UNIVERSITÀ DEGLI STUDI FIRENZE

UNIVERSITÉ DE REIMS CHAMPAGNE-ARDENNE

DOTTORATO DI RICERCA IN SCIENZE AGRARIE E AMBIENTALI - CICLO XXX

SSD: PATOLOGIA VEGETALE - AGR/12

DOCTORAT EN PHYSIOLOGIE ET BIOLOGIE DES ORGANISMES - POPULATIONS - INTERACTIONS

SPÉCIALITÉ: PHYSIOLOGIE VÉGÉTALE

COORDINATORE: Prof. Giacomo Pietramellara

Developing an innovative tool to enhance the biological activity of active substances for the control of fungal diseases in *Vitis vinifera* L.

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Abstract

Title: Developing an innovative tool to enhance the biological activity of active substances for the control of fungal diseases in *Vitis vinifera* L.

The research investigates the application of biomimetic calcium phosphate as innovative delivery system for grapevine (*Vitis vinifera* L.) protection purposes. This smart material was successfully studied in the biomedical field, from the functionalization of biomimetic calcium phosphate with anti-cancer molecules for localized releases, to the development of an innovative toothpaste for oral hygiene. Preliminary assays to implement the control of the grapevine fungal diseases, have revealed promising results.

In this framework, the biomimetic inorganic hydroxyapatite was investigated as potential delivery system of bioactive substances allowed in organic agriculture for plant protection.

Through a multidisciplinary approach, the study was aimed to evaluate the efficiency of hydroxyapatite in enhancing the biological activity of copper(II) compounds, on the control of relevant common diseases, like downy mildew, and complex fungal diseases, such as the grapevine trunk diseases. This aim is related to further ambitious goals: the significant reduction of the fungicides amounts applied in plant protection and the optimization of the distribution and persistence of the bioactive substances in the plant tissues, including the vascular ones, where harmful pathogens can develop.

Overall, the experimental activities allowed: (i) to understand the interaction between delivery system, functional substance and grapevine tissues; (ii) to demonstrate the mechanism on which the higher efficacy of the functional substance is based; (iii) to collect new information on the mechanisms involved in the symptoms expression by studying the plant defense reactions induced by the treatments.

Keywords: sustainable viticulture; fungal diseases; grapevine protection;

Résumé

Titre : Développement d'un outil innovant pour optimiser l'activité biologique des substances actives afin de contrôler des maladies fongiques chez *Vitis vinifera* L.

Ce travail a consisté en l'étude l'application du phosphate de calcium comme système de transporteur (« drug delivery ») pour la protection de la vigne (*Vitis vinifera* L.). Ce biomatériau a été étudié avec succès dans le domaine médical, de la fonctionnalité du phosphate de calcium avec des molécules anticancéreuses, à la mise au point d'un dentifrice innovant. Des essais préliminaires dans le contrôle des maladies fongiques de la vigne ont révélé des résultats prometteurs.

Dans ce contexte, l'hydroxyapatite inorganique et biomimétique a été étudié en tant que système transporteur potentiel de substances bioactives autorisées en agriculture biologique pour la protection des plantes. À travers une approche multidisciplinaire, l'objectif de l'étude était d'évaluer l'efficacité de l'hydroxyapatite dans l'amélioration de l'activité biologique des composés de cuivre(II) pour lutter contre des maladies fongiques telles que le mildiou et les maladies du bois. Cette étude a pour ambition de contribuer à l'optimisation de la distribution et de la persistance des substances bioactives dans les tissus végétaux, y compris vasculaires, où des pathogènes nocifs peuvent se développer ainsi qu'à la réduction des quantités de fongicides.

Cette recherche a ainsi permis de (i) comprendre l'interaction entre le système transporteur, la substance fonctionnelle et les tissus de la vigne ; (ii) démontrer le mécanisme sur lequel l'efficacité supérieure de la substance fonctionnelle est basée ; (iii) recueillir de nouvelles informations sur les mécanismes impliqués dans l'expression des symptômes des maladies du bois en étudiant les réactions de défense des plantes induites par les traitements.

Mots clés : viticulture durable ; maladies fongiques ; protection de la vigne ;

Riassunto

Titolo: Sviluppo di un mezzo tecnico innovativo per ottimizzare l'attività biologica di sostanze attive nel controllo delle malattie fungine in *Vitis vinifera* L.

La presente ricerca studia l'applicazione del fosfato di calcio biomimetico come sistema «delivery» innovativo per la protezione della vite (*Vitis vinifera* L.). Questo materiale intelligente è stato studiato con successo nel campo biomedico, dalla funzionalizzazione del fosfato di calcio con molecole anti-cancro, allo sviluppo di un dentifricio innovativo. Saggi preliminari per implementare il controllo delle malattie fungine della vite hanno rivelato risultati promettenti. In questo contesto, l'idrossiapatite inorganica biomimetica è stata studiata come potenziale sistema «delivery» di sostanze bioattive consentite nell'agricoltura biologica per la protezione delle piante.

Attraverso un approccio multidisciplinare, lo studio è stato finalizzato a valutare l'efficienza dell'idrossiapatite nel potenziare l'attività biologica dei composti di rame(II), sul controllo di malattie comuni, come la peronospora, e le malattie complesse, come le malattie del legno della vite. Questo scopo è legato a ulteriori obiettivi ambiziosi: la riduzione dei fungicidi applicati nella protezione delle piante e l'ottimizzazione della distribuzione e persistenza delle sostanze bioattive nei tessuti vegetali, compresi quelli vascolari, in cui possono svilupparsi agenti patogeni dannosi.

Complessivamente, le attività sperimentali hanno consentito: (i) di comprendere l'interazione tra sistema «delivery», sostanza funzionale e tessuti della vite; (ii) dimostrare il meccanismo su cui si basa la maggiore efficacia della sostanza funzionale; (iii) raccogliere nuove informazioni sui meccanismi coinvolti nell'espressione dei sintomi, studiando le reazioni di difesa delle piante indotte dai trattamenti.

Parole chiave: viticoltura sostenibile; malattie fungine; protezione della vite;

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1. Sustainable control of grapevine fungal diseases

Grapevine (*Vitis vinifera* L.) is one of the most important crops worldwide, due to the production of both wine and table grapes. Based on the most recent OIV estimations, grapevine covers 7.5 million ha worldwide and 5 countries represent 50 % of the world vineyard surface area (Spain, France, China, Italy and Turkey). Approximately half of the global harvested grape, is destined for wine production, making an economical annual turnover estimated around 29 milliard euros [1]. Unfortunately, most of the cultivars used for winemaking are highly susceptible to several pathogens [2].

Since their diffusion from the American continent in the 19th century *Plasmopara viticola* (the causal agent of downy mildew) and *Erysiphe necator* (the causal agent of powdery mildew) are the most important grapevine fungal diseases, and together with *Botrytis cinerea* (the causal agent of grey mold) are the cause of the largest number of treatments in vineyards [3].

In Europe, the oomycete *P. viticola* was initially identified in France in 1878 after its presumable accidental introduction by the importation of American *Vitis* species required for breeding program of rootstocks resistant to phylloxera [4]. In the following years the pathogen was rapidly found in several other European countries and the fast diffusion was later attributed to the oospores distribution through soil and propagating material [5]. During the 20th century, downy mildew caused by *P. viticola*, became the most severe, spread and economically important grapevine fungal disease in middle Europe. Downy mildew affects leaves, fruits, and shoots of grapevines. It causes losses through damaging the leaves bringing to a partial or total defoliation, damage or loss of the young shoots, and causing a low-quality production up to the full loss of the crop. Under optimal weather conditions and when no protection is provided, downy mildew can actually easily destroy 50 to 75% of the crop in one season [6]. Despite the firsts control treatments based on lime-copper in the form of the better-known Bordeaux mixture were revealed as an efficient and widely used fungicide, a significant part of the vineyards had to be explanted and the more susceptible *V. vinifera* cultivars were substituted with several hybrids obtained by crossing *V. vinifera* with American species [7].

However, powdery mildew was the very first and disastrous grapevine fungal disease appeared in Europe, as the related pathogen was detected in a glasshouse in England by Tucker in 1847. Massive epidemics over several years, especially in warm and dry southern regions, caused staggering losses that often approached 100% and extremely changed the grape growing and vineyard management. Fortunately, early applications of sulphur powder revealed a significant disease control, later integrated by a mixture of sulphur, lime and water [4, 8].

In temperate climates worldwide, grey mold (or botrytis bunch rot and blight) caused by the ascomycete *Botrytis cinerea* (Pers.: Fr), is a further important disease of grapevines responsible of extensive economic losses through grape desiccation, rot and biochemical changes that reduce wine quality [9]. Economic losses may also derive from the costs associated with manually sorting diseased clusters pre- or postharvest, and from the lower value of the affected fruit [4].

From the second half of the 20th century, chemical disease management was the most effective measure used worldwide to control grapevine pathogens especially in modern viticulture. Considering that the fungal infections are mainly responsible for damages in grape quality and yield losses, the most used pesticides in viticulture are fungicides [10].

In this context, the progressive increase of the production standards, often requires an intensive pesticide schedule. A precise quantification of the synthetic organic pesticides used for the grapevine protection is not available and the amounts can be highly variable depending on the year, location and the relative importance of each disease. In general, fungicides account for the largest share of pesticide applications in most vineyards (with an average of 12-15, up to 25-30 treatments in the most problematic conditions). Consequently, over the years, several and specific synthetic organic fungicides were developed by the agrochemical industry and applied to enhance the control of downy mildew, powdery mildew, grey mold and several other pests. More recently, problems associated with old synthetic active substances and consumer demand for residue free products have stimulated research into new tools for disease management [3].

In the same years, sustainability has become a recurrent term in viticulture and thus attention towards environmentally friendly practices in the wine industry has increased. Sustainability attempts to integrate practices that are sensitive to the environment, socially equitable and economically feasible into the wine growing context [11].

In this perspective, agrochemical companies are aimed to develop new molecules with a lower toxicological profile for human health and the environmental pollution, and new mechanisms of action with lower risk of developing resistant pathogens populations. On the other side, the grape growers are stimulated to combine several different actions in order to reduce the input of synthetic organic pesticides on the crop system. This approach was recently defined and promoted as integrated viticulture, which principle consists to evaluate and employ all suitable techniques and methods in the most compatible manner to maintain the pest population levels below those causing economic damage [12].

The integration of agronomic practices, such as rational management of the canopy, soil cover grass and plant nutrition, is a common example of practices implemented to reduce the disease inoculum, by improving the microclimate of the plant and avoiding conditions favorable for the pathogens development.

A new frontier on the reduction of the fungicide application in viticulture is represented by the varieties that are resistant/tolerant to grapevine diseases, in particularly downy mildew and powdery mildew. Since the introduction of *P. viticola* and *E. necator* from America to Europe, breeding programs were initially developed in France by crossing resistant American *Vitis* species with traditional European *V. vinifera*, creating resistant hybrids, which, however, were no longer cultivated because of the undesired off-flavor in wine (foxy taint) [13, 14]. A more successful breeding program was developed in the second half of last century in Germany and the new cultivars were this time classified for quality wine production, besides to be accepted by the grape growers for their high quality and good resistance. Currently, the such called "new generation's hybrids" show an average reduction of plant protection treatments of about two thirds [15, 16].

The sustainable control of the major grapevine fungal diseases is also being performed by the farmer through the application of natural molecules and biocontrol agents based on microorganisms. These tools represent a concrete alternative to synthetic chemicals, despite that several of the existing solutions have still drawbacks or limiting factors, which prevent a fast uptake by the farmers [17]. At the same time, the sustainability is also achieved by the rational use of the chemical fungicides. This is even more important for copper-based fungicides, which are still the most effective products allowed for organic viticulture in several countries [18].

Nevertheless, as supposed by Pertot *et al.* (2017), a significant change in the grapevine cropping system is unlikely to take place rapidly in traditional areas, such as Europe, for both, economic (e.g. traditional varieties are used for the production of most of the more profitable wines) and environmental reasons (e.g. high visual negative impact on the traditional landscape). For the same reasons, the integrated pest management (IPM) appears the most applicable approach in short term for the reduction of the pesticide use and the efficient disease control. Besides the agronomical practices, the resistant/tolerant cultivars, the bio-pesticides, the IPM toolbox also includes several computer-based forecasting systems, which simulate the pathogen cycle and predict the onset and progress of the related diseases allowing to optimize the timing of fungicide applications [19].

Novel studies are more and more addressed to new potential tasks related to the increasing diffusion of complex diseases, the possible invasions of alien species and the occurrence of pathogen strains resistant to pesticides. In this framework, the development of innovative tools to enhance the biological activity of active substances may lead to crucial purposes of sustainability, such as the amplification of the activity spectrum of such molecules and the significant amount reduction of the applied pesticide.

1.1. From copper to innovative natural substances

For a long time, wide-spectrum contact fungicides have been the only compounds available for controlling grapevine diseases [20]. Especially on downy mildew control, from the discovery of the antifungal activity of copper by Millet in the 19th century till the first successful greenhouse trials with non-copper products in the middle of the 20th century, Bordeaux mixture was the only oomycete fungicide available in phytomedicine [21].

After World War II, several synthetic organic fungicides were successfully developed and applied in viticulture, thanks to their technical stability, low cost and high efficiency on the grapevine disease control [22]. Moreover, fewer problems with phytotoxicity were observed in comparison to the copper-based fungicides [23]. However, later, the high efficiency and selectivity of such synthetic organic molecules were also associated to side effects on the human health and the environment. As a consequence, by the years several fungicides have been removed from the market according to the guidelines for plant protection products in the European Union (Directive 2009/128/EC, Regulation EC No 1107/2009).

Today, the most important fungicide in organic agriculture is copper, being a non-synthetic compound with a wide activity spectrum [24].

The biological activity of a cupric fungicide is linked to copper ions Cu(II) that are able to denature proteins and blocking various enzymes of the fungal spores (as well as of the bacterial cells) [25]. Copper based fungicides have preventative action and therefore the treatments have to be applied, for example in the case of downy mildew, before an infective rain, by closely observing weather forecasts. Beside the direct toxic effect, copper also causes a delay in plant growth and a hardening of foliage and berries, which has additional benefit such as protection against secondary pathogens and climatic stress [26].

However, among the side effects related to the copper use, the extensive copper accumulation in soils is responsible of serious environmental problems [27], resulting in laws which limit its use. For this reason, the efficacy of reduced copper doses (lower than the dosages

recommended on cupric fungicide labels) for the control of downy mildew, is object of investigations, in order to adapt to legislative restrictions [24, 28].

Natural alternatives to copper-based fungicides and to pesticides in general are commonly suitable for use in organic agriculture. Such compounds come from many different sources (e.g., plant, animal, microbial and mineral) and present different modes of action (e.g., antibiosis, induction of resistance, hyper-parasitism, competition for space and nutrients). The majority are microbial and botanical active ingredients presented as early-stage experimental compounds (proposed by research institutions), compounds in the advanced stages of development (commercial manufacturers) and substances available in the markets (commercial products).

Zanzotto *et al.* (2016) have extensively reviewed the biological activity of the substances that among the inorganic compounds or the organic chemical inducers and natural extracts (plant extracts, fungal extracts and compost extracts), were found to be effective on the control of the major grapevine diseases caused by fungi or oomycetes. For many of these compounds, the role in the plant disease resistance mechanisms were highlighted and they were eventually considered for exploitation in crop protection [29]. The possibilities for the practical application of induced resistance in disease management are discussed by Walters *et al.* (2013) [30]. However, as reported in the same review, natural compounds alone are mostly incapable of ensuring sufficient protection under high levels of disease pressure.

1.2. Biocontrol agents

An alternative strategy to reduce the use of chemical pesticides for a sustainable viticulture is represented by the application of biocontrol agents (BCAs). Such approach is being widely applied in organic farming systems as well as within the integrated pest management (IPM) strategy, thanks to their very low environmental impact [24].

Plants interact with many microbial organisms living inside the plants, as endophytes, or on the external surface, as epiphytes. Special attention has been given to fungi and bacteria, acting as biocontrol agents, plant growth-promoting agents and resistance inducers. A certain number of BCAs have already been investigated and promoted for their antifungal activity and the stimulation of the plant defense responses, especially on the control of the grapevine mildews and the grapevine trunk diseases [29].

Moreover, many BCAs with good protection capacities under laboratory conditions did not have their performances confirmed in field tests [20]. Several factors, such as abiotic and biotic

stresses, plant–soil interactions, climatic conditions, may contribute to the results variability, limiting the exploitation of the antagonistic capacities of BCAs [31, 32]. Furthermore, the specific microbial strain or, as for the natural compounds, the type of formulation can significantly affect the protective efficacy, while the effectiveness of the same BCAs can differ depending on the grape cultivars [33].

In this context, considering their preventative activity and their inconsistent efficacy, most of the biological products have to be applied by the grape growers with particular attention and in the meantime, they require the development of specific protection strategies in vineyards [30].

2. Sustainable control of the diseases related to the decline of grapevine

The grapevine protection is even more challenging in the control of diseases that are still poorly understood like the ones responsible for the grapevine young decline, which is severely affecting the vineyard productivity worldwide. It is the case of the grapevine trunk diseases (GTDs) that are becoming a serious problem in viticulture, in both Europe and around the world [35]. The worldwide economic cost for the replacement of dead grapevines is roughly estimated to be over 1.5 billion dollars per year [35]. GTDs are major diseases affecting both young and old vineyards, reducing their productivity and longevity, thereby causing considerable economic loss to the industry [36].

Wood diseases have been known and well described in literature for tree crops, but their current diffusion and increase in severity worldwide [37, 38] in many different crops may be hypothesized to be somehow linked to climate changes and undoubtedly related to modern practices of the intensive viticulture [39].

GTDs affect the main trunk and cordons of the vine and appear as a complex of several diseases caused by pathogens that were associated to specific related symptoms: Botryosphaeria dieback and Eutypa dieback, caused respectively by *Botryosphaeriaceae* or *Diatrypaceae* fungal pathogens are becoming increasingly important, Phomopsis dieback caused by *Phomopsis viticola*, and other *Phomopsis* species, the black foot disease caused by *Dactylonectria* and *Ilyonectria* species that typically affects the roots and collars of young vines, and the most common and damaging complex of diseases in Europe, the esca disease complex, which involves vascular fungal pathogens, decay agents and their likely interaction with canker pathogens [3, 34]. In this complex frame, a crucial aspect for the development of the GTDs is the interaction between several pathogens, including their factors of pathogenicity and the physiological status of the plant [40].

Until some decades ago, GTDs normally damaged mainly old plants, and the substitution of affected vines with healthy ones was a common and efficient way of restricting the dissemination of the disease [41]. Recently, these diseases have been observed to be in rapid diffusion and to affect even 2- or 3-year-old plants. Nowadays, GTDs appear often in vineyards that are over 7-year-old [42].

Regarding the sustainable control of GTDs, over the years a wide range of research activities has been internationally promoted, producing a deeper understanding of the diseases, their aetiology, biology, epidemiology but still a lot of questions remained open on disease management [43].

This is linked to several critical aspects, beside the still incomplete knowledge of the symptoms expression mechanisms, especially in the most serious esca disease complex and the related grapevine leaf stripe disease (GLDS).

2.1. Complexity of the grapevine trunk diseases

A first critical aspect on the sustainable control of GTDs, is related to the lack of products with low toxicity but good efficacy for the control of these diseases. Sodium arsenite was considered the only chemical agent effective on esca disease complex, acting by its accumulation in treated tissues [44]. Anyway, due to its high toxicity, sodium arsenite is not allowed any more in viticulture [45].

A promising – though low – reduction in disease incidence was obtained recently in field trials with copper formulates that could partially penetrate the wood [46], probably impairing the growth and metabolism of the involved fungal pathogens. This finding opened a new field of research on the application of wood penetrating products, rather than systemic ones, as up to now the only systemic product that gave some consistent and prolonged efficacy in reducing GLSD incidence is fosetyl-aluminium, a fungicide used to control *P. viticola*; its activity appeared linked to its ability to activate defence responses [47].

A second critical aspect is related to the ineffective protection of pruning wounds, a major point of penetration for all wood fungal agents. The commercially available products were effective in vitro [48], but were not able to ensure persistent protection compared with the prolonged susceptibility of pruning wounds [49]. To this aim, the application of BCAs based on species of *Trichoderma* species was studied. Results achieved in several years of experiments did reveal some positive effects on GLSD symptoms as the BCA colonizes the wound, allowing a prolonged interaction with the plant [50].

A third critical aspect, behind the difficulties in the control of these wood diseases, and in particular of GLSD, is that the pathogens are localized in the wood tissue but the causes of the symptoms expression in the leaves are linked to the defence reaction activated in the foliage by the production in the wood of phytotoxins translocated via the vascular tissue, under the influence of others - not well determined - environmental cofactors. On this view, some positive results were obtained with foliar nutrient applications, whose efficacy could be strongly improved by a better distribution and penetration of the product [51].

In this direction, a research group from the University of Poitiers (France) recently investigated two types of compounds with different and complementary modes of action. A first study was

performed using modified existing molecules to obtain products able to move, after a foliar application, into the grapevine trunk where pathogens are localized. A second study associated these mobile substances to molecules able to stimulate plant defences. [52].

2.2. Control of grapevine trunk diseases in both vineyard and nursery

The control of GTDs represents a big challenge for winegrowers, nurserymen, technicians and scientists mainly because of their complexity compared to other grapevine diseases like powdery- and downy-mildew, for instance. The control of GTDs is a clear example of the need for an integrated management approach. In fact, the high infection risks in nurseries and vineyards and the lack of curative treatments have encouraged the idea of a transversal strategy for the GTDs control along the grapevine growing sector [3, 53].

Despite the lack of developed tools or protocols with specific efficacy against GTDs pathogens, the meticulous management of source mother blocks is the first step to prevent infections in grapevine propagating material at the nursery stage. The most effective practices in nursery are the application of good hygienic measures and the prevention of the canes contamination whenever the propagating material is wounded and cut. Recognizing the critical importance of these two factors raises the issue of a possible redesign of the entire nursery production process [54, 55, 56].

Sustainable control tools, already developed for nursery applications, include primarily the use of BCAs, in particular several *Trichoderma* species, which on several studies were found to be efficient against some of the main pathogens related to GTDs [57, 58].

The better known hot water treatment is frequently used for obtaining commercial plants in good sanitary conditions. Such treatment is generally performed at 50°C for 30 min, but it is stressful for the plant [56] and still variable results in terms of efficacy and plant quality were reported [36]. Nevertheless, further experimentation is needed to validate the applicability and efficiency of innovative and low impact control tools such as a range of sanitization products [54] and the use of ozonized water [58].

Among the approaches aimed to reduce the symptoms related to GTDs in vineyard, foliar treatments based on minerals and sea weed extracts [59] or defense inducers such as fosetyl-Al [44] were found to be efficacy on the nutrition and defense-inducing factors associated to GLSD (esca disease complex). However, such applications should be further explored to verify the long-term efficiency on reducing mortality and maintaining productivity in existing GTDs affected vineyards.

Novel potential curative tools are more oriented to interact with the activity of the trunk pathogens colonizing wood and their virulence factors. Recently, formulations based on copper and co-formulants were proposed to be applied directly to the wood in order to simulate the same mechanism that was probably activated by winter spraying of the carcinogen sodium arsenite, no longer applicable [46]. On plants affected by severe symptoms it is also suggested to apply measures of trunk surgery, consisting on the trunk renewal, re-grafting and "curettage", which led to significant removal of the affected wood. It is an invasive technique but frequently useful for prolonging the productivity of the vineyard [60, 61].

A preventative approach in vineyard is also highly recommended. First of all, removing and burning branches, dead/dying vines, pruning residues, are essential practices to limit the spread of inoculum. It is also important to limit and protect pruning wounds and to remove the dead part of the trunk or arms or if necessary, to replace completely the symptomatic plant [62]. Moreover, disease management practices based on soil improvement and plant-based resistance to infection, should be investigated in order to maintain the vine in its best condition ready to react to infection and symptom development [63].

However, the pruning wound protection (mainly applying formulations based on strains of *Trichoderma* spp.) against infection, actually appears the more promising preventive measure to control the GTDs diffusion that is available in the market [29, 57]. Further trials are being performed with wound protectants based on physical barriers, which may represent innovative tools for the future [64].

The integrated control strategy against GTDs should consider all the factors potentially responsible for dysfunctions of the water and sap transport, as well as the plant resilience, including pruning, training, nutrition, protection and soil management. For instance, the two first methods may not only create infection niches for pathogens, but also perturb the water flow and balance in the vine [65]. Consequently, water flow deficiency has a primary, but not exclusive, effect on the pathogen virulence factors (i.e. phytotoxins), leading also to a reduction in photosynthesis activity [40] and in most acute conditions, to the vine decline, loss of production and vine death.

2.3. Innovative approaches for grapevine trunk diseases monitoring

The perspectives for efficient GTD control could take advantage of both the GTD knowledge reservoir and the ongoing new insights. A holistic view of the problem could be the key to define winning strategies for the control of GTDs, whose model could also be applied for the control

of other complex plant diseases [53]. In this context, the development of new tools to detect and monitor constantly and efficiently the vines health status is a real challenge, especially in vineyard heavily affected by GTDs.

Among GTDs, one of the most widely spread in European vineyards is GLDS [66], whose symptoms include typical foliar interveinal necrosis, giving the leaves a typical tiger-stripe appearance that may not appear in every growing season. This discontinuity of leaf symptoms on each single vine makes it almost impossible to determine the true incidence of esca in a vineyard at any given time, as many infected vines may not show symptoms every year [67]. Many authors clearly demonstrated a drastic alteration of photosynthetic functions as well as a stimulation of defense responses in affected grapevines several days before the appearance of the first visible symptoms on leaves [68, 69].

Di Gennaro *et al.* (2016) proposed an innovative approach for GLSD monitoring, based on the correlation between Normalized Difference Vegetation Index (NDVI) images acquired by an UAV (Unmanned Aerial Vehicle) platform, and symptomatic plant monitored by ground-based observation. The innovative methodology was aimed primarily to conduct a quantitative and qualitative analysis of the symptom spread, and then to realize a predictive model of the esca symptoms onset. The study has produced valuable results that indicate new possibilities for the use of remote sensing technologies for precision viticulture. UAV features, such as low cost, capability of timely provision of high resolution images, provide an approach that can be easily applied for investigation and disease control strategy planning in vineyards. These methods are likely to be useful, both for research and for practical applications in viticulture.

Early detection could also support the planning and testing of economically rewarding field treatments, or the application of specific control methods on the single vines before they show the symptoms. The applications of such a predictive tool could open new perspectives in the knowledge and control of this important grapevine disease, within the increasing development of the precision agriculture approaches in viticulture [70].

3. Aim of the thesis

The present research arises from the perspectives opened by previous applications of inorganic crystals based on a specific phase of the biomimetic calcium phosphate, the hydroxyapatite (HA), which has been extensively studied and applied in many fields [71, 72, 73].

Based on the successful HA applications performed in the biomedical field, such innovative smart material can potentially act as a delivery system of active substances, including metal ions, such as copper(II), and organic compounds. One of the main investigation was performed in medicine, to replace chemotherapy in the treatment of a bone cancer, thanks to the functionalization of HA with an anticancer molecule [74, 75], while a second effective application was achieved through the development of a toothpaste based on the same material, functionalised with zinc ions, in order to replace fluorine, SLS, titanium dioxide and parabens [76].

In this framework, the research is aimed to assess, through a multidisciplinary approach, the development and application of HA to enhance the biological activity of active substances for the control of fungal diseases in *Vitis vinifera* L.

This aim responds to crucial goals: (i) the reduction of the amount of the chemical fungicides usually employed on the grapevine protection, (ii) the optimization of the active substance distribution, penetration and persistence within the plant tissues, (iii) the potential application in pathogen control even in vascular and complex diseases.

The experimental activities and the related results are presented and discussed according to the following actions: (i) characterization and stability of hydroxyapatite functionalized with copper(II) compounds, applied to leaf tissues of *Vitis vinifera* L. for plant protection purposes; (ii) *in vitro* and *in vivo* evaluation of the biological activity of hydroxyapatite functionalized with copper(II) compounds on the grapevine fungal pathogens control; (iii) evaluation of the distribution and efficiency of hydroxyapatite functionalized with two copper(II) compounds in grapevine woody tissues colonized by a vascular fungal pathogen and characterization of the defense responses activated by the plant.

Chapter 1

Characterization and stability of hydroxyapatite functionalized with copper(II) compounds, applied to leaf tissues of *Vitis vinifera* L. for plant protection purposes

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1. Abstract

Over the years, the research in nanotechnology has led to the development of revolutionary smart materials in many fields. In agriculture, the nanotechnological approach is often applied to improve plant productivity, crop quality and disease management, through nano-sized materials. In particular, the development of slow-release systems for pesticides open new perspectives to reduce the amount of active substance applied leading to efficient plant protection and disease control.

Taking into consideration the possible phytotoxicity of such innovative materials, which still remains unclearly in literature, the present study considered a biocompatible smart material for plant protection. In this case we used a biomimetic synthetic hydroxyapatite (HA) that has been studied extensively due its unique bioactivity and biocompatibility. Aggregation between the nano-particles of a particular HA and four copper(II) compounds, applied in *Vitis vinifera* L. leaves as pesticide was studied. Several formulations were prepared and characterized to determine shapes and dimensions of the aggregates by XRD, DLS and electron microscopy. The same formulations were applied *in planta* in to verify particle aggregation and efficiency in protecting the plant against the fungal pathogen *Plasmopara viticola*.

X-Ray analysis showed a different interaction between HA and the copper(II) compound, possibly based on the solubility and pH of the different formulations. The DLS analysis showed a granular distribution ranging globally out of the nanometer range. Further observations by TEM and ESEM microscopy showed, in all formulations, large aggregates partially nanostructured, which were recognized as aggregates and not clusters, and thus stable in their micrometric dimension. The detected particles, based on calcium, phosphorous and copper, did not show any phytotoxic effect after their application *in planta*. A formulation based on HA and the soluble copper(II) compound showed promising results in the control of the fungal pathogen, confirming the potential role of HA an as innovative delivery system of Cu(II) ions. The present work indicates the possibility to improve the biological activity of a bioactive substance by modifying its structure through a specific and achievable formulation with a biocompatible material.

2. Introduction

Nanotechnological research has led to innovative solutions in many fields, including electronics (nano-sensors), medicine (drug release) and in agriculture (nano-silver pesticides). Through the development of revolutionary smart materials, nanotechnology has provided solutions in the fields of agriculture and food sciences to improve plant productivity, crop quality, food with nano-sized nutrients and new tools for molecular and cellular biology in this field.

In agriculture, the nanotechnological approach has significant potential in the development of slow release systems for pesticides [77, 78]. This opportunity arises from several critical aspects, mainly linked to the very low concentration of the applied pesticide that generally reaches the plant target site and the consequent eco-toxicological impact of the remaining pesticide. In this context, several studies have been done ranging from the nano-encapsulation of agrochemicals [79] to the synthesis of nanoparticles in biological systems [80, 81]. The most promising results were achieved by the engineered nanoparticles (NPs) based on carbon, metal and metal-oxide nanomaterials, among which are nano-TiO_2, nano-Al, nano-ZnO, Cu NPs and Ag NPs [82–86]. Ghormade *et al,* confirmed that nanoparticle technology can provide innovative solutions for plant nutrition or protection by improving the distribution and transportation of bioactive molecules, such as fertilisers, fungicides, insecticides, plant hormones, elicitors and nucleic acids through the plant vascular system to the targeted sites [87]. Nonetheless, numerous authors have reported the need for further evaluations on the cytotoxicity and genotoxicity within the plant tissues. Several electron microscopy studies confirmed the damage caused by metal-based nanoparticles (such as ZnO) to cells of plant meristems, while the same approach showed the uncontrolled uptake of Cu NPs across the cell membrane in mung bean [88, 89]. As underlined by Nel *et al,* the higher the surface area of nanomaterials combined with a bioaccumulation of their residues in crops and in the environment, may cause toxic effects to the agricultural ecosystem [90]. Furthermore, international organizations for organic agriculture, such as Naturland and the International Federation of Organic Agriculture Movements (IFOAM), forbade the organic certification for food products grown with artificial nanomaterials, proposing at the same time the definition of the nanoscale range (approximately 1–300 nm) and the properties or compositions (e.g. shape, surface properties and molecular composition) of manufactured nanomaterials [91, 92]. These aspects suggest an accurate evaluation and promotion of materials that are non-toxic, biocompatible and biodegradable. To this purpose, the biomedical field may represent a reliable source of safe materials.

In the last twenty years, nanotechnology has been successfully applied in medicine, improving diagnostic methods and the efficiency of treatments against human diseases including several forms of cancer. Those intentions focused the research and development of organic and inorganic biocompatible nano-carriers for drugs [93].

Biogenic calcium phosphates from teeth and bone have been studied to design new models of inorganic synthesis. Biomimetic synthetic hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, has been studied extensively due its unique bioactivity and biocompatibility properties [94]. Research in biomaterials was mainly focused on the carbonated hydroxyapatite (CHA), $Ca_{10}(PO_4)_{6-x}(CO3)_x(OH)_2$ where x represents the carbonate content of approximately 4–8% wt. in human bone mineral [71]. HA is based on a structure of tetrahedral PO_4 groups and the carbonate group can partially substitute both hydroxyl and phosphate ions. Based on this mechanism, the inorganic synthesis of CHA has been intensively investigated for applications as a bone substitute [72].

For both HA and CHA, several mechanisms of incorporation and release have been described including metal-based molecules and organic drugs. The exchange rate between HA and a wide variety of ions such as B^{3+}, Zn^{2+}, Pb^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Ag^{+} and Co^{2+} has been studied extensively [73, 95–97]. Ceramics based on Ag^{+}, Cu^{2+} and Zn^{2+} substituted HA were applied as antimicrobials which revealed the bactericidal effect of Ag^{+}HA on *Escherichia coli* [98]. In a recent study, HA was synthesized in presence of Zn^{2+} and Cu^{2+} by the neutralisation method, allowing the incorporation of the metal ions in the HA structure [99]. *In vitro* tests in both solid and liquid media, confirmed the antibacterial activity of HA doped with both metal ions against pathogenic bacteria, *E. coli*, *Staphylococcus aureus* and the pathogenic yeast *Candida albicans* [100].

In the last decade, through a nanotechnological approach, it has been possible to enhance the physical and chemical characteristics of HA and CHA, revealing innovative biomedical applications, especially for drug delivery purposes [101, 102]. In fact, calcium phosphate nanoparticles have shown advantageous properties as drug carriers, which are strongly influenced by the chemical characteristics of the drug molecule and by the chemical and structural properties of the nanoparticles. Roveri *et al*, reported the dissolution mechanism of these particles at low pH (around 4.00), thereby releasing incorporated drugs or biomolecules [94]. Specific biomedical studies concerned the functionalisation of HA with organic antitumoral molecules (cisplatin and alendronate), demonstrating how the adsorption and release kinetics of these molecules are influenced by the HA surface area and surface charge (Ca/P ratio), as well as the charge on the adsorbed molecules [103, 104].

The aim of the present research was to investigate a biocompatible drug delivery system applicable in plant protection to enhance the distribution and potentially the antimicrobial activity of common bioactive substances. For this purpose, HA previously developed for applications in agriculture and copper(II), widely applied to protect grapevine (*Vitis vinifera* L.) against the fungal pathogen *Plasmopara viticola*, were chosen as a case study.

P. viticola is certainly responsible of one of the most harmful disease, causing the largest number of treatments in vineyards [3]. Since the discovery of the copper antifungal activity, cupric fungicides were widely applied to control this pathogen and still today copper is the most efficient fungicide available in organic viticulture, being a non-synthetic compound with a wide activity spectrum [24]. However, among the side effects related to the copper use, the extensive accumulation in soils of this metal is responsible of serious environmental problems,[34] resulting in enacting laws which limit its use. For this reason, the efficacy of reduced copper doses, achievable by optimizing its biological activity and persistence *in planta*, is looked with particular interest, in order to adapt its use to legislative restrictions [24, 27, 28].

The HA used was synthesized by a specific method that allows the aggregation of the nanostructured particles in micrometric clusters, thus reducing the presence of single nanoparticles [105]. According to the previous applications concerning the HA functionalisation with metal ions, four different compounds based on copper(II) which are commonly used for disease management in organic agriculture, were chosen to dope the micro structured HA with copper(II) ions [106]. This study was focused on investigating the aggregation of the HA particles *in planta*, in terms of shape and dimension, in order to verify the micrometric aggregation of the HA. Through electron microscopy, the same aspect was verified on the formulations obtained by functionalising HA with copper(II) ions and on leaves of *Vitis vinifera* which were infected by the fungal pathogen, *P. viticola,* and treated by spraying the same formulations on the abaxial and the adaxial surfaces.

3. Experimental methods

3.1. Functionalisation of hydroxyapatite with copper(II)

An aqueous suspension containing 30% (w w^{-1}) of nanostructured hydroxyapatite (NDG Natural Development Group Srl, Italy), was obtained according to a patented process of synthesis [105]. Four copper(II) compounds (CNR-IBIMET, Bologna, Italy) were prepared to functionalize HA: copper sulfate pentahydrate (CuSPHy), tribasic copper sulfate (CuTBS), copper oxychloride (CuOxCl) and copper hydroxide (CuHyOx). Functionalization of the products was carried out by maintaining the HA slurry with the respective copper(II) compound and water complex. The various copper(II) compounds were pre-solubilized or dispersed in water and then added to the HA slurry, keeping in slow agitation for 4 h. The amount of each copper compound used was calculated so that the final formulation contained 5 ± 0.1% (w w^{-1}) of copper(II). The obtained formulations (Table 1) were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (Arcos-Spectro, AMETEK, Kleve, Germany) to verify the copper concentration.

Table 1. Samples based on the pure compounds or on the formulations, prepared and characterised.

Formulation	HA % w w^{-1}	Cu % w w^{-1}
HA	30	0
CuSPHy	0	5
CuTBS	0	5
CuOxCl	0	5
CuHyOx	0	5
HA-CuSPHy	3	5
HA-CuTBS	3	5
HA-CuOxCl	3	5
HA-CuHyOx	3	5

During the synthesis, the pH was measured at 20°C on the samples based on the pure copper(II) compounds and the ones formulated with HA to evaluate the stability of HA with a specific copper(II) compound (Table 2).

The samples listed in Table 1 were prepared for the characterization analyses and the application *in planta*. Before application to foliar tissues, the formulations needed to be diluted. In that respect stability of the formulations suspended in distilled water was previously observed at five concentrations (0.25, 0.5, 1, 2 and 4 w v^{-1} %). At concentrations greater than 1% (w v^{-1}) all formulations tended to sediment after being shaken, so a concentration of 0.5% (w v^{-1}) was arbitrarily chosen for the foliar applications.

Table 2. pH values measured at 20°C on the samples based on the pure compounds or on the formulations.

Formulation	pH
HA	12.17
CuSPHy	2.47
CuTBS	6.99
CuOxCl	6.98
CuHyOx	6.85
HA-CuSPHy	2.24
HA-CuTBS	10.19
HA-CuOxCl	11.58
HA-CuHyOx	10.58

3.2. Structural characterisation of the HA-copper suspensions

3.2.1. X-Ray Diffraction (XRD). The formulations were characterised by XRD to evaluate the crystalline phases and the species obtained following the functionalization process. Each copper(II) compound and all the formulations obtained by functionalizing the HA were first examined (Table 1). The samples based on HA and copper(II) compounds were centrifuged and the solid phase was allowed to dry at 30°C for 24 hours. The analyses were carried out with an analytical X'Pert³ Powder Diffractometer (Cu Kα) at 40Kv and 40mA (PANalytical, The Netherlands) with divergent slides of 1° and 0.1 mm slits. The measurements were carried out at an angular range of 2θ between 5° and 65°, a step size of 0.03° with 20 s of exposure per step.

3.2.2. Dynamic Light Scattering (DLS). The granulometric analysis was performed to estimate the size of particles and aggregates formed in the studied samples (Table 1). Granulometric distribution was investigated using a Mastersizer 3000 and a Dispersion Unit Hydro MV (Malvern Instruments, UK). The analyses were performed using a wet dispersion method using the Hydro EV dispersion unit. Samples based on HA and copper(II) compounds were added to demineralised water directly in the sampler until it reached a dimming of between 6 and 10%. Five measurements were performed for each sample both before and after 100% ultrasonic bath treatment for a duration of 5 minutes. During the measurements, a circulation speed of 2000 rpm was set. The granulometric distribution of the particles was obtained from particle scattering data using Mie theory, setting as the refractive index of the sample 1.52 and the 0.1 absorbance index. The granulometric distributions and the dimensional statistical parameters provided by the software are defined in terms of the volume equivalent ballast diameter.

3.2.3. Transmission Electron Microscopy (TEM). The morphology of the HA particles and their surface structure contained in (Table 1) the aqueous suspensions functionalised with copper(II) ions were studied with a Transmission Electron Microscope (CM 12 TEM, PHILIPS, The Netherlands), equipped with an OLYMPUS Megaview G2 camera. Drops of each suspension (prepared as described above) were placed on carbon coated 200 mesh gold grids and dried at room temperature. An X-ray microprobe in the TEM chamber of an Energy Dispersion System (EDS by EDAX, software EDAX Genesis, AMETEK, Mahwah, NJ, USA), verified the elemental composition. The EDS spectra showed the presence of elements like Ca, P, Cu, S and Cl. Values of the accelerating voltage 100 KeV (for TEM) related to each element are shown in the Table 3.

Table 3. Accelerating voltage values (25KeV in ESEM and 100Kev in TEM) of the elements traced on the X-ray spectra.

Element	Kα	Lα
Ca	3.690	--
P	2.013	--
Cu	8.040	0.930
S	2.307	--
Cl	2.621	--

3.3. Application and characterisation *in planta*

3.3.1. Treatments. 1-year-old grapevine plants (*Vitis vinifera*, cv. Chardonnay grafted on Kober 5BB rootstock) were selected in individual 2.5-liter pots of peat rich, pre-fertilized (NPK: 14:16:18) soil (Topfsubstrat D450; Stender, Schermbeck, Germany) and grown under natural light at temperatures ranging from 18–28°C. Six potted vines with at least 7 developed leaves were selected as replicates for each treatment.

Distilled water was used as the control treatment to be compared with the suspensions of each formulation based on HA doped with copper(II) and a non-functionalised HA (Table 1). In laboratory conditions (temperature 20 ± 1°C, relative humidity 65 ± 5%, artificial light), 1.25 g of each formulation were suspended in 250 mL of distilled water (5 mg mL^{-1}). The pH of the suspensions and the distilled water (Table 4) was measured with a pH meter (pH 538; WTW GmbH, Weilheim, Germany) and compared with the pH of the pure formulation.

About 30 mL of each suspension were applied to the plants with a gardening spray bottle (Sprayer 0.6 L, Leroy Merlin, Italy) taking care to cover homogenously both the adaxial and abaxial leaf surface. After the treatment, leaves were left to dry for one hour before the microscopic and ultramicroscopic observations.

Table 4. pH values measured at 20°C on samples after dilution with distilled water (pH 7.50).

Formulation	pH
HA	7.88
CuSPHy	5.57
CuTBS	7.52
CuOxCl	7.61
CuHyOx	7.65
HA-CuSPHy	5.70
HA-CuTBS	7.79
HA-CuOxCl	7.80
HA-CuHyOx	7.80

3.3.2. Environmental Scanning Electron Microscopy (ESEM). The distribution of the treatments on the plant foliage and the structural features of the particles suspended in the applied formulations, were assessed by direct analyses *in planta* with an optical microscope (Stereo Microscope, Nikon, Japan) and an Environmental Scanning Electron Microscope (Fei Quanta 200 ESEM, FEI Corporation, Eindhoven, The Netherlands), operating in low-vacuum mode, at 25 kV, without pre-treatment of the samples. X-ray (EDS) microanalysis by an Energy-Dispersive System, coupled to the ESEM (EDAX, software EDAX Genesis, AMETEK, Mahwah, NJ, USA) was used to investigate the composition of the particles and the distribution of the related elements on the leaf surface, in this case, Ca, P, Cu, S, Cl. Table 3 shows the accelerating voltage values 25 KV for the traced elements. Observations were performed on the leaves of treated potted vines 24 hours post treatment. Foliar samples were collected in each replicate plant of all the conditions by counting from the basal part of the primary shoot and sampling the fourth leaf. Leaves were cut into small portions (10 mm x 10 mm) and transferred to a conductive adhesive carbon mounted on aluminium stubs.

3.3.3. Disease severity on the treated leaves. To evaluate the *in-planta* efficiency of the formulations against a fungal pathogen, a suspension of *P. viticola* sporangia was applied to the leaves 6 hours after the foliar treatments. Sporangia were washed from freshly sporulating lesions (infected leaves provided by Fondazione Edmund Mach, San Michele A.A., Italy) with cold (4°C) distilled water. An aqueous suspension of sporangia was diluted to a concentration of 10^5 sporangia mL^{-1}. Plants were inoculated by spraying the abaxial surface of each leaf with the sporangial suspension (approximately 40 mL per plant), using the air compressor system working at 200 kPa at the nozzle. Inoculated plants were kept in the dark in a growth chamber for 12 hours at 20 ± 1°C and relative humidity (RH) of 80–99%. Plants were then kept for 10 days in the greenhouse (temperature of 18–25°C, RH of 60–80%, and natural light regime). After this time, the plants were placed overnight (12 h) in a growth chamber at 20 ± 0.5°C and

99% RH to promote *P. viticola* sporulation. The sporulation area covering the leaf surface was estimated by an optical microscope and reported as a percentage of leaf surface area. Disease severity was evaluated for each treatment as the mean visual percentage of total leaf area covered by sporulating lesions per plant. The experiment was a randomized complete block designs. Data were normalized and subjected to analysis of variance and Tukey's test ($\alpha = 0.05$). Statistical analysis was performed using IBM SPSS Statistics 24.0 (IBM Corp, Armonk, NY, USA).

4. Results

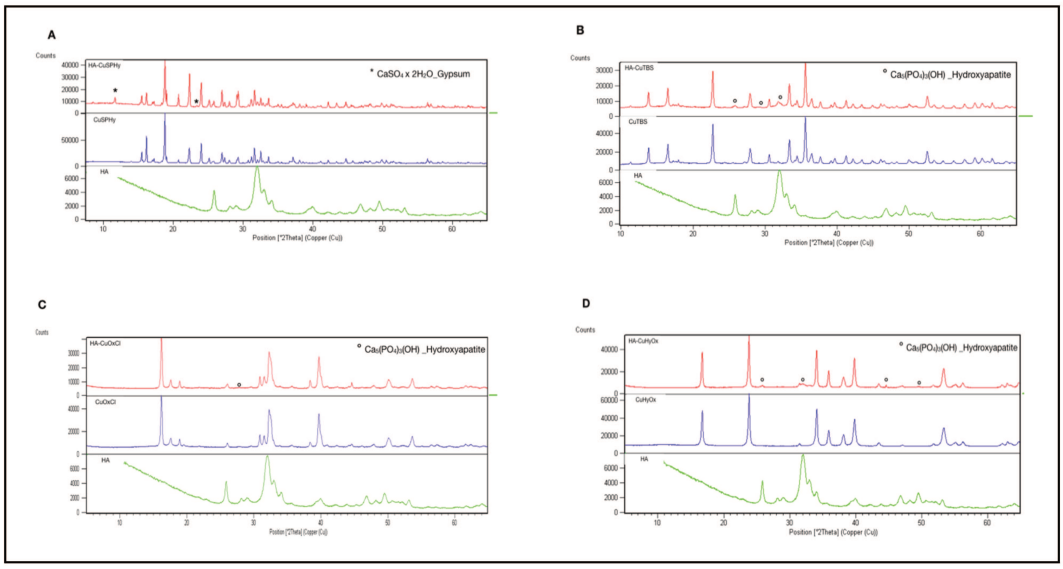
4.1. Functionalisation of hydroxyapatite with copper(II)

During the synthesis, in order to determine HA stability, pH of the samples was measured at 20°C (Table 1). The pH measures of the CuSPHy aqueous solution revealed an acid pH, while the suspensions of the other copper(II) compounds revealed a basic pH. The formulations based on HA-CuTBS, HA-CuOxCl, and HA-CuHyOx showed pH values similar to that of pure HA (12.47); while the formulation based on HA-SPHy showed the same pH as the pure copper(II) compound.

4.2. Characterisation

4.2.1. X-Ray Diffraction. XRD analysis (Fig. 1) shows that the formulation HA-CuSPHy (1A) mainly consists of two phases. One is a CuSPHy crystalline phase and the other a calcium phosphate phase. In addition, a small and insignificant amount of a copper phosphate phase was detected (liberthenite, whose signals were obscured by the prevalent presence of CuSPHy signal). However, there was no typical signal of the HA phase, which implies that this phase was completely dissolved in the acidic solution of the product and the calcium and phosphate ions had been rearranged with copper and sulphate ions of the CuSPHy.

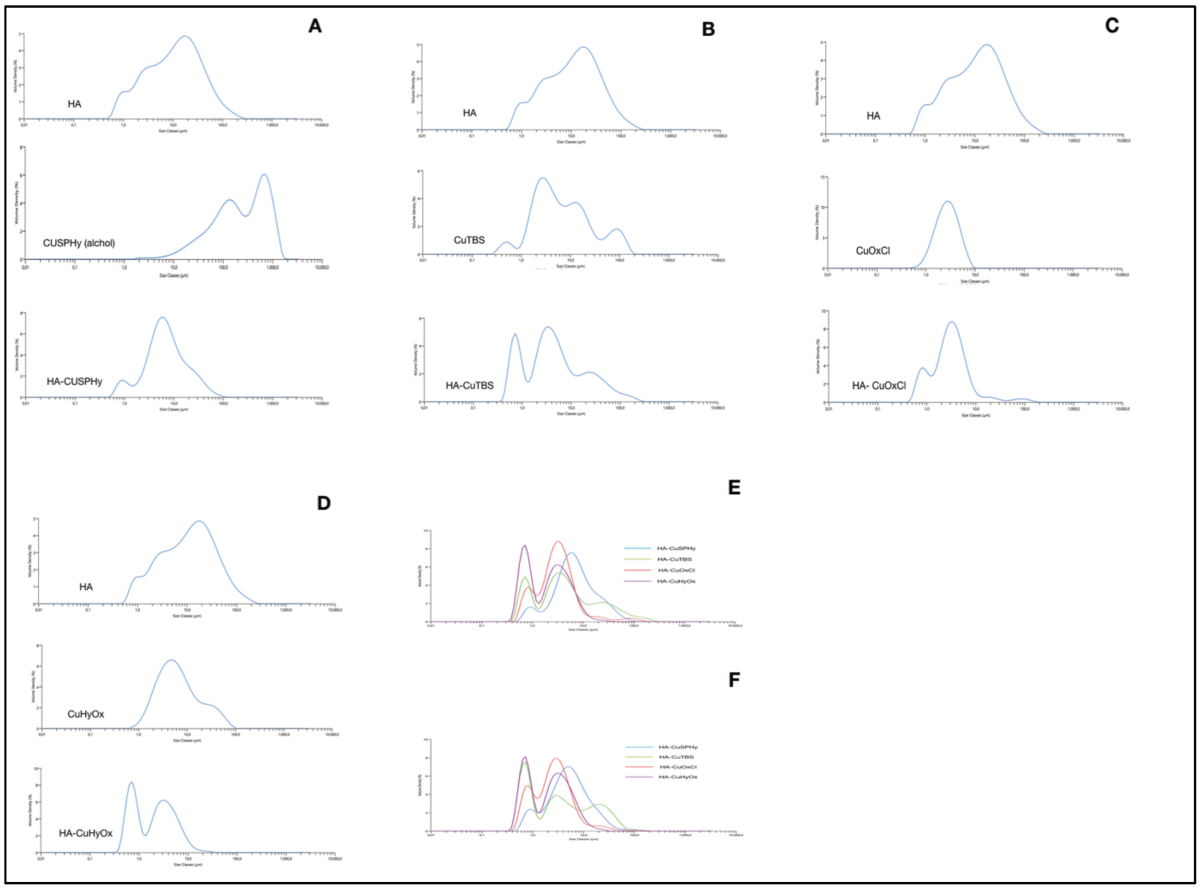
Figure 1. The XRD pattern related to HA-CuSPHy (A) mainly consists on a CuSPHy crystalline phase and a calcium phosphate phase, revealing also the presence of calcium sulphate (gypsum). In the XRD patterns of HA-CuTBS (B), HA-CuOxCl (C) and HA-CuHyOx (D), the signal of the original Cu(II) compound phase and those of the HA phase were visible with a different crystallinity degree.



The XRD of the water insoluble copper(II) compounds with basic pH show how these compounds, given their pH, do not dissolve HA when it is brought into association with them. HA phase is found in each of the three compounds, HA-CuTBS (1B), HA-CuOxCl (1C) and HA-CuHyOx (1D) and for each one, the signal of the original copper(II) compound phase and those of the HA phase used are clearly visible. On the other hand, the presence of other crystalline phases is not found in any of them. Based on these observations, it can be assumed that given the basic pH of these compounds, HA remains stable and does not dissolve.

4.2.2. Dynamic Light Scattering. The graph of the granulometric distribution (Fig. 2) shows a regular trend of HA-CuTBS (2B), HA-CuOxCl (2C) and HA-CuHyOx (2D) formulations.

Figure 2. Size classes (µm) of the detected particles of: formulation HA-CuSPHy (A), formulation HA-CuTBS (B), formulation HA-CuOxCl (C), formulation HA-CuHyOx (D), non-ultrasonicated samples (E), ultrasonicated samples (F). The formulation HA-CuSPHy (A) shows a singular trend of the granulometric distribution in contrast to a more regular trend shown by HA-CuTBS (B), HA-CuOxCl (C) and HA-CuHyOx (D) formulations, which expose two peaks: one of which was around 0.6 µm and one around 3 µm. The ultrasonication of the samples (F) did not reveal the presence of smaller particles than the ones spotted by non ultrasonicated samples (E).



All have the same pattern of 2 peaks; one of which is around 0.6 µm and one around 3 µm, in addition to a small peak after 30 µm. The trend of HA-CuSPHy formulation (2A), is different. The HA-CuTBS formulation shows an increase population percentage in both 0.6 µm area and around 8 µm area, with a decrease of the maximum around 100 µm. That is in line with the amount of added HA in the functionalization process. In the HA-CuOxCl formulation, compared to the original compound CuOxCl, there is an increase in population percentage at values greater than 10 µm, and according to the addition of HA, it also increases to a maximum value of around 0.7 µm, which is not reported in CuOxCl but reported in HA. The same trend is shown for the HA-CuHyOx compounds.

4.2.3. Transmission Electron Microscopy. The morphology of the particles is shown in Fig. 3, according to the pure HA (3A) and to the singular copper(II) compound functionalising HA: CuSPHy (3B), CuTBS (3C), CuOxCl (3D) and CuHyOx (3E). In the suspension of pure HA (3A) the characteristic needle morphology of the HA particles can be observed, but they are linked together forming aggregates and were never seen as single particles. From the elemental analysis, the calcium and phosphorus signals are denoted in the typical Ca/P ratio for this type of material, which is about 1.67.

The HA-CuSPHy formulation, shows uniform particles with needle morphology but with a different size from those of the HA, while they are more similar to the size of the CuSPHy particles, which also exhibit a needle morphology (3B). The figure shows that there are single and no aggregated particles as in the case of HA particles. From microanalysis, little visible calcium is present and there is no longer the typical calcium phosphate ratio of HA confirming that in this case, the original HA structure has disappeared during the association with CuSPHy, which is a consequence of its acid pH. This was further detailed and confirmed in Fig. 3A.

In the case of HA functionalized with CuTBS, two different morphologies of particles were observed, namely agglomerated needle particles attributable to HA and larger particles attributable to CuTBS (3C). The microanalysis confirms that in this case both HA and CuTBS elements are well distinguishable and show that the Ca/P ratio remains similar to that of HA. It also reveals that HA remains stable and, in this functionalization, it does not dissolve, since the CuTBS has a basic pH. This is confirmed in Fig. 4 by a further comparison of HA-CuSPHy (4A) and HA-CuTBS (4B). A uniform morphology distribution was observed in the formulation based on HA-CuSPHy (Fig. 5A) in which particles with the same morphology and similar size to those of HA particles were never found confirming that CuSPHy, with an acid pH, solubilizes the HA

that loses its structure. Comparing the HA-CuTBS formulation (Fig. 5B), both the morphology of the CuTBS particles and that of HA particles are recognized, confirming that in the basic pH of CuTBS, HA stays stable and retains its structure.

Figure 3. Transmission electron microscope images of the detected particles in: a suspension of pure HA (A), the formulation HA-CuSPHy (B), the formulation HA-CuTBS (C), the formulation HA-CuOxCl (D), and the formulation HA-CuHyOx (E), with X-ra Q37 y spectra of elements. The scale bar corresponds to 200 nm (A, C, D), 1000 nm (B) and 500 nm (E).The characteristic needle morphology of the HA particles is spotted in the suspension of pure HA (A), reporting the calcium and phosphorus signals in the typical Ca:P ratio for HA. The HA-CuSPHy formulation (B) shows uniform particles with needle morphology and different size from those of HA. Two different morphologies of particles were observed in the HA-CuTBS formulation (C): agglomerