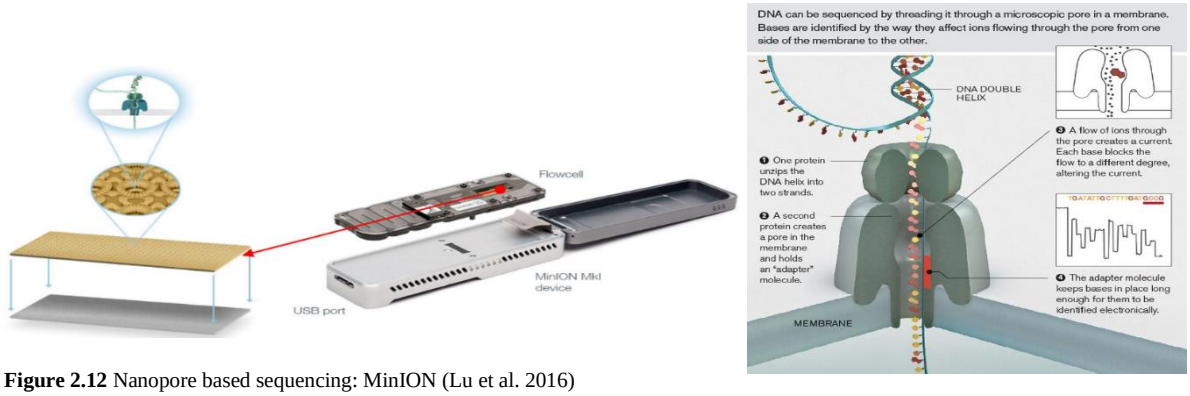


Figure 2.12 Nanopore based sequencing: MinION (Lu et al. 2016)

In this work the PCR Sequencing Kit SQK-PSK004 and the Direct RNA Sequencing Kit SQK-RNA002 were tested. The first system allows sequencing starting from 100ng of DNA and obtaining 2-3 + Gb in 6 hours of running. The required input is genomic DNA or DNA derived from a PCR amplification. On the contrary, the SQK-RNA002 kit provides direct sequencing of RNA, without going through steps such as retro-transcription (RT) and (PCR) amplification. The starting material is 500ng of RNA and the output is <1 Gb in 6 hours.

2.4.6 RNA ligase-mediated amplification of cDNA ends (RLM-RACE)

Sequencing systems such as Ion Torrent and MinION based on PCR Sequencing Kit SQK-PSK004 do not allow to obtain reliable information about the ends of the sequenced nucleic acids. In collaboration with the Nagoya University (Japan) the 5' and 3' ends of viral RNA genomes, including untranslated regions (UTRs), were determined using a RACE protocol based on T4 RNA ligase 1. The ligation of a 5′ phosphoryl-terminated DNA adapter (DNA oligomer-5′-PO4-CAATACCTTCTGACCATGCAGTGACAGTCAGCATG-3′) to a 3′ hydroxyl-terminated ssRNA was performed then the 3RACE-^{1st} primer (5'CATGCTGACTGTCACTGCAT-3'), complementary to the 3′ half of 3RACE-adaptor, was used for RT reaction. The resulting cDNA

was amplified by PCR with the primer set 3RACE-^{2nd} (5'-TGCATGGTCAGAAGGTATTG-3') to obtain the ends of the unknown viral genome (Coutts and Livieratos 2003; Chiba et al. 2009). Sequences obtained were cloned into pGEM®-T Easy Vector Systems and confirmed through Sanger-based sequencing.

2.4.7 Viral purification and protoplast transfection

Up to 30gr of mycelium were harvested and ground to powder in the presence of liquid nitrogen then the fungal material was homogenized with 90 ml of 0.25 M potassium phosphate buffer (pH 7.0), 0.5% thioglycolic acid and 10 mM EDTA. After filtration through cheesecloth, 1% Triton X was added, and the solution was stirred for 1 h at 4°C. Fungal homogenate has been clarified through differential ultracentrifuges and the separated particles through sucrose gradient as reported by Turina et al. 2007. Purification of viral particles by PEG was used for protoplast transfection (Chiba et al. 2009). Protoplast of *Fusarium* spp. were prepared starting with the generation of fresh conidia. Mycelial plugs were moved into a Mung bean medium and incubated for 5 days at 20°C then conidial suspension was inoculated in Potato Dextrose Broth (PDB) for 24h at 20°C under dark condition. Mycelial suspension was centrifuged and washed twice with 1M NH_4Cl, before addition of 1.0% lysing enzyme and 0.4% Kitalase (w/v). The degradation reaction was left for 3-4 h (60 rpm, 37°C) then protoplasts were harvested by centrifugation (14,000 x g, 15 min, room temperature). Pellet was clarified twice using wash buffer (1.0 M NH4Cl, 1.0 M sorbitol, 10 mM Tris HCl pH 8.0, 50 mM CaCl2) collecting protoplasts by centrifugation as described before. Pellet was resolved in STC (1.0 M sorbitol, 10 mM Tris HCl pH 8.0, 50 mM CaCl2) and added by ¼ in volume of PEG solution (60% PEG 4,000, 50mM CaCl2, 10 mM Tris-HCl pH 8.0).

2.4.8 Internal Ribosome Entry Site (IRES) activity evaluation

In collaboration with the Nagoya University (Japan), bicistronic, dual-luciferase assay system was developed for the identification of IRESs in the 5' UTR of the genomes of diverse positive-sense single stranded RNA and double stranded RNA. The translation system consists of reporter genes encoding codon-optimized genes for firefly luciferase (OFluc) and Renilla luciferase (ORluc) with a multiple cloning site in between, thus viral genomes has been cloned into a fungal expression vector pCPXHY3 with a hygromycin resistance to have a complete bicistronic vector pCH-DLst3 (Chiba et al. 2018).

2.5 RESULTS

2.5.1 Growth rates of *Fusarium* spp.

Grown rate test showed differences in the growing speed of fungi subjected to hypovirulence analysis, although strains were grown in the same nutritional and dark conditions. The fungi selected for further analysis (Figure 2.13, blue arrow) showed a slower growth when compared to the other strains of the same genus.

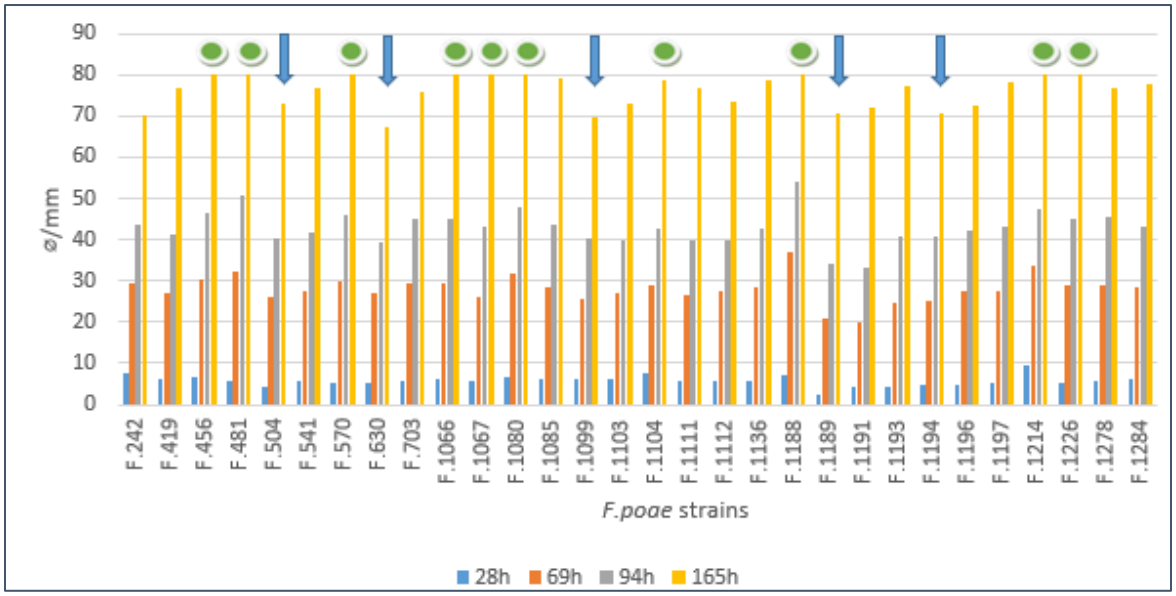


Figure 2.13 Graph showing the growth rate (diameter/mm) of 30 *F. poae* strains at different time. With green balls are highlighted strains full grown, while fungi with inconsistencies in growth are indicated with a blue arrow.

In particular, strains F.504, F630, F1099, F1189, and F1196 exhibited a reduction of the growing rate up to 20% measured on the average area of growth (Ø / mm). Although, even if the growth rate of F.242 resulted slightly slower than F.456 and F481 (full growth) at 165h, at 28 and 69 hours this strain recorded a growth rate equal to that of the strains classified as full growth. To avoid distortions of the results due to initial variation of the fungal inoculum, three replications have been performed for each strain (Table 4) and therefore the comparison has been formulated on the average value. Strains such as F.456, F.481, F.570, F.1066, F.1067, F.1080, F.1104, F.1188, F.1214 and F.1226 not only presented a greater speed of growth compared to the other strains, but their morphology was not affected by the presence of viruses. The strains marked by the blue arrow (Figure 2.13) were selected based on the growth rate parameters, morphology alteration (compared to full growth strains) and dsRNA profiles.

Table 4 Growth rate measurements carried out on 30 *F. poae* strains

	28h Ø/mm			28h average	69h Ø/mm			69h average	94h Ø/mm			94h average	165h Ø/mm			165h average
F.242	13.5	13.2	11	7.57	35.53	32.33	34.7	29.19	49.27	46.97	49.53	43.59	67.13	80.13	78.47	70.24
F.419	11.93	11.17	10.8	6.30	30.87	32.1	33.3	27.09	44.23	45.63	48.83	41.23	80.67	79.27	85	76.65
F.456	11.5	11.53	11.63	6.55	35.37	35.03	35.3	30.23	53.67	51.27	49.8	46.58	85	85	85	80.00
F.481	10.67	9.93	12.23	5.94	37.33	34.9	40.1	32.44	54.17	53.57	59.33	50.69	85	85	85	80.00
F.504	11.1	10	6.67	4.26	35.7	29	28.8	26.17	53.83	42.27	39.7	40.27	85	79.83	68.67	72.83
F.541	11.2	10.67	10.03	5.63	33.3	31.83	32.8	27.64	47.23	46.7	46.07	41.67	80.4	80.27	85	76.89
F.570	9	10.97	10.17	5.05	32.83	34.83	37.73	30.13	49.4	50.17	53.37	45.98	85	85	85	80.00
F.630	9.8	10	10.4	5.07	34.17	30.43	31.67	27.09	44.63	42.83	46	39.49	76.33	62.5	77.63	67.15
F.703	10.37	8.23	13.07	5.56	33.77	31.4	38.67	29.61	50.17	45.83	53.57	44.86	80.67	80.67	81.17	75.84
F.1066	11.33	12.03	10.83	6.40	33.53	36.1	33.6	29.41	50.67	50.6	48.8	45.02	85	85	85	80.00
F.1067	11.43	10.67	10.03	5.71	31.07	30.83	31.33	26.08	48.03	48.33	48.47	43.28	85	85	85	80.00
F.1080	11.17	11	12.17	6.45	35.5	36.73	37.83	31.69	52.63	53.17	53.1	47.97	85	85	85	80.00
F.1085	11.67	11.07	11.23	6.32	34.93	32.6	32.9	28.48	50	47.93	47.5	43.48	82.63	85	85	79.21
F.1099	9.63	11.83	11.87	6.11	27.83	31.33	32.87	25.68	43	45.83	46.47	40.10	77.13	80.73	66.07	69.64
F.1103	10.53	12.53	10.5	6.19	29.3	32.83	33.8	26.98	41.5	45.97	47.17	39.88	77.27	77	80.47	73.25
F.1104	13.07	13	11.7	7.59	32.93	33.33	36.17	29.14	48.37	47.67	46.83	42.62	85	85	80.97	78.66
F.1111	10.83	10.67	10	5.50	33.17	31.33	29.83	26.44	48.5	44.33	42.17	40.00	85	79.5	80.47	76.66
F.1112	9.33	10.83	11.33	5.50	31.5	33.8	32.7	27.67	43	40.67	50.27	39.65	73.17	81.5	81	73.56
F.1136	10.83	10.77	11	5.87	33.77	33.43	32.63	28.28	49.33	47.57	45.9	42.60	85	85	81.27	78.76
F.1188	10.67	13.43	12.77	7.29	38.67	43.47	43.5	36.88	55.17	60.37	61.13	53.89	85	85	85	80.00
F.1189	6.5	7.63	8.33	2.49	25.8	25.07	27.5	21.12	40.67	37.57	39	34.08	79.87	70.83	76.17	70.62
F.1191	10.5	9.9	6.97	4.12	27.7	25.23	22.5	20.14	41.87	38.4	33.87	33.05	79.83	77.87	73.63	72.11
F.1193	10.03	9.37	8	4.13	29.7	27.97	32	24.89	46.03	44.63	46.83	40.83	85	80.73	81.33	77.35
F.1194	10	9.23	10.67	4.97	28.83	26.76	34.9	25.16	37.86	45.9	53.6	40.79	69.27	72.1	85	70.46
F.1196	11.33	10.53	7.97	4.94	33.17	30.1	34.9	27.72	46.67	49.4	45.53	42.20	76.2	77.07	79.07	72.45
F.1197	8.13	11	11.33	5.15	30.03	34.17	34.03	27.74	46	49.33	49.43	43.25	80.33	85	85	78.44
F.1214	12.17	16.5	15.5	9.72	36.33	39	40.5	33.61	50.67	52.93	54.17	47.59	85	85	85	80.00
F.1226	9	11	10.33	5.11	32.5	35.5	34.17	29.06	49.03	53.67	47.33	45.01	85	85	85	80.00
F.1278	10.33	12.17	10.17	5.89	35	35	31.33	28.78	50.63	51.67	48.93	45.41	85	82.67	77.33	76.67
F.1284	11.37	11.17	10.7	6.08	33.27	33	33.83	28.37	48.73	47.17	49.1	43.33	85	78.97	85	77.99

2.5.2 Exploring dsRNA patterns of *F. poae* strains

The strains F.504, F630, F.1099, F.1189 and F.1194 showed similar dsRNA profile (Figure 2.15), but different morphology in Petri dishes, from most of the other fungi. Up to 9 different dsRNA profiles were found for each individual strain. After the electrophoretic analyses, in order to interpret the dsRNA profiles, the GelAnalyzer software was used (Figure 2.14) referring to the fragments from the marker 1kb DNA Ladder (Promega, USA). The marker was first acquired by GelAnalyzer software setting a contrast threshold to clearly identify even the faintest profiles. The software automatically detects the electrophoretic profiles in each run generating peaks which are then translated into numerical values using the marker as a reference for the size.

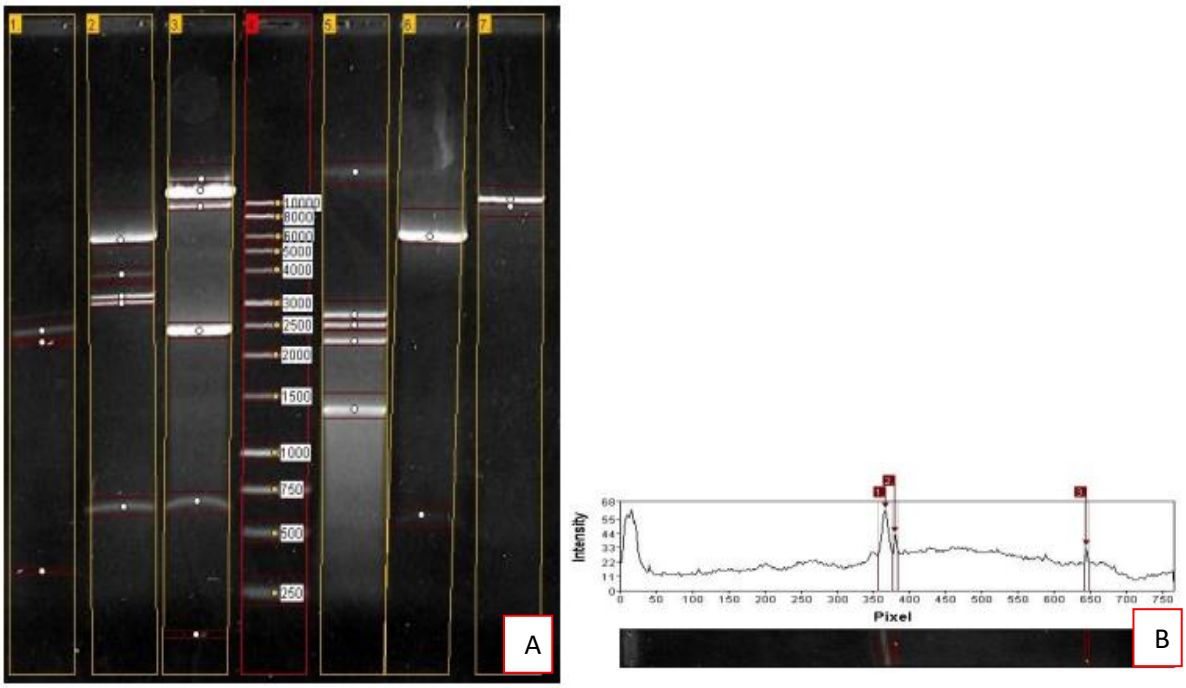


Figure 2.14 (A) Lines 1 to 3 and 5 to 7 represent profiles of F.1066, F.1067, F.1103, F.1111, F.456 and F.1085 respectively. (B) The image represents processing of the GelAnalyzer software.

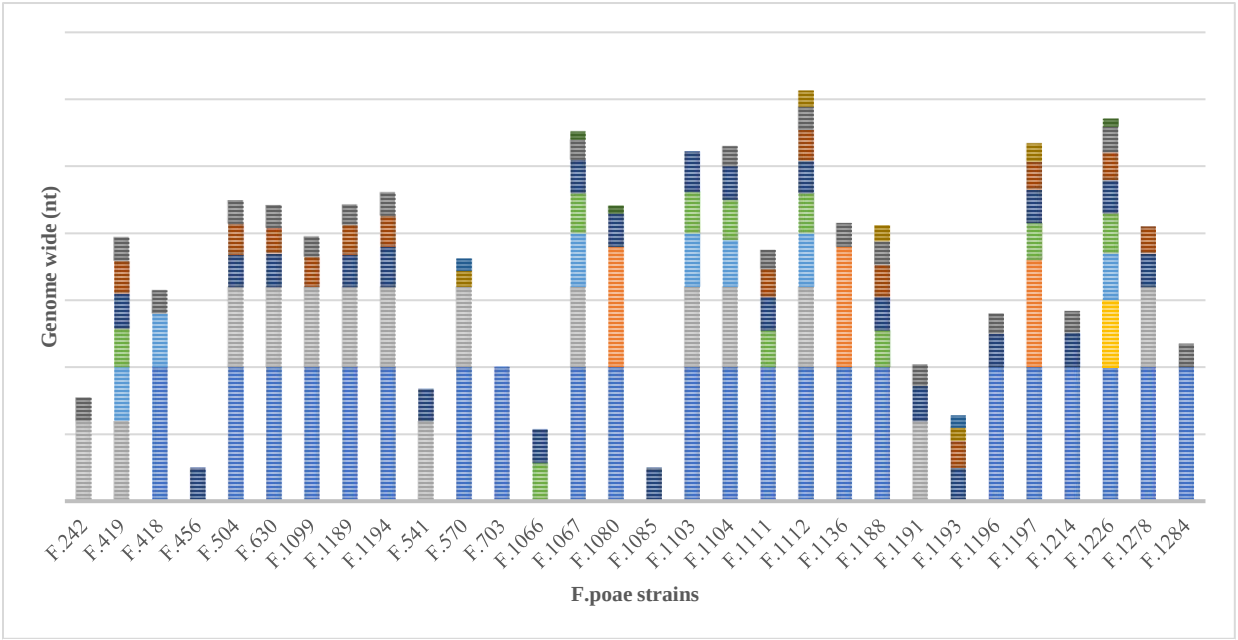


Figure 2.15 Genome wide expressed in nucleotides identified in the 30 strains of *F. poae*

Table 5 The table shows the measurements of the mycoviral genomes used for the construction of the graph in Figure 2.15.

F.242			6000						1750				
F.419			6000		4000	2900	2600	2450	1750				500
F.418	10000				4000				1750				
F.456							2500						
F.504	10000		6000				2400	2300	1750				
F.630	10000		6000				2500	1900	1700				
F.1099	10000		6000					2250	1500				
F.1189	10000		6000				2400	2250	1500				
F.1194	10000		6000				3000	2300	1750				
F.541			6000				2400						
F.570	10000		6000							1200	900		
F.703	10000												
F.1066						2900	2500						
F.1067	10000		6000		4000	3000	2500		1500			600	
F.1080	10000	9000					2500					600	
F.1085							2500						
F.1103	10000		6000		4000	3100	3000						
F.1104	10000		6000		3500	3000	2500		1500				
F.1111	10000					2750	2500	2100	1400				
F.1112	10000		6000		4000	3000	2400	2300	1750	1200			500
F.1136	10000	9000							1750				
F.1188	10000					2750	2500	2400	1750	1200			
F.1191			6000				2600		1600				
F.1193							2500	2000		1000	900		
F.1196	10000						2500		1500				
F.1197	10000	8000				2750	2500	2100		1350			
F.1214	10000						2600		1600				
F.1226	10000			4000	3500	3000	2450	2100	1900			600	
F.1278	10000		6000				2500	2000					
F.1284	10000								1750				

The analyzed resulted indicate a widespread and heterogeneous presence of dsRNA in *Fusarium* spp., characterized by 1 to 9 dsRNAs for each strain and summarized in a graph correlation between genomic size, incidence of the viroma and the fungus strains (Figure 2.15). Each colored fraction forming a bar was associated with a different dsRNA profile analyzed. For example, the strains F.504, F.630, F.1189 and F.1194 show a very similar electrophoretic profile and common fragments of 10,000bp, 6,000bp, 3,000-2,400bp, 1,900-2,300bp and 1,750bp (Figure 2.15 and Table 5). Looking at the most repeated fragment around 6,000bp, the analysis allows to hypothesize the viral family of the related virus as in the literature usually a genome of the same size is associated to a members of the *Totiviridae* family (Niu et al. 2016). All 30 strains of *F. poae* analyzed resulted infected by mycoviruses, *F. tricintum* species complex shown 1 infected strain out 8 and *F. culmorum* shown 7 strains containing dsRNA out of 9. Obviously, only after molecular investigations is it possible to confirm the identity of each virus.

2.5.3 Evaluation of different NGS approach

2.5.3.1 Library preparation

The quality of the generated library was optimized before the sequencing performed by NGS techniques. In particular, a method for generating long amplicons from a random RT-PCR reaction was established. The integrity of the starting dsRNA or ssRNA was previously evaluated by electrophoretic then a random RT-PCR was used to ensure homogeneous distribution of virome information in the amplification products. To enhance the efficiency on long amplicons generations, different strategies have been evaluated, such as the use of high temperatures and different concentrations of Dimethyl sulfoxide (DMSO) (Figure 2.16).

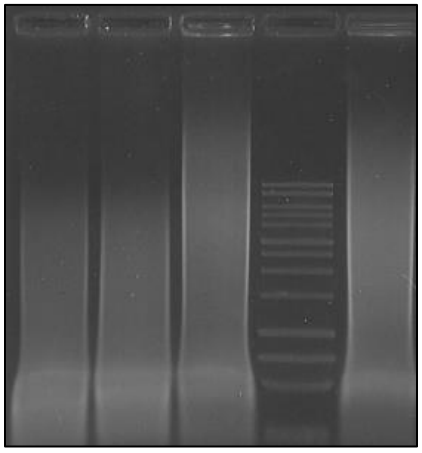


Figure 2.16 Picture shows different denaturation strategies for RT-PCR. Firstline dsRNA are not denaturated, second line shows the action of heat-treatment, in third and fourth line 50% and 90% of DMSO were used. 1kb marker (Pomega) was used.

2.5.3.2 IonTorrent sequencing

Sequencing using the IonTorrent based system produced 460,566 reads with an average length of about 150 bp. The chip was loaded for about 80% of the sequencing surface, optimizing system performance (Figure 2.17).

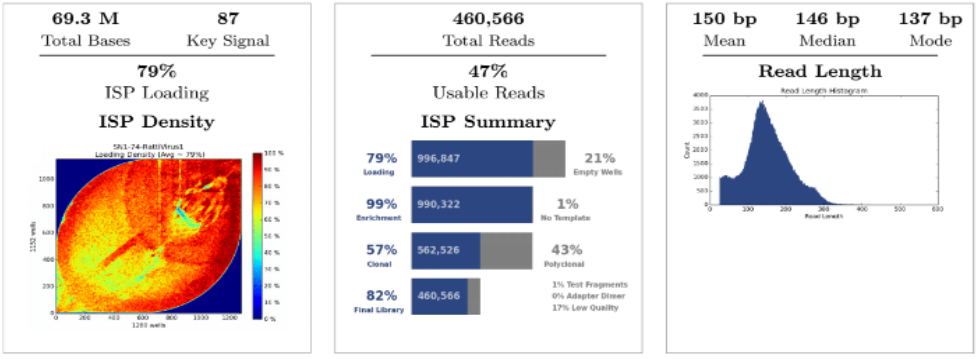


Figure 2.17 The image shows the results obtained from the run through IonTorrent sequencing. The chip was successfully loaded for 79% of its available surface, thus obtaining up to 460.566 reads of average length 150bp.

The raw reads were qualitatively trimmed by Nanofilt command using the Anaconda platform and then *De novo* assembling of fasta files was executed using wtdbg2 command. To increase the amount of long consensus sequences, the contigs generated by the assembly were filtered by their length (> 500bp) using seqkit tool and long reads were mapped to draft assembly sequence using minimap2 and samtools. The graphical layout of the results was elaborated using the Geneious 10.0.9 software.

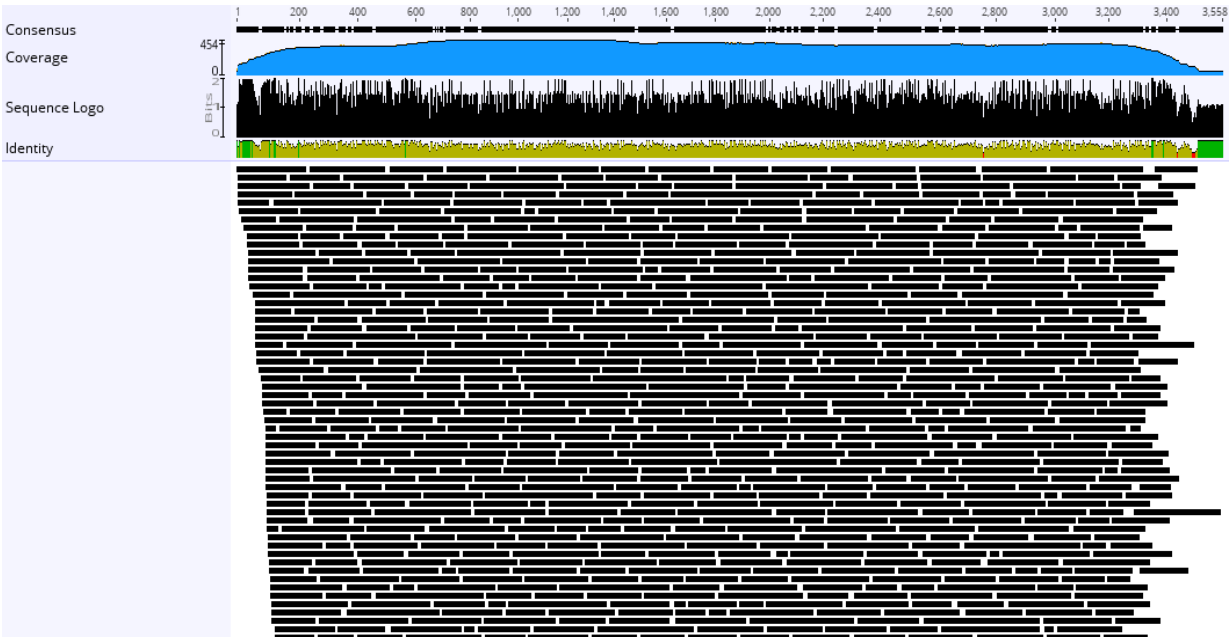


Figure 2.18 Sequencing result of the mycoviral genome obtained via IonTorrent technology. In the example the assembled genome of Fusarium poae alternavirus 1 genomic RNA, segment 1 (LC150613) was shown.

The coverage obtained with the Ion Torrent sequencing system was distributed throughout the mycoviral genome except for the 3 'and 5' ends, areas where this system showed low or null coverage (Figure 2.18). Analysis generated 5,956 contigs with length ranging from 500 to 13,000 nucleotides and the consensus sequences were processed with the online bioinformatics tool ViroBLAST (https://indra.mullins.microbiol.washington.edu/viroblast/viroblast.php) using the database Viral Genbank and setting an AMtch / Mismatch scores to " 2, -3 " and Gap costs to " Existence: 5, Extension: 2 ". The results obtained were then remapped using the same settings against each identified reference and the viral accessions were reported in the Table 6.

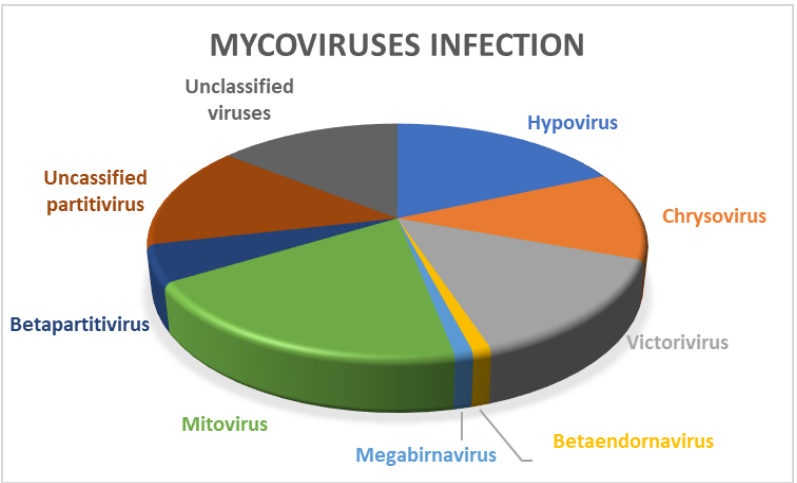
The results suggest a very high and diverse viral infection rate. Up to 50 viral accessions have been reported to infect 30 different strains of *F. poae*. Viruses belonging to the genus *Betaendornavirus,*

Betapartitivirus, *Chrysovirus*, *Hypovirus*, *Megabirnavirus*, *Mitovirus*, *Partitivirus*, *Totivirus* and *Victorivirus* have been identified together to other mycoviruses, not yet classified.

Table 6. The table shows all the results obtained from the different sequencing techniques (IonTorrent and MinION). The pie chart shows how each viral genus is represented in the screened *F.poae* population.

,Genus	Virus	Accession numbers	Identity (%)
Betaendornavirus	*Sclerotinia sclerotiorum endornavirus 2*	KU299046	78.4
Betapartitivirus	*Fusarium poae virus 1*	NC_003883; NC_003884; LC150606; LC150607	69.1-100
Chrysovirus	*Botryosphaeria dothidea chrysovirus*	KJ573505	71.9
	Penicillium chrysogenum virus	NC_007539; NC_007540; NC_007542	96.2-97.5
Hypovirus	*Fusarium graminearum hypovirus 1*	NC_023680	64.2-84.3
	Fusarium graminearum hypovirus 2	KP208178	78.3-86.1
	Fusarium langsethiae hypovirus 1	KY120321	81.7-85.3
	Fusarium poae hypovirus 1	LC150612	73.9-87.1
Megabirnavirus	*Megabirnavirus 1*	KT601119	89.7
	Rosellinia necatrix megabirnavirus 1	NC_013462	80.0
Mitovirus	*Botrytis cinerea mitovirus 1*	LN827940.1	93.5
	Botrytis cinerea mitovirus 2	LN827941; LN827945	71.2 - 74.2
	Fusarium poae mitovirus 1	LC150564	68.6-91.2
	Fusarium poae mitovirus 2	LC150565	83.3-96.4
	Fusarium poae mitovirus 3	LC150566	91.1-97.2
	Fusarium poae mitovirus 4	LC150567	77.3-96.1
	Ophiostoma novo-ulmi mitovirus	NC_004054	90.9
	Sclerotinia sclerotiorum mitovirus 18	KP900925	82.5
	Sclerotinia sclerotiorum mitovirus 4-A	MF444237	85.1
	Soybean leaf-associated mitovirus 4	KT598246	71.5-77.9
Unclassified	*Aspergillus fumigatus tetramycovirus-1*	HG975305; HG975302	75.1-80.2
	Beauveria bassiana polymycovirus 2	LN896311	70.9
	Diplodia scrobiculata RNA virus 1	EF568774	87.5
	Fusarium poae alternavirus 1	LC150613; LC150614; LC150615	72.2-100
	Fusarium poae dsRNA virus 3	KU728181	67.8-100
	Fusarium poae fusarivirus 1	LC150611	72.4-87.3
	Fusarium poae mycovirus 1	LC150616	68.5-100
	Fusarium poae mycovirus 2	LC150617	77.1-86.2
	Fusarium virguliforme dsRNA mycovirus 1	JN671444	90.9
	Rosellinia necatrix fusagravirus 2	LC333738	83.3
	Sclerotinia sclerotiorum dsRNA mycovirus	LN827951	74.6

,Genus	Virus	Accession numbers	Identity (%)
Partitivirus	*Botrytis cinerea partitivirus 2*	MG011707; MG011708	77.4-96.8
	Fusarium poae partitivirus 2	LC150608; LC150609	70.3-100
	Penicillium aurantiogriseum partiti-like virus 1	KY595973	83.3
	Pittosporum cryptic virus-1	LN680393	75.8-85.1
Totivirus	*Botryotinia fuckeliana totivirus 1*	NC_009224.1	75.4
Victorivirus	*Beauveria bassiana victorivirus 1*	KR011117; HE572591	74-81.5
	Beauveria bassiana victorivirus NZL/1980	NC_024151	77.0
	Bipolaris maydis victorivirus 1	MH396496	74-83.3
	Chalara elegans RNA Virus 1	NC_005883	66.3-93.1
	Fusarium poae victorivirus	LC150610	72.2 - 100.0
	Gremmeniella abietina RNA virus	NC_003876	78.4
	Helminthosporium victoriae virus	NC_003607	81.5
	Magnaporthe oryzae virus 1	NC_006367	90.9
	Magnaporthe oryzae virus 3	KP893140	76.0
	Nigrospora oryzae victorivirus 1	KT428155	78.2-96.6
	Phomopsis vexans RNA virus	NC_026135	84.9
	Tolypocladium cylindrosporum virus 1	NC_014823	64.4-90
	Ustilaginoidea virens RNA virus 3	NC_023547	76.4-76.7
	Ustilaginoidea virens RNA virus L	NC_025366	71.6-81.3



2.5.3.3 Nanopore based sequencing: MinION

In order to identify a rapid NGS methodologies to screen mycoviruses infecting *F. poae*, the MinION sequencer was also tested. The Nanopore system allows different solutions for nucleic acid sequencing. In this work it was to exploit the DNA sequencing capability through the Minion PCR Sequencing Kit SQK-PSK004, but also the MinION Direct RNA Sequencing Kit SQK-RNA002 were used for the purpose of sequencing genomic RNA.

MinION PCR Sequencing Kit SQK-PSK004

The MinION sequencing system based on the SQK-PSK004 kit allows the sequencing of the viral genome after retro-transcription and amplification of the cDNA and to obtain information about the DNA viral genome through direct DNA sequencing. In the Figure 2.16 the generation of long random amplicons has been shown. To take advantage of the kit, long amplicons have been generated and selected based on size. Not all samples exhibited homogeneous amplification and long amplicons, according with the different virome of each fungal strain. The sequencing run quickly produced around 100,000 reads in the first 3h, reaching 200,000 reads after 8h. At the end of the run the 512 available pores were 50% saturated, but the instrument was ready for another run. Unexpectedly an average of 500bp of length of the reads were obtained, below the ~ 134kb limit reached by the nanopore system (Tyson et al. 2018). The inefficiency of the system reflects what has also been observed in terms of genome coverage. Indeed, unlike IonTorrent sequencing, some gaps have been identified during the generation of contigs, thus not achieving high coverage in some areas of the sequenced mycoviral genomes (Figure 2.19).

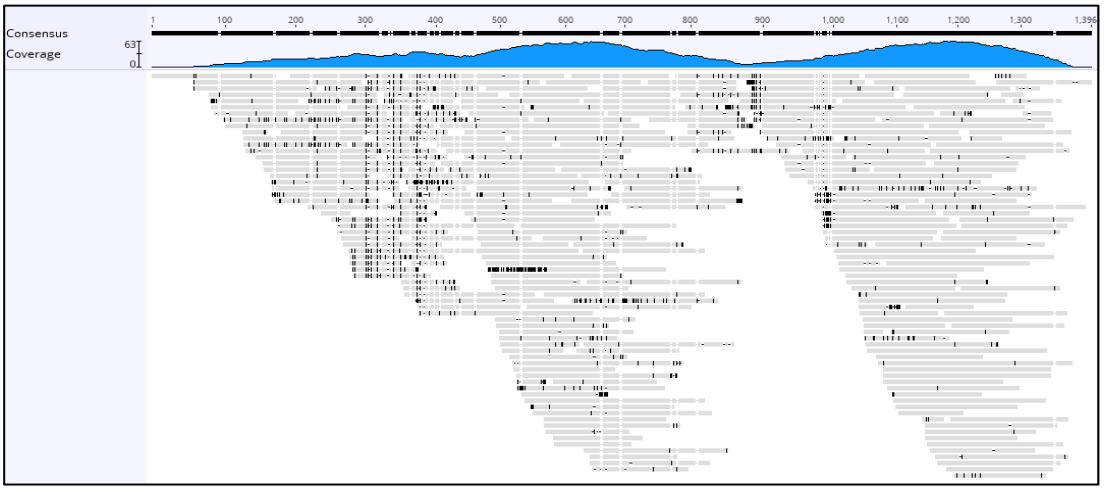


Figure 2.19 shows the results obtained with the PCR Sequencing Kit SQK-PSK004. The genome coverage of Penicillium aurantiogriseum parties-like viruses 1 strain MUT4330 (segment RNA2) (KY595973) was not deep and had gaps along the entire genome assembly

The low yield in terms of amplicon length was associated with the presence of several critical steps such as retro-transcription (RT) and PCR affected by secondary RNA structures, unlike when direct genomic DNA sequencing is performed. As expected also by SQK-PSK004 kit the 3’ and 5’ ends were sequenced only after using the classic RLM-RACE approach.

MinION Direct RNA Sequencing Kit SQK-RNA002

Unlike the previous kit, the SQK-RNA002 allowed to directly sequence the viral RNA without using step amplification. In this case the preparation of the library started from RNA as input material as required by the protocol, a poly (A) tail was attached to the 3 'end making library homogeneous and ready for loading through the pores. After total denaturation of dsRNAs in ssRNAs, E. coli Poly (A) Polymerase was used to catalyze the addition of AMP from ATP to the 3’ end of each RNA strand. This system, as previously mentioned, does not offer an amplification of the cDNA and therefore the yield in terms of number of reads is expected to be lower than in IonTorrent and MinION with SQK-PSK004 protocol. The instrument efficiently directly sequenced RNA providing 92,320 reads with length ranging from 200bp to 10,000 nucleotides. The coverage of small genomes (up to ~ 2,500bp) was comparable to the sequencing obtained by IonTorrent, while the system was able to generate long contigs even in the case of longer genomes (~ 13,000bp). The genome of Fusarium poae dsRNA virus 1 has been full sequenced, despite its genome consists in about 10,000bp (Figure 2.20). In the latter case the coverage was not comparable to the IonTorrent system. In 2 h of time the MinION device processed the 500ng of RNA introduced providing real-time results, through the basecalling system used by the MinKNOW software. MinKNOW is an operating software that drives nanopore sequencing devices allowing to acquire data, to have real-time analysis, local basecalling and data streaming.

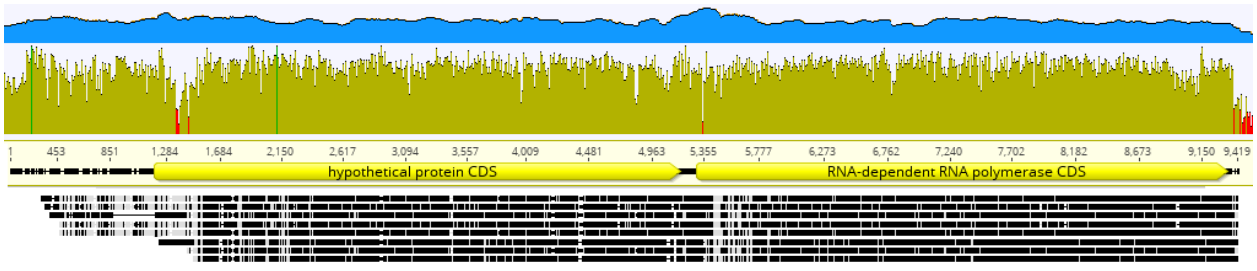


Figure 2.20 The mycovirus Fusarium poae dsRNA virus 3 isolated SX63 (KU728181) was completely sequenced starting from the 3 'to 5' ends, although its genome was 9419nt. The kit used in this sequencing was Direct RNA Sequencing Kit SQK-RNA002.

MinKNOW is used regardless of the kit used, allowing to produce FAST5(HDF5) files and/or FASTQ files, according to the preference of the user. Furthermore, SQK-RNA002 allowed to obtain also the 3 'and 5' ends without using RLM-RACE, because the sequencing starts from the 3' end and continues uninterruptedly until the 5' end, if the RNA has an acceptable quality.

2.5.4 Viral purification of *F. poae* strains and protoplast generation

The purification of the viral particles allowed to apply different strategies of preparation of the library, as well as to study in detail the morphology of the identified mycoviruses. As an example, the observed particles of F.541 (Figure 2.21), are characterized by an icosahedral structure and measure around 40 nm. Viral particles were mounted on carbon coated copper grid and negative stained using 2% uranyl acetate using a CM100 (Philips) electron microscope for observations.

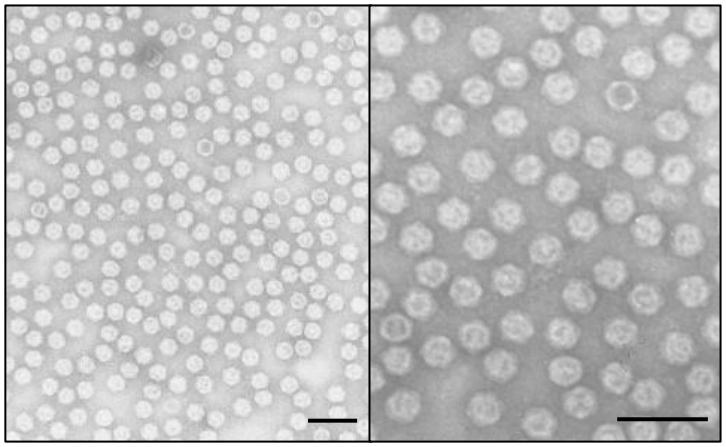


Figure 2.21 The particles of the Fusarium poae alternavirus 1 (FpAV1) virus are shown after observation through Transmission electron microscopy (TEM). The bar represents 100nm.

In addition, the purified viral particles were used for the transformation of *F. poae* protocols not infected with viruses, but no attempt has yielded positive results. On the other hand, the generation of *Cryphonectria parasitica* spherotoplasts has been successful, allowing the screening of IRES activities within different viral genera (Figure 2.22).

2.5.5 IRES activities

In collaboration with the University of Nagoya viruses identified with NGS techniques, as previously described, have been analyzed for IRES activities. Untranslated regions of mycoviruses at 5 'end involved in the initial mechanism of cap-independent translation, have been screened. A common vector for fungal expression pCPXHY3 was modified by inserting an expression cassette for Renilla and Firefly luciferase upstream and downstream of a multiple cloning site in which were inserted the 5'UTRs sequence and the first 72 nucleotides of the impending open reading frame (Figure 2.22). Subsequently the vector pCH-DLst3 was transformed into spheroplasts of *Cryphonectria parasitica* and then the fluorescence emitted by each sample was evaluated. Different viruses were analyzed, grouped into 4 groups (Figure 2.22), the first group (red columns) includes Yado-kari virus 1 and Yado-nushi virus, while the second group (green) contains all Botybirnaviruses. The third group (light blue) includes viruses belonging to the genus *Mitovirus* and the proposed genera *Phlegivirus* and *Megatotivirus*. Finally, the last group is the largest one (orange) of Fusagraviruses and close relatives. Many of those exhibited positive IRES activities, and more interestingly, some viral sequences showed potential host-fungal specificity for IRES functioning.

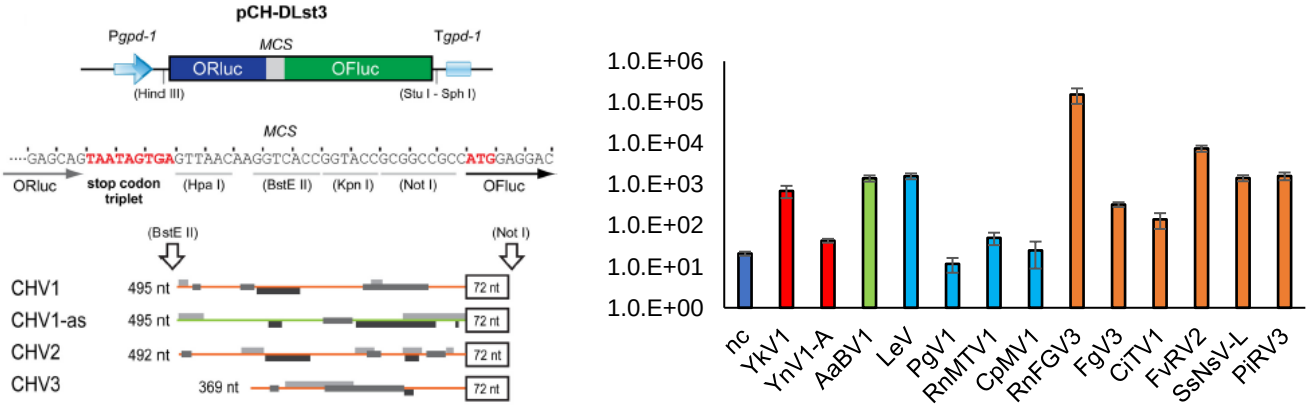


Figure 2.22 In the image (left) pCH-DLst3 vector is shown. pCH-DLst3 consists of a mutilated cloning site (MCS) and expression cassettes for Renilla (ORluc) and Firefly (OFluc) luciferase Source (Chiba et al., 2018). The 5'UTR ends of the selected viruses were inserted into MCS and the fluorescence of IRES activity was measured (imagine right).nc = pCH-DLst3 empty vector, without any insert in MCS, used as a control.

2.6 DISCUSSION AND CONCLUSIONS

The purpose of this work was to exploit the recent sequencing technologies (IonTorrent and MinION) but also the tools of classical virology to analyze the characteristics of mycoviruses infecting *Fusarium* spp. with the aim to select viral strains candidate for further studies aimed to identify possible biocontrol agents.

The growth rate of different fungal isolates was first assessed, using homogeneous growth conditions to avoid physical-chemical variation capable to affect the results. The experiments were thus repeated three times, always using the same methodology for the growth area measuring. Strains that were slower in growth exhibited homogeneous and common dsRNA profiles. This interesting data could support the hypothesis that a common virus or multiviral complex can interact with the normal growth of the fungus (Figure 2.13). The results obtained suggest that viruses infecting the fungal strains that reached the maximum growth area in less time may can confer benefits to their hosts and can therefore be excluded for further work. On the other hand, the mycovirus that infect the slow growing *F. poae* strains, may reduce their performances therefore virus recovery experiments can focus on those selected viruses in order to confirm hypovirulent induction on the fungus. Indeed, after the removal of viruses from the fungal strains it will be possible to perform an effective comparison between infected and uninfected strain.

In any case, the observation of growth parameters served to carry out a quick screening to identify fungi that could interfere with fungal fitness. The most interesting fungi have been selected and studied at the molecular level. The extraction of nucleic acids and the subsequent analysis provided an identity to the viruses infecting the selected strains. Both replicative intermediates (dsRNA) and VANA were isolated allowing to obtain information on every type of DNA and RNA viruses infecting *Fusarium* spp. The purified viral genomes were dissected by different NGS approaches. Such innovative methods as IonTorrent and MinION have been decisive in screening mycoviruses, because they allow to screen up to 30 fungi strains in each run. Each fungus can present a mixed infection and the study of viral populations with a classic approach is not economically and timely sustainable. IonTorrent-based sequencing was the most reliable system compared to the MinION method. The first has an error rate of approximately 0.5-1%, while the MinION can reach errors of up to 5-6% with the cell version R9. Recently the R10 version of the MinION chip appears very

promising: consensus accuracy has been achieved on a small genome sample, this translates to 1 error in every 100,000 bases, or 99.999% accuracy (Fu et al. 2019). Moreover, as advantage the MinION is able to sequence long molecules up to 134kb, compared with the 150bp reads length of IonTorrent. Furthermore, the SQK-RNA002 Direct RNA Sequencing Kit chip has revolutionized the concept of home RNAseq as even the 3 'and 5' ends can be sequenced without passing through the critical steps of RT and PCR reactions. Despite the efficient sequencing of the 3 'and 5' ends, this kit is limited only to the 3' end polyadenylated RNA as it do not includes the enzyme E.coli Poly (A) polymerase to reconstruct the poly (A) tail. In our work, unknown viral genomes were investigated, therefore E. coli Poly (A) polymerase was used to enrich the non-polyadenylated 3' ends of viral segments even if the non-polyadenylated 3' ends of a viral genome already presents adenine residue, the addition of a further poly (A) tail impair the exact reconstruction of the 3' end. For this reason the original kit has been modified replacing polyadenylation step with ligation of an oligomer DNA adapter used in RLM-RACE directly to 3' end of the RNA segments. In this way the developed system resulted no longer limited to polyadenylated RNAs. The viroma identified in the 30 strains of *F. poae* shows a great heterogeneity in terms of viruses belonging to many different mycoviral genera. Mainly viruses belonging to genera such as *Hypovirus, Chysovirus, Victorivirus, Betaendornavirus, Megabirnavirus, Mitovirus and Betapartitivirus* have been identified. A quarter of the viruses identified on the total are not yet classified and some of these are not yet present in GenBank. Some of the obtained contigs have been associated with viruses belonging to the *Totivirus* genus such as Chalara elegans RNA Virus 1 (NC_005883) and Tolypocladium cylindrosporum virus 1 (NC_014823) respectively with a nucleotide similarity of 66.3% and 64.4%. These values confirm that the viruses identified are too different to be the considered the same viruses present in GenBank and therefore can be considered new species. On the other hand, members of quadripartite dsRNA viruses, isolated from the phytopathogenic filamentous fungus, *Rosellinia necatrix*, has been associated to hypovirulence and shows traits very similar to members of the *Totiviridae* family (Lin et al. 2012). Furthermore, an interesting result was found in the identification of viruses similar to Fusarium graminearum hypovirus 1 (NC_023680) with 64.2% similarity. Indeed, hypoviruses have been deeply studied for their ability to confer hypovirulence to the host, based on *Cryphonectria parasitica* hypovirulence model. Finding new viruses that may be associated with virulence also in *Fusarium spp.* could be very effective about the diseases that

this fungus causes. Finally *F. poae* has been confirmed as a fungus hosting a wide variety of viruses that are very different each other (Osaki et al. 2016).

Furthermore, the IRES activity was evaluated in the fungal strains subjected to sequencing. Translation in eukaryotes usually begins at the 5' cap so only a single translation event occurs for each mRNA. However, some bicistronic vectors take advantage of an element called as Internal Ribosome Entry Site (IRES) to allow for initiation of translation from an internal region of the mRNA. This tool is suitable to understand the initial regulation mechanisms of the translation of viruses in the host, a key step for the interaction virus-fungus. Indeed, some of these mycovirus genera have been studied as inducers of hypovirulence in some phytopathogenic fungi. To verify the result obtained *in vitro*, *in planta* experiments will be carried out.

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CHAPTER 3: ''A NEW *EX-VIVO* APPROACH TO EVALUATE THE ACTIVITY OF SUITABLE COMPOUNDS TO CONTROL PHYTOPLASMA DISEASES''

3.1 INTRODUCTION

The identification of new phytoplasmas and phytoplasma-associated diseases increased dramatically in the last years and, consequently, also their economic impact on the wide range of infected hosts (Hosseini et al. 2017)(Wei et al. 2011)(Saeed et al. 2016)(Al-Subhi et al. 2017)(Zhu et al. 2017). The peculiar obligatory parasite condition makes phytoplasmas difficult to isolate in axenic conditions and constricted to the phloem of the plants where the pathogen can replicate. The pleomorphic nature of these wall-less bacteria makes them able to colonize efficiently the phloemic cells of host plants resulting in the damage of the nutrients translocation and of the biochemical plant balance (Wei et al. 2008)(Gai et al. 2014)(Giorno et al. 2013).

Measures as mandatory uprooting and vectors control become an unavoidable requirement to limit the progress of these diseases, as well as the use of new molecular techniques that play a fundamental role in the early detection of infected seedlings or propagated cuttings (Bertaccini et al. 2019) (Firrao et al. 2007). Over the years many strategies have been tried to stem the use of insecticides such as the recently and promising research concerning the use of acetic acid bacteria *Asaia* as a biocontrol agent of phytoplasma. These specific symbionts would be able to interfere during the phytoplasma-vector acquisition phase, because of their ability to invade almost all the organs of the vectors reported up to now (Gonella et al. 2018)(Favia et al. 2008). Unlike vector-control methods, several approaches to restore plants affected by phytoplasmas was proposed such as CO_2 insufflation (Converse and George 1987), thermotherapeutic treatments (Viswanathan and Rao 2011), tissue cultures for the production of phytoplasma-free clones (Dai et al. 1997) also coupled with heat therapy or hot water treatments (Chalak et al. 2013). The use of microorganisms from the host rhizosphere or of external control agents, such as fungi and bacteria, also represent an interesting biocontrol approach to hinder the proliferation of diseases caused by phytoplasmas (Lingua et al. 2002) (Gamalero et al. 2010) (Kamińska et al. 2010) (Bulgari et al. 2011).

Indeed, the interaction between the apple endophyte *Epicoccum nigrum* and the pathogen "*Candidatus* Phytoplasma mali" is a promising approach to the use of microorganisms such as

BCA against diseases caused by phytoplasmas. In the complex relationship between fungus-plant-phytoplasma, Musetti *et al.* (2011) demonstrated that *E. nigrum* stimulates a structural change in the tissues of the model plant *Catharanthus roseus* and in the morphology of the phytopathogen resulting in a significant increase in plant defence mechanisms and decrease in phytoplasma levels. More recently, the arbuscular mycorrhizal fungus (AM) *Rhizophagus irregularis* BEG 72 has been exploited for its action of attenuation of the damages caused by phytoplasmas. Furthermore, the combined use of AM and BP100 antimicrobial peptide appears to increase periwinkle plants resistance against *Ca.* Phytoplasma rubi associated to the rubus stunt disease and *Ca.* Phytoplasma solani associated to the stolbur disease (Rufo et al. 2017).

Although, the most active compound able to affect phytoplasma life cycles are the antibiotic class of oxytetracyclines (Singh et al. 2007) but other groups of synthetic and eco-friendly molecules widen the scenario on the rehabilitation of plants affected by phytoplasmas, encouraging the development of new screening systems (Tanno et al. 2018) (Askari et al. 2011).

Many variants of products based on biopesticides and botanical pesticides have been tried in all years to control diseases caused by phytoplasmas. The secondary metabolites of fungal as cercosporin, cladosporol and spirolaxin and metabolites with plant origin as pulegone and carvone were tested for their biocidal activity. These substances could act according to different mechanisms of action respectively, stimulating the production of reactive oxygen species (ROS), inhibiting β-glucan biosynthesis or through the inhibition of DNA topoisomerase (Chiesa et al. 2007).

The evaluation of commercial products described as resistance inductors against *Ca.* Phytoplasma solani associated to the grapevine diseases Bois noir (BN) has been performed in the work of Romanazzi *et al*. (2009). The bio-formulations tested showed a successful induction of resistance in BN-infected plants. The best results have been obtained with Bion, a product based on Acibenzolar-S-methyl, and with Kendal and Olivis both based on a mixture of glutathione and oligosaccharines.

In this work a system for a rapid *ex-vivo* evaluation of compounds proposed against uncultivable pathogens has been developed. The "study model" for this purpose was found in the *Ca.* Phytoplasma vitis associated to the Flavescence dorée (FD) disease of grapevine, related to the taxonomic elm yellow group (16 SrV) of phytoplasmas with sizes variable from 200 to 800 nm (EPPO, 2016). FD is transmitted mainly through the grafting of infected material on healthy plants

and by the vector *Scaphoideus titanus* Ball (Homoptera: Cicadellidae), a leafhopper with north American origin that feeding on grapevine phloem can spread the phytoplasma. Due to the high economic damages caused, FD is classified into quarantine species and it is inserted in the EPPO (European Plant Protection Organization) A2 list N ° 94; EU Annex designation II/A2. The typical symptoms of FD are leaves discolorations (turn yellowish/reddish) that can be limited by leaf veins, downward rolling of edges often resulting in an angular shape of leaves blade that becomes brittle, lack of lignification of the infected canes, death of clusters and vine stock decline sometimes very fast, total loss of leaves may occur resulting in plant death. The economic impact of this disease can include the substantial loss of wine grapes and the numerous plants dead that need to be replaced requiring great economic efforts by farms. Indeed, in 2005 Italy was indemnified with 34 million euros, from the European Union, to compensate for losses due to FD to which needs to be added the enormous budget that the regions affected by this disease have to spend to control the leafhopper vector (Chuche and Thiéry 2014).

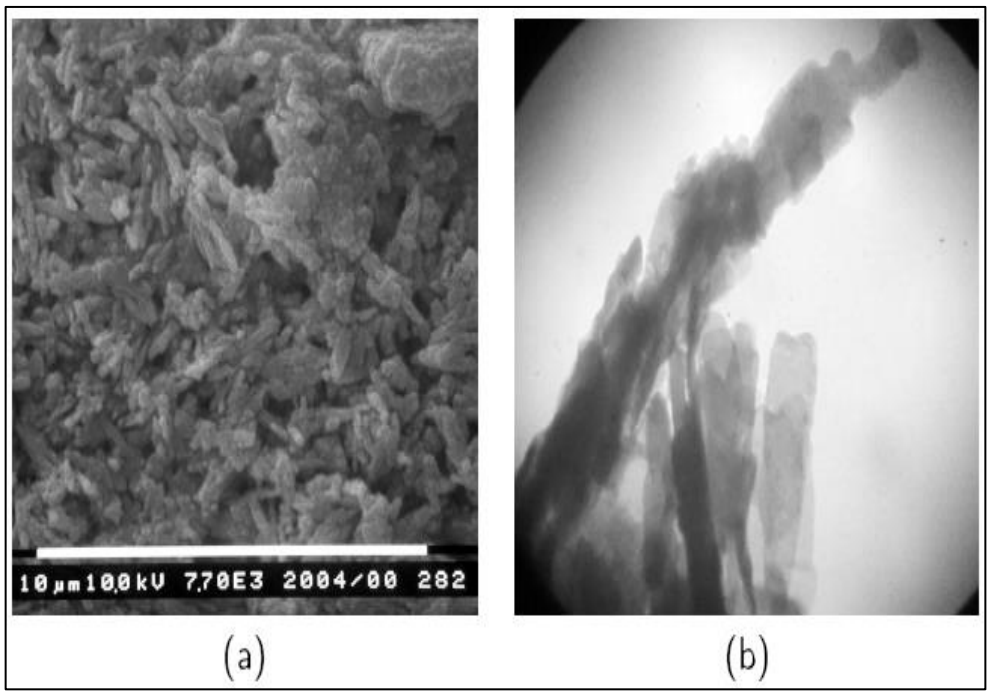


Figure 3.1 In the figure (a) hydroxyapatite crystals with a size of 0.5-2.0 µm seen by scanning electron microscope (SEM); In the figure (b) the same crystal seen by transmission electron microscope (TEM) (Baldo et al. 2019).

Taking advantage from the system developed in this work, the nanoparticles-based VITIBIOSAP® FI PLUS compound (patent Nr. 102015000015570 25 October 2017) has been studied for its direct action against phytoplasmas. This formulate is composed by a nano-carrier synthetic hydroxyapatite (HA) $Ca_{10}(PO4)_6(OH)_2$ (Figure 3.1), patented as MICROSAP®.
The MICROSAP® crystals have a size of 0.5-2.0 micron, and one gram can cover a surface from 90 to 120 square meters, leading to high efficacy of the coverage together with a low run-off. In addition to the HA, copper (Cu(II)-HA) and zinc (Zn(II)-HA) the formulation is completely miscible with essential oils that are widely proven to possess many proprieties such as antimicrobial and insecticidal activity (Ibrahim et al. 2008) (Šupová 2015).

3.2 AIM OF THE STUDY

The aim of this chapter was to evaluate the potential of botanical pesticides for the biocontrol of phloem limited phytopathogens such as the phytoplasmas causing flavescence dorée disease. The only active compounds able to control phytoplasmas are the antibiotic oxytetracyclines but *in vitro* direct and fast screening of new antimicrobials compounds on media is almost impossible due to the difficulty to culture phytoplasmas. For this reason, a simple and reliable screening method was developed to evaluate the effects of antimicrobials directly on phytoplasmas by an “ex-vivo” approach. Using scanning electron microscopy (SEM) in parallel with molecular tools (ddRT-PCR), the direct activity of tetracyclines on phytoplasma cells was verified, identifying also a promising compound showing similar activity.

3.3 MATERIALS AND METHODS

3.3.1 FD-infected plant material

Experiments were carried out using 3-month-old *Catharanthus roseus* (periwinkle) plants (var. albus G. Don) infected by FD. The propagation of the infection was performed by apical graft and growing plants under greenhouse condition at 24 ± 2 °C and 16 h photoperiod. Plants showing symptoms were tested 20 days post grafting by crude RNA/DNA extraction and RT-qPCR based method (Minguzzi et al. 2016) to confirm phytoplasma infection. The plant tissues with high concentration of phytoplasmas were identified by a rapid screening using DAPI (4′,6′-diamidino-2-phenilindole, 2HCl) at 1 μg/ml in 0.01M phosphate buffer pH 7.2 (Figure 3.6) (Poggi Pollini et al. 2001). The tissue areas with phloem vessels rich in phytoplasmas have been transverse micro-sectioned in presence of 5% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, obtaining slices from stem, petiole or larger leaf veins. The slices were immediately transferred into a 1.5 ml tube with 500 μl of the same fixative solution and subjected to 2 bar pressure for 5-10 minutes, using an adapted Critical Point Dryer (CPD) specimen holder coupled with an air compressor, to ensure the fixation of the most internal tissues. The samples were then rinsed twice with 0.1M phosphate buffer, pH 7.2, before preparation for electron microscopy.

3.3.2 Transmission Electron Microscopy (TEM)

Freeze substitution protocol was followed for rapid embedding of TEM specimens using TAAB Araldite 502/812 kit. A perforated aluminium block was cooled in liquid nitrogen and immediately samples, soaked in a solution of 1% osmium tetroxide and 0.1% uranyl acetate in acetone, were loaded. On reaching the room temperature gradually, the samples were quickly embedded in 25-100% resin through microspin centrifuge and moved to a stove for 2h at 100 °C (Mcdonald and Webb 2011). The resultant resin samples were trimmed by hands to have trapezoidal shaped pyramid from which ultrafine sections (60 to 100nm in thickness) were obtained by Leica C.Reichert Om U3 Microtome (Type 700141) equipped with DiATOME Histo knife (Figure 3.2).

Slices were loaded on EMS grids and poststained for 15 min in 5% uranyl acetate in water followed by 2 min in Reynold's lead citrate. Samples were then observed by a PHILIPS CM10 TEM at 80kV.

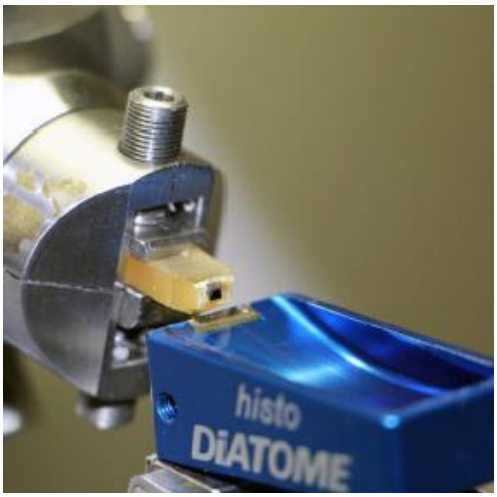


Figure 3.2 Preparation of samples of samples embedded in resin by microtome

3.3.3 Scanning Electron Microscopy (SEM)

Sampled sections were dehydrated twice by 20% ethanol in distilled water then once in 30%, 50%, 75%, 95% and 100% ethanol solutions maintaining the tubes 8 minutes in a rotator device for each step. Samples were subjected to Emitech K850 Critical Point Drying (CPD) using liquid CO_2 as a transitional fluid. The dried sections were placed on the 1/2" slotted head-1/8" pin stubs covered with a conductive 3M 465kp scotch tape and coated by gold particles using Emitech K500 sputter at 40mA for 3 minutes.

Resin from embedded TEM samples was removed, after ultrafine sections trimming, using sodium ethoxide obtained from an ethanolic NaOH solution after precipitation with acetone (Olson, Edgar T. 1934). The precipitated sodium ethoxide was then dehydrated under vacuum and the resulting powder resuspended in 30% ethanol. Trimmed resin blocks were submerged in the solution for 12 hours, rinsed twice in 100% ethanol and processed with critical point dryer and sputter as described. Specimens were imaged using HITACHI SEM S510 at 15kV and images acquired with Point Electronic DISS 5 system.

3.3.4 Phytoplasma RNA and DNA quantification

Total nucleic acids were directly extracted from samples previously prepared for SEM observation and subjected to ddRT-PCR (Figure 3.3). Each section was removed with sterile tweezers from the stub and homogenized with a motor-driven tissue grinder G50 (MoBiTech) in 1.5 ml Eppendorf tubes using 100µL of MacKenzie extraction buffer (MacKenzie et al. 1997). The resulting homogenate was diluted in 400µL of nucleic acids-free water and an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) was added. After centrifugation at 16,000 g for 10 minutes the supernatant was precipitated with 2 volumes of absolute EtOH, 16µL of 5M sodium chloride and 1µL of 10mg/ml glycogen solution. After incubation for 15min at -20°C and centrifugation at 16,000g for 20 min, total nucleic acids were washed with 70% EtOH, vacuum dried and then resuspended in 20 µL of nuclease-free water.

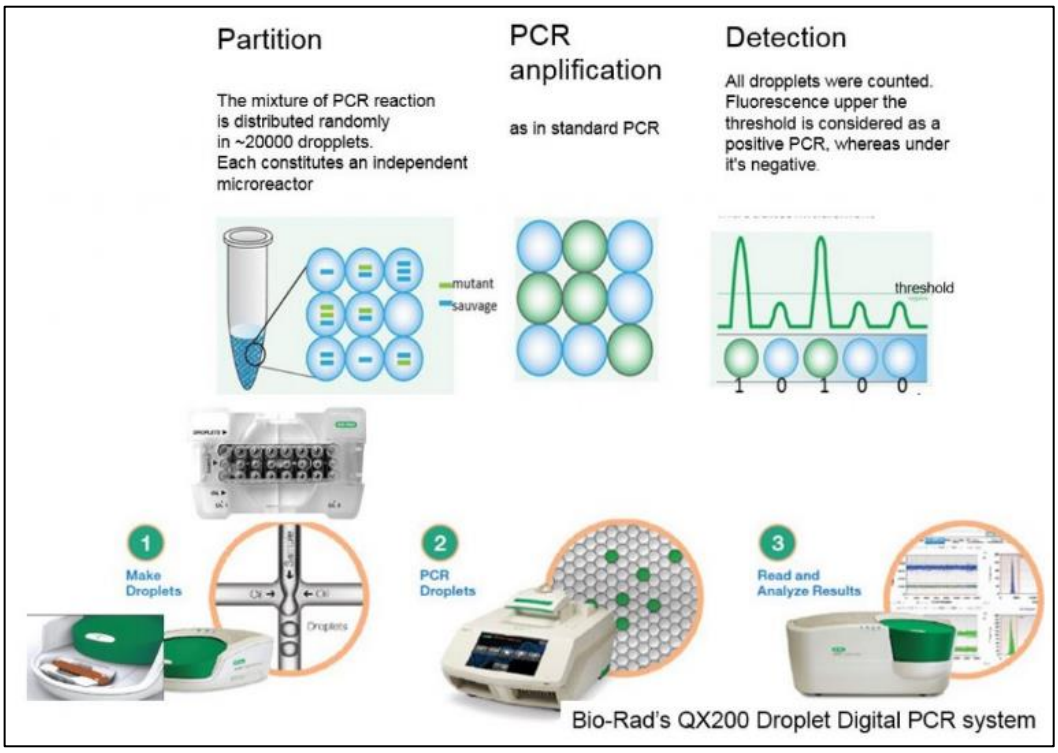


Figure 3.3 The picture shows the essential steps of a ddPCR analysis. In this type of analysis there is no calibration curve as in qPCR, but the quantification is absolute. Source (www.bio-rad.com)

Droplet Digital PCR (ddPCR) and one-step ddRT-PCR analyses were performed (Figure 3.3) using ddPCR™ Supermix for Probes (#1863024, Bio-Rad Laboratories) as indicated by manufacturer protocol and added by 2 units of M-MLV Reverse Transcriptase (#M1705, Promega, USA), respectively.

Previously published primers and probe, specific for *Candidatus* Phytoplasma vitis and for plant 18S rRNA (Ratti et al. 2019) were used in a total reaction volume of 20µL. After droplet production by a QX200™ Droplet Generator (Bio-Rad Laboratories) the mixture was subjected to the following amplification protocol: 48°C for 30 min, 95°C for 5 min, 40 cycles of 95°C for 30sec and 60°C for 1 min, followed by 4°C for 5min, and 90°C for 5 min. After amplification droplets were streamed and analysed by a QX200 Droplet Reader and QuantaSoft Software (Bio-Rad Laboratories) to identify fluorescent positive and negative droplets and calculate target nucleic acid concentration.

3.3.5 Calibration of VITIBIOSAP® FI PLUS concentration

Since VITIBIOSAP® FI PLUS was used for the first time on the *Catharanthus roseus* test plant, the concentrations application methods of the product were tested. Previous experiment evidenced that periwinkle plants cannot uptake the formulate when it is sprayed on leaves or when it is applied to the root system (Baldo et al. 2019). Then, periwinkle plants were exposed to the formulate using a modified falcon sealed to the stem of the plants to obtain a liquid-container where a solution containing VITIBIOSAP® FI PLUS at different concentrations was loaded (Figure 3.4). In order to allow fluid to enter the phloem district, several small wounds were caused on the stem. Once the non-phytotoxicity limit concentrations were identified, the experiment was hijacked with the aim of evaluating the effect of VITIBIOSAP® FI PLUS on periwinkle plants affected by FD.

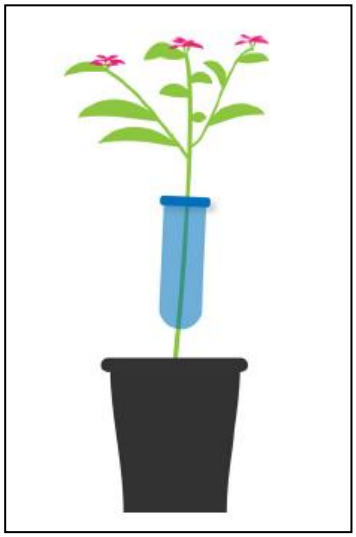


Figure 3.4 Rapid test to evaluate non- phytotoxicity limit. concentrations

3.3.6 Ex-vivo plant treatment assay

Cuttings (10 cm long) from infected periwinkle plants were left to feed for 48 hours or 10 days in different solutions under continuously stirring to ensure homogeneous concertation of each compound. For this purpose, tap water and a solution at 150mg/L of Tetracycline hydrochloride ≥95% (#T3383, Sigma-Aldrich) were used for both exposition times, while 0.1 and 1% solutions of VITIBIOSAP® FI PLUS were used for 10 days and 48 hours experiments, respectively. Tetracyclines solution has been protected from sunlight for all experiments to prevent photodegradation. The experiments have been replicated twice and for each exposition time 5 cuttings have been used in each solution for a total of 60 cuttings. From all of those, 2 stem sections have been collected 3cm upper the feeding point and used to assess qualitative and quantitative phytoplasmas infection by SEM and ddPCR analyses (Figure 3.5).

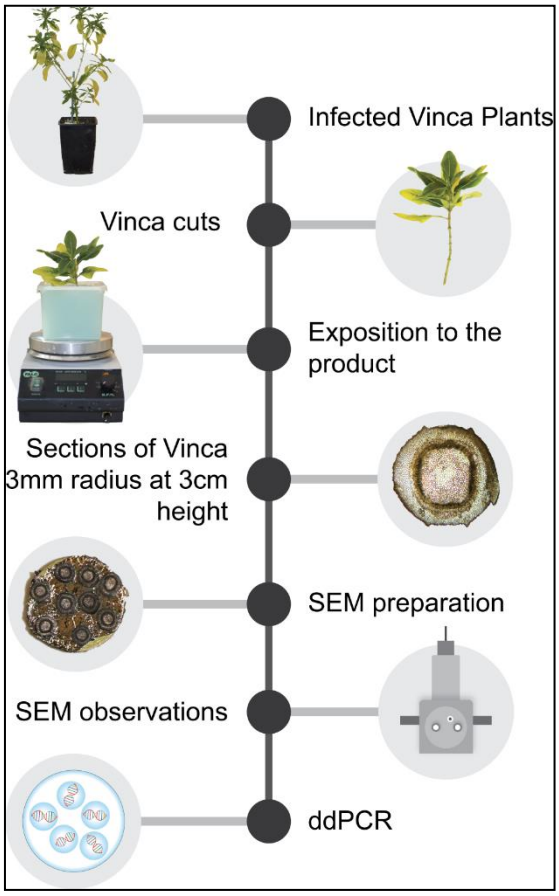


Figure 3.5 Representative scheme of *ex-vivo plant treatment assay*.

3.3.7 Data analysis

Data obtained by morphological observations at SEM and by molecular analyses performed by ddRT-PCR were statistically analysed by Tukey method using the IBM SPSS Statistics for Windows package (IBM Corp. Released 2017, Version 25.0. Armonk, NY: IBM Corp.).

3.4 RESULTS

3.4.1 Plant material

Leaf yellowing, significant reduction in leaf size and zig-zag deformation of veins appeared on periwinkle plants 20-25 days after graft-inoculation with FD-infected shoot.

All symptomatic plants tested positive to FD by RT-qPCR analyses while not inoculated or not symptomatic plants tested negative.

Observations on stem, petiole and leaf veins sections, using DAPI stain, revealed bright fluorescent spots in the sieve tubes of infected periwinkle plants, absent in the not inoculated plants, indicating phloem cells completely colonized by phytoplasmas. As expected, small light spots, highlighting the cellular DNA, have been observed inside the parenchyma cells on sections from both infected and not infected plants (Figure 3.6)

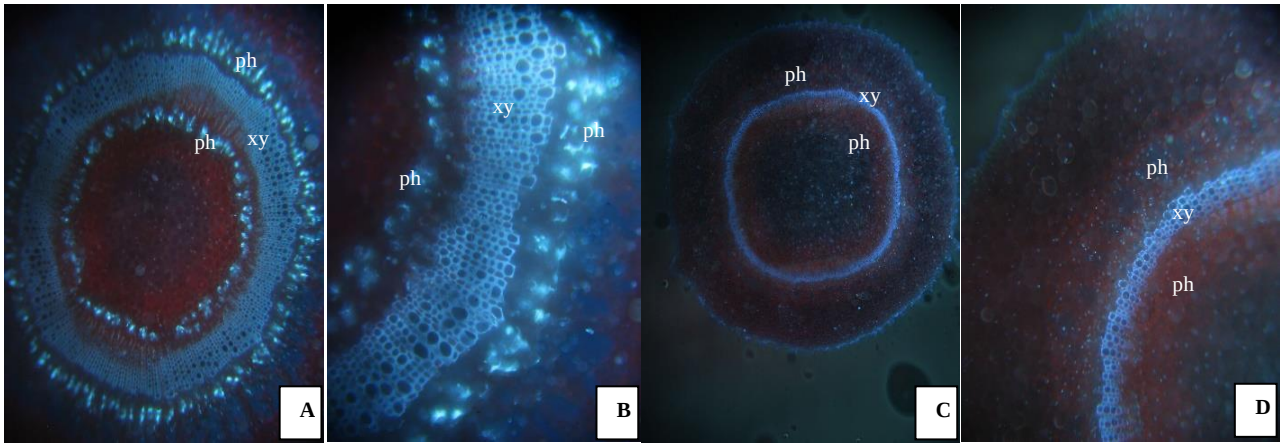


Figure 3.6 The pictures show cross sections of periwinkle plants prepared with DAPI stain and observed by fluorescence microscopy. In A-B phloem elements (ph) have fluorescent spots due to the presence of phytoplasmas. Sections of healthy plants B-C show only the auto-florescence emitted by the xylem elements (xy), but no luminous spots are present in the phloem.

3.4.2 Electron microscopy analyses

Ultrathin sections of resin-embedded samples from infected plants shown, under TEM observation, the typically pleomorphic, spherical or filamentous structures surrounded by a trilaminar membrane of 6-7nm. Heterogeneous morphologies of phytoplasma were observed varying in electronic density and size (from 100nm to up 900nm) according to the growth stage, stationary phase and sectioning layers (Figure 3.7A and B). After ultrafine sections trimming, in

order to address further SEM observation to the certain identification of phytoplasma cells, the resin was removed from 10 embedded samples of stem sections from infected (8) and not infected (2) plants. Observation of the resin-free specimens by SEM, at the same phloem cellular districts previously analyzed by TEM, allows to compare the structures visualized by the two microscopic techniques. Plant cells, appeared completely occupied by phytoplasma under TEM observation, showed a compact mass under the surface served for ultrathin sectioning. Where the mass resulted less compact, three-dimensional spherical structures, with diameter of approximately 1μm, were clearly distinguished (Figure 3.7 C and D). No similar structures were observed in plant cells resulted empty by TEM except in some samples, only from infected tissues, where SEM analyses allowed to identify spherical structures (phytoplasma cells) not interested by the sectioning layer therefore not visible on the ultrathin sections under transmission observation (Figure 3.7 A and C).

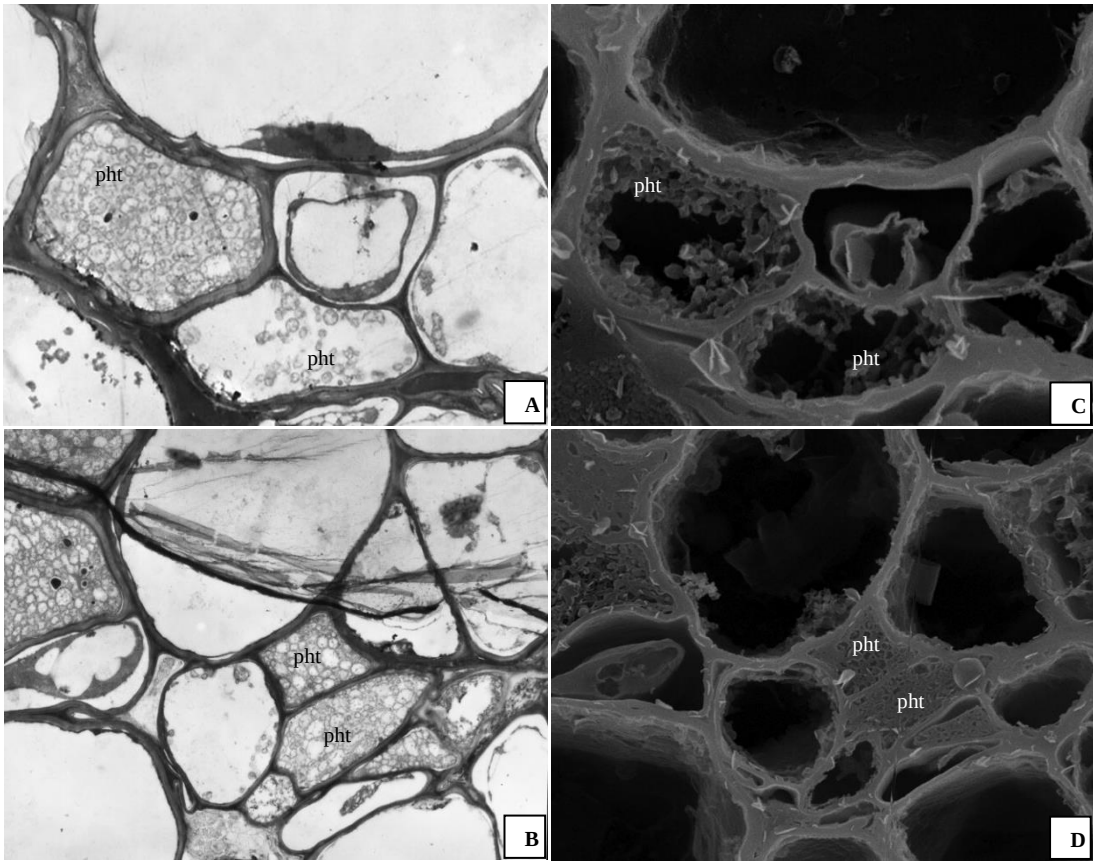


Figure 3.7 In figure A and B TEM sections of infected plants by FD; in figure C and D SEM observation of the typical structure of phytoplasmas (pht) after resin disassembly.

Sections from fresh infected stem, petiole and leaf veins observed by SEM revealed the presence of phloem cells completely clogged by a dense mass that, at higher magnification, appear composed by spherical cells, with dimension ranging from 400 to 900 nm, packed inside the cell

walls. None of the described structures have been observed on samples from not infected plants (Figure 3.8 A, B and C).

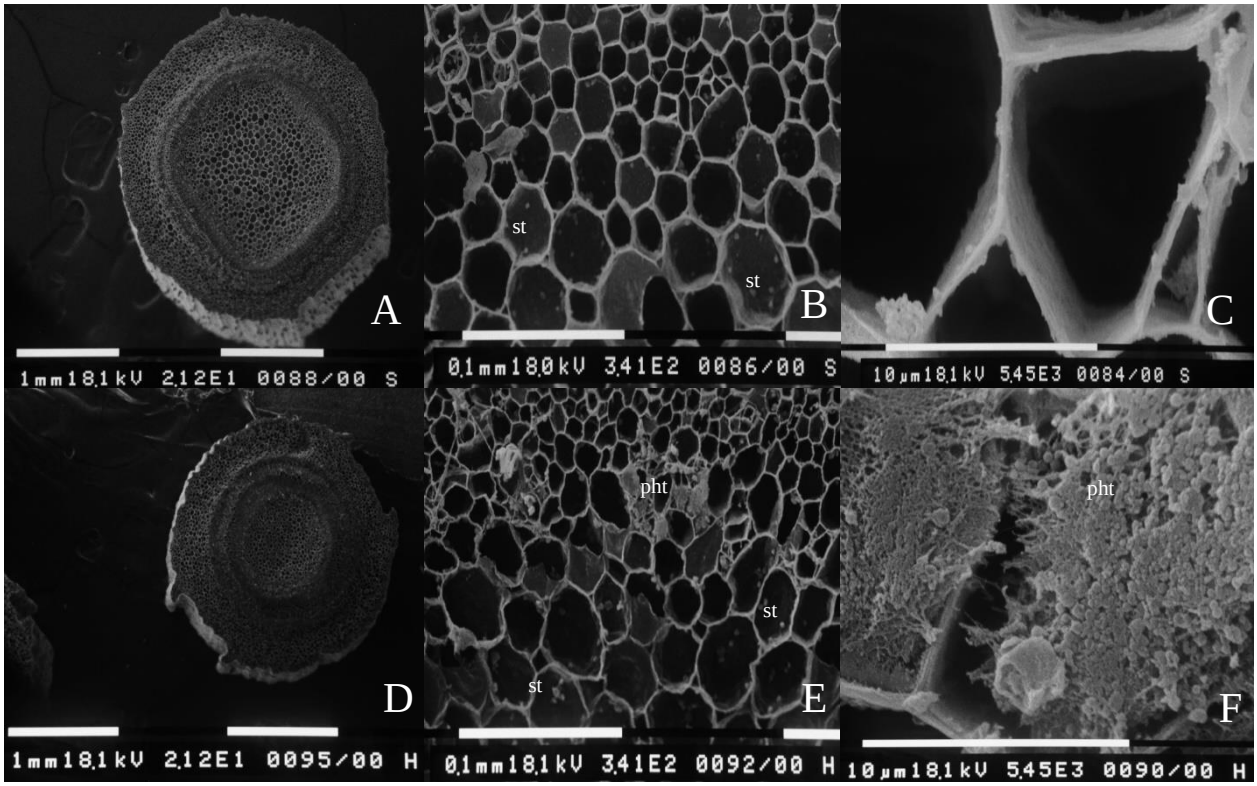


Figure 3.8: In pictures A, B and C healthy periwinkle plants are shown throughout SEM, while in pictures D,E and F the infected ones by Grapevine flavescence dorée phytoplasma (FD).pht: phytoplasma; st: starch grains.

3.4.3 Ex-vivo plant treatment assay

A total of 120 sections from the cuttings exposed to the different treatments were analyzed by SEM. In each section, 60 plant cells containing phytoplasmas were randomly selected on the phloem ring and the status of the pathogen has been evaluated by a scale ranging from 1 to 3. The class 1 corresponds to high concentration of undamaged pleomorphic round-shaped structure of the phytoplasma (Figure 3.10A and B), 2 indicates structural irregularities and appreciable reduction in the phytoplasma cells concentration (Figure 3.10C and D) while 3 means that the pathogen structure is seriously compromised and spherical cells cannot be recognized (Figure 3.10E and F).

The classification of the 7,200 phloem cells observed showed that pathogen status corresponding to class 1 was observed in sections exposed to tap water (94% after 48 hours and 31% after 10 days) and to tetracyclines (84% after 48 hours) but not in samples exposed to VITIBIOSAP® FI PLUS or to tetracyclines after 10 days (differences were significant at $P < 0.001$). Phytoplasma

cells showing class 2 status were observed with low incidence after 48 hours (6% in tap water, 16 % in tetracyclines and 1% VITIBIOSAP® FI PLUS) but with higher incidence after the 10 days experiment (69%, 50% and 21%, respectively) ($P < 0.005$). Finally, status corresponding to the class 3 was observed in samples treated by VITIBIOSAP® FI PLUS for 48 hours and 10 days

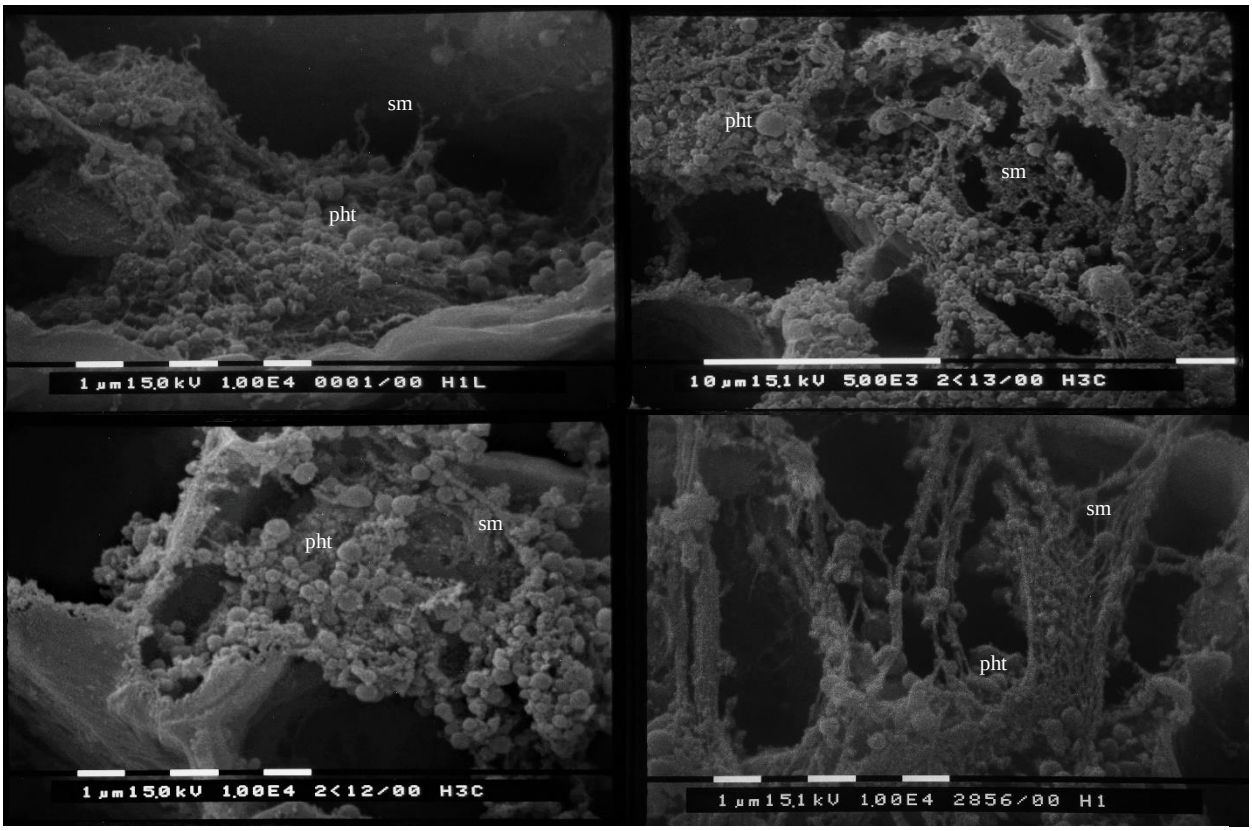
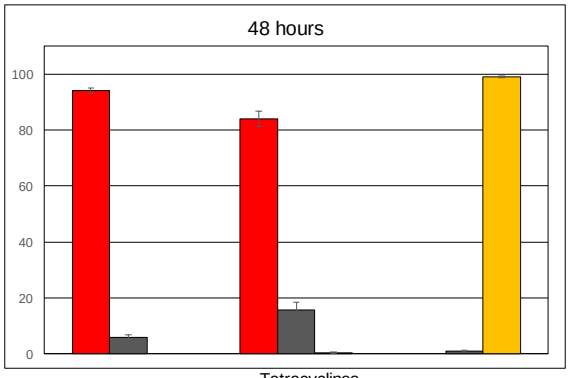


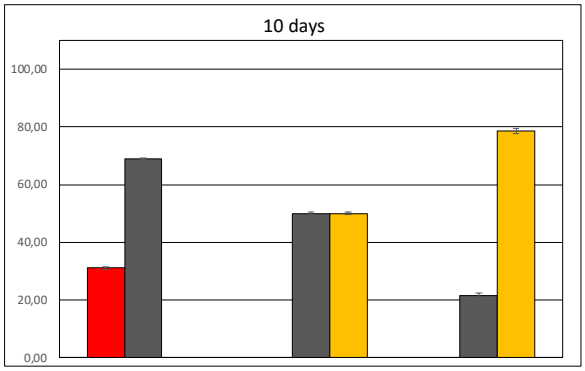
Figure 3.9: Images at SEM of phytoplasmas (pht); it is possible to observe the typical round shape of the pathogen and supporting matrix (sm)..

(99% and 78%, respectively) and by tetracyclines for 10 days (50%) ($P < 0.001$) (Table 3.1).

Table 3.1 Summary of the data obtained by the morphological observation of the samples.



	Tap water	Tetracyclines (150mg/L)	FI PLUS (1.0%)
Type 1 (%)	94.20±0.89	84.00±2.74	0.00
Type 2 (%)	5.80±0.89	15.67±2.66	1.00±0.35
Type 3 (%)	0.00	0.33±0.24	99.00±0.35



	Tap water	Tetracyclines (150mg/L)	FI PLUS (0.1%)
Type 1 (%)	31.00±0.39	0.00	0.00
Type 2 (%)	69.00±0.39	50.00±0.51	21.33±1.02
Type 3 (%)	0.00	50.00±0.51	78.67±1.02

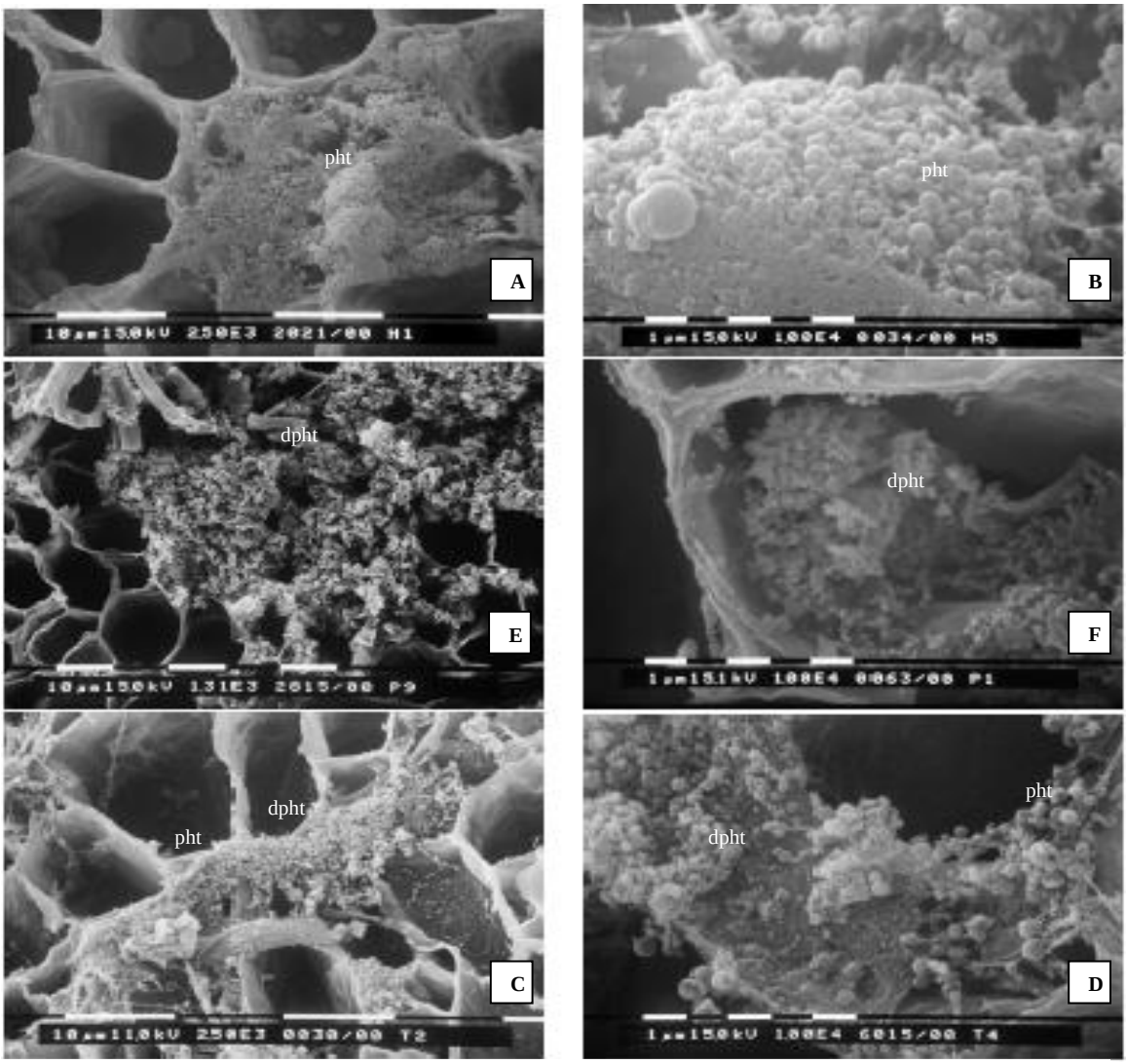


Figure 3.10 Images of the classes identified during the study corresponding to the status of phytoplasmas. pht: phytoplamas; dpht: phytoplasmas structure seriously compromised.

3.4.4 Phytoplasma RNA and DNA quantification

Samples from the second replicate of the *ex-vivo* experiments were also subjected to the quantification of FD DNA and RNA content. The quantification of 18S rRNA from plant tissues allows to normalize data according to the size of the analysed samples.

Phytoplasma DNA, after 48 hours, halved in samples treated by VITIBIOSAP® FI PLUS if compared with samples exposed to tap water while treatment with tetracyclines resulted in about 1.8 folds decrease (differences were significant at $P \leq 0.001$) (Table 3.3A). After 10 days a 3.5 folds reduction of DNA concentration has been observed in samples treated with VITIBIOSAP® FI PLUS ($P \leq 0.001$) with not statistically significant difference in concentration ($P = 0.984$) to that of the samples treated with tetracyclines (Table 3.3B).

Table 3.2. *Ca.* Phytoplasma vitis DNA and RNA concentration on periwinkle tissues exposed to different treatments for 48 hours or 10 days.

Cuttings feeding time	Treatment	DNA	RNA
48 hours	Tap water	3.92±0.38	187.43±36.55
	Tetracyclines (150mg/L)	2.97±0.32	77.82±12.63
	VITIBIOSAP® FI PLUS (1%)	1.65±0.25	110.15±23.56
10 days	Tap water	5.06±0.88	227.17±58.17
	Tetracyclines (150mg/L)	1.56±0.25	58.36±12.37
	VITIBIOSAP® FI PLUS (0.1%)	1.42±0.31	26.05±6.87

The FD RNA concentration also varied according to treatment and exposition time. RNA content in samples treated with tetracyclines resulted nearly 2 and 4 times lower than the concertation in samples treated with tap water respectively after 48 hours ($P = 0.019$) and 10 days ($P = 0.007$) exposure. Samples treated with VITIBIOSAP® FI PLUS showed, with low significance ($P = 0.116$), RNA content after 48 hours 1.5 folds lower than samples treated with tap water while, after 10 days, the corresponding reduction has been calculated of about 8.5 times ($P = 0.003$) (Figure 3.3).

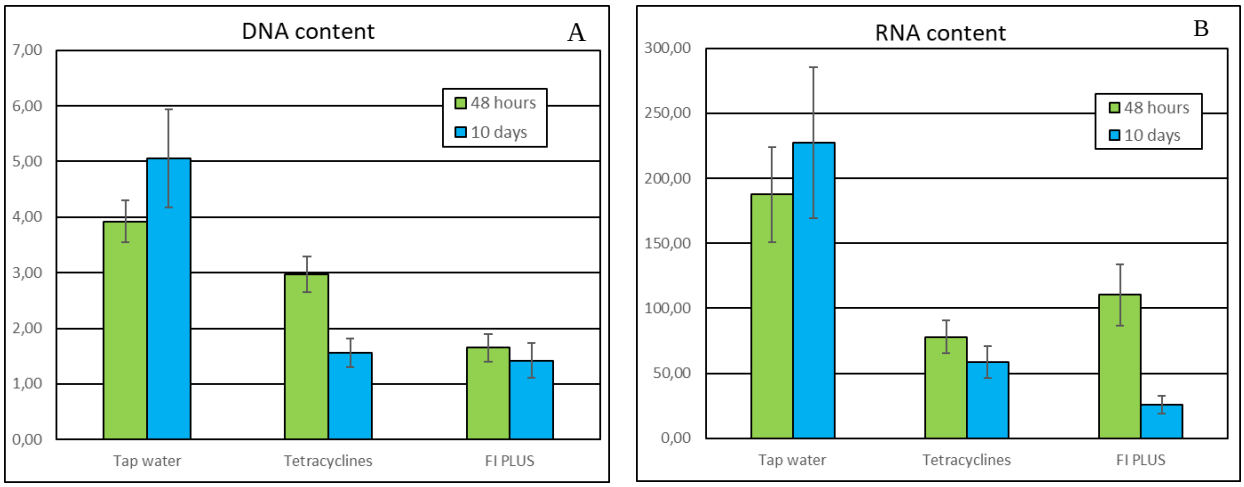


Figure 3.3: graphs obtained from the data of the table 3.2. A) DNA content; B) RNA content.

3.5 DISCUSSION AND CONCLUSIONS

Since the first TEM detection of phytoplasmas bodies into phloem cells (Doi et al. 1967), the electron microscopy has been largely used to analyse the ultrastructural details of this pathogens within the infected plant tissues. Transmission techniques, the most common and used methods for phytoplasma studies, obtain excellent results for morphological investigations but require chemical fixation and dehydration, with final embedding in a resin followed by ultrathin sectioning (Devonshire 2013) only providing 2D images. On the other hands, the techniques for SEM are less laborious and allow to appreciate a 3D view of phytoplasmas permitting to better evaluate the biological vitality and the infection rating within the host cells. Even though SEM observations of phytoplasmas have been employed since long time (Haggis, G. H., & Sinha 1978)(Poghosyan et al. 2004)(Devonshire 2013) this approach has been rarely used maybe as considered less reliable than other methods. In this work we demonstrated the consistency of SEM analyses observing, for the first time, the same phytoplasmas-infected cells first by TEM and then by SEM imaging. Scanning observation under the cutting surfaces of embedded samples (Figure 3.7) clearly resemble the situation that appears under SEM observation of samples prepared from fresh-infected tissue (Figure 3.8F). According to that we can argue that following the SEM preparation described above we minimized the removal or the modification of cell contents as well as the creation of artifacts on the specimen analysed (Devonshire 2013).

Thanks to this comparison we identified SEM analyses as a robust method to perform a fast-morphological screening of plant tissue, monitoring the status of the phytoplasmas saving time for samples processing. This approach, coupled with the use of the rapid-growing and phytoplasmas high-susceptible periwinkle plants and of ddRT-PCR quantification, allow us to develop a new approach for the screening of compounds suitable for the control of uncultivable pathogens such as phytoplasmas.

It is well known and confirmed that tetracyclines are specific antimicrobials active in the cure of plants infected by phytoplasmas but many studies have been performed investigating new possibilities to identify other compounds active in this type of control (Singh et al. 2007) (Chiesa et al. 2007) (Bianco et al. 2013) (Rufo et al. 2017) (Tanno et al. 2018).

For this purpose, in the last years, many nanotechnological approaches have focused on the functionalization of biocompatible molecules thus giving rise to ‘’the biocompatible drug delivery system’’. These compounds, initially developed as biomimetic carriers for human medical science,

are now applicable in plants protection improving bio-availability, preventing premature degradation of active molecules and enhancing the uptake of not specific compounds expressing antimicrobial activity (Chen et al. 2016)(Battiston et al. 2018). One of these innovative compounds, the VITIBIOSAP® FI PLUS, based on the drug delivery system named MICROSAP® developed by the NDG Natural Development Group Srl, was included in our experiments.

This compound, under our experiments, showed promising performances altering the phytoplasmas status and the DNA-RNA content when compared with results obtained using tap-water and tetracyclines, respectively, as negative and positive control.

First of all, the developed *ex-vivo* protocol allowed to discriminate between the effect of the experimental controls. Regarding the tap-water treatments, as expected, no morphological modifications have been observed after 48h and only minor changes appeared after the 10 days exposure, probably due to the unavoidable changes in the phloem composition caused by the continuous feeding of the cutting by water. These observations are confirmed by a comparable content of DNA and RNA (not significant differences) in both the timing of the experiments (Table 3.3).

When the treatments with tetracyclines are considered, a consistent modification of the phytoplasmas bodies status and an appreciable reduction of DNA content have been observed only after the 10 days experiment while the RNA content is reduced in both 48 hours and 10 days exposures (Table 3.2) suggesting an early activity of tetracyclines on phytoplasmas metabolism (replication, physiology) that, later, results in a reduction of the number of phytoplasma cells.

Surprising results have been obtained after the VITIBIOSAP® FI PLUS treatments. The compound, applied at the dilution of 1%, seriously compromised the phytoplasma bodies just after 48 hours as demonstrated also by the significant reduction of DNA content but in particular, in the 10 days experiment, by the drastic reduction of both RNA and DNA contents (8.5 and 3.5 times, respectively) even with more than 20% of phytoplasma bodies showing intermediate (class 2) modification, probably due to the applied dilution (0.1%) of the formulate (Table 3.1).

Our results demonstrated that we developed a fast and reliable *ex-vivo* assay that allows to verify the activity of compounds directly on phytoplasma cells. Moreover, as electron microscopy (SEM) and molecular tools (ddRT-PCR) provided consistent conclusions the efficiency evaluation can be achieved even using a single approach according to the laboratory facilities. The use of periwinkle plants ensures the possibility to perform the experiment at any time independently by seasons and

availability of infected grape plants.

The assay results therefore particularly useful for a fast and preliminary screening of potential antimicrobials and to define their treatment conditions before conducting more expensive and time-consuming field trials.

Finally, this work also identified a formulate, the VITIBIOSAP® FI PLUS, that demonstrated to reduce the phytoplasmas concentration, slow down replication and change their morphology. Further studies will focus on the application of the formulate to grape plants first by the ex-vivo assay on leaf petiole then investigating its ability to work under field conditions.

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CHAPTER 4

4. CONCLUSIONS

The biological control offers an ecological and economic alternative, if compared to the use of pesticides, for the control of phytopathogenic agents. Usually the effectiveness and the costs of BCA use are expressed in comparison to the use of pesticides, but very often the former have greater stability over time associated with a lower development and production cost (Bale et al. 2008). The level of diseases control obtained by biocontrol agents can be compared with that achieved through fungicides. In an example taken from the work of Alexander and Stewart (2001) the treatment with fungicides had a 100% success against *Phytophthora cactorum* infecting the apple tree, compared to 79-98% of that obtained with different BCAs. Moreover, even there are often comparative results in field trials and greenhouses, in the open field suppressions of only 24% of the control level achieved by fungicide, were recorded using BCAs (Fu et al. 2010). These examples demonstrate the difficulties of some BCA to act in the open field and often this problem is linked to the relationships between control agents and the set of microorganisms that are present in the phyllosphere or in the rhizosphere. Indeed, extreme specificity of a BCA makes it less efficient in pathogen control. This is one of the reasons explaining why different BCAs are often combined in a single formulation. Except for BCAs used immediately after production, cells or biomass are usually dried and formulated as products suitable for storage but compatible with distribution and application. In order to increase the shelf life of the formulate air and freeze drying, drying on silica gel and spray and fluid bed drying are used at industrial level. Thanks to these processes the water is eliminated preserving the vitality of the BCA. After that the stabilization of the inoculum is carried out by adding carriers, bulking agents, diluents and nutrients. BCAs have been formulated as dusts, gels, emulsions, prills, pellets and granules for seed treatments, dips, wettable powders, sprays for application to aerial plant parts and drenches for incorporation into soil or growing media (Fravel, 2005).

Economically sustainable mass production of stable inoculum, appropriate formulation cost-effective of commercial product as well as ease of use are fundamental parameters for the successful development of BCAs. Currently, many fungicides are relatively cheap and more effective than BCAs and are unlikely to be substituted for by BCAs unless they are withdrawn

from the market. Another aspect that affect spreading of BCAs use is the fact that just few growers are informed about how to appropriately store and use BCAs, this lack often results in an inadequate disease control and subsequent poor satisfaction of the user. Clearly, there is need to train growers on how to use BCAs effectively and integrate their use into crop protection programmers. Methods such as pseudo-soil fumigation, solarization and biosolarization are organically compatible and have the advantage of exploiting physical and chemical parameters such as solar radiation and volatile biocidal compounds, instead of using live microorganisms requiring precautions as described above. However, in the age of intensive cultivation and continuous crop rotations these methods have limitations. For example, the field cannot be cultivated for the period during or after the treatment, such as 1-2 months during the summer months when the maximum solar radiation is used. For the disinfection of pathogens in the depth of the soil it is necessary to irrigate and keep the soil wet and this requires adequate supplementary irrigation systems. Moreover, in the current Anthropocene era, where the decrease of plastic residues are the subject of continuous discussion, these polluting methods could be at the end of their use (Elmore et al. 1997).

Natural development of hypovirulence has allowed researchers to focus on the biological use of viruses, opening a new field of study regarding the biological protection of crops and forests by using viruses as natural antagonist of fungi which represent the major plant pathogens. It has been nearly 50 years since the first evidence of this beneficial relationship between viruses and fungi has been described. Awareness that a virus could naturally reduce the incidence of a virulent strain of fungi saving a large amount of plant species has inspired studies and experimentations to reach a final practical application of this method. The study model on which the research is mainly conducted is the Chestnut blight, disease caused by *Cryphonectria parasitica*, it is the first case in which a mycovirus-based biocontrol technique has been satisfactorily implemented. It is taken in account especially in the development of new preventive and therapeutic measures centered on several tree species. Despite the apparent abundance of mycoviruses in nature, studies on these infective agents is relatively scarce and performed mainly in the last years. In the past, technical difficulties limited such studies of mycoviruses, because of the impossibility to investigate their biology and structure, but these obstacles are decreasing with the advent of new research approaches and technologies. Several hypovirulence-associated mycoviruses, such as *Sclerotinia sclerotiorum hypoviruses*, *Helminthosporium victoriae* viruses, and *Rosellinia necatrix* viruses,

have been detected and studied using reverse genetic and NGS approaches (Chiba et al. 2009; Pearson 2014; Marzano et al. 2016). Because viruses lack an extracellular phase, researchers have investigated transfection methods using purified virus particles, full-length viral cDNA clones and *in vitro* RNA transcripts. These infection assays will facilitate identification of viral and/or host factor(s) involved in symptom induction or virus replication for many mycovirus–host systems. New possibility has been opened by the discovery of mycoviruses able to be transmitted through an extracellular phase like the case of *Sclerotinia sclerotiorum hypovirulence-associated* DNA virus 1 (SsHADV-1) (Yu et al. 2013). The approaches that have been deepened in chapter 2 based on the cross analysis of data regarding the growing speed of fungal strains and the study of replicative intermediates/viral genomes (dsRNA), represent an effective and economically feasible biological assay for the identification of morphological changes in fungus due to the presence of viruses. Furthermore, this information has been linked to the results obtained from molecular assays based on the latest new-generation sequencing technologies (NGS), thus allowing to provide an identity to the viral agents that caused morphological changes in the fungal host. Technologies such as MinION and Ion Torrent were then compared by identifying characteristics suitable for rapid identification of viruses with RNA or DNA genome. In the logic of rapid screening, even systems such as MinION pcr sequencing kit sqk-psk004, which worked less efficiently, can be considered useful as it allows the sequencing of only small portions of the viral genome sufficient for mycoviral identification. Once clarified what is the viroma present within a fungal population, in the case of this work *F. poae*, it was possible to proceed with the choice of viruses known in the literature as possible hypovirulent agents. Rapid screening of more fungal strains gives therefore the opportunity to increase the knowledge of the lifestyle of the host virome, but also to find new ways of control not yet completely understood.

Unlike biopesticides, naturally derived compounds could be characterized by a direct and easy method of application and by a highest stability of formulate at commercial level. Botanical pesticides are mainly chemicals that are naturally extracted from plants with a fast mechanism of action, exhibiting, in most cases, an action to control plant diseases, a repellent activity against insects, unlike some synthetic compounds that inhibit the growth of even "noble" insects. Unlike against pesticides, phytopathogenic microorganisms do not immediately develop resistance and in the soil these compounds are rapidly degraded avoiding accumulation in the environmental but also in the food products. On the other hand, even natural compounds are not all safe for humans,

some of those have been reported as carcinogenic similar to compounds with synthetic origin even if they are usually quickly degradable under solar UV rays. Another important aspect is that up to now the legislation do not provide clear rules for their use (Regnault-Roger and Philogène 2008; Regnault-Roger et al. 2012; El-Wakeil 2013; Sabir et al. 2017).

In the case of essential oils, for example, one of the major problems is the transportation and distribution of these hydrophobic substances within the treated plant tissues, as a root treatment or leaf sprinkling are not effective. The new nanotechnologies in recent years have offered numerous alternatives to increase the adsorption of active compounds to the target. The technology screened in chapter 3 was based on hydroxyapatite microcrystals (with patent name of MICROSAP®) completely miscible with essential oils that are widely known to possess many properties such as antimicrobial and insecticidal activity (Ibrahim et al. 2001; Šupová 2015; Battiston et al. 2018; Baldo et al. 2019). For this reason, in this work the bioformulate VITIBIOSAP® FI-PLUS was tested against phytoplasmas associated to FD disease, known as living pathogens within the plant's phloem vessels and therefore immune to external pesticide treatments. This concrete example of drug delivery system capable to target hidden pathogenic organisms, suggest its possible application in open field even in organic farming, reducing the use of synthetic pesticides to control vectors. Biological control is therefore an inviting alternative for the control of plant diseases, offering advantages and disadvantages depending on the crop management method and the needs of growers. However, within the framework of Integrated Pest Management (IPM), the potential of biological control is at the moment confined to research laboratories. To overcome this problem, new formulations based on nanotechnologies offer a new possibility for improving the selection and characterization of biocontrol agents. Systems such as those presented in this thesis work aim to generate new models for biocontrol tool screening, trying to speed up and target research on promising products to be tested directly in open field.

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ABSTRACT

Choosing natural enemies to suppress pest population has been for a long the key of biological control. Overtime the term biological control has also been applied to the use of suppressive soils, bio-disinfection and biopesticides. Biological control agents (BCA) and natural compounds, extracted or fermented from various sources, are the resources for containing phytopathogens. BCA can act through direct antagonism mechanisms or inducing hypovirulence of the pathogen. The first part of the thesis focused on mycoviruses, which are viruses that infect fungi, have the potential to control fungal diseases of crops when associated with hypovirulence. The development of new approaches capable of faster dissecting the virome of filamentous fungi samples was performed. The semiconductor-based sequencer Ion Torrent™ and the nanopore-based sequencer MinION have been exploited to analyze DNA and RNA referable to viral genomes. Comparison with GeneBank accessions and sequence analysis allowed to identify more than 40 putative viral species belonging to the genera *Partitivirus, Betapartitiviruses, Victoriviruses, Totiviruses, Betaendornaviruses, Mitoviruses, Megabirnaviruses, Chrysoviruses, Hypoviruses*, to the proposed new genera Polymycoviruses, Fusaguraviruses and Alternaviruses or to unclassified mycoviruses. Some of these mycovirus genera have been studied as inducers of hypovirulence in several phytopathogenic fungi, therefore future works will focus on the comparison of the morphology and physiology of the fungal strain infected and cured by the viruses identified and their possible use as a biocontrol agent. In a second part of the thesis the potential of botanical pesticides has been evaluated for the biocontrol of phloem limited phytopathogens such as phytoplasmas. The only active compounds able to control phytoplasmas are the antibiotic oxytetracyclines and *in vitro* direct and fast screening of new antimicrobials compounds on media is almost impossible due to the difficulty to culture phytoplasmas. For this reason, a simple and reliable screening method was developed to evaluate the effects of antimicrobials directly on phytoplasmas by an "*ex-vivo*" approach. Using scanning electron microscopy (SEM) in parallel with molecular tools (ddRT-PCR), the direct activity of tetracyclines on phytoplasma cells was verified, identifying also a promising compound showing similar activity.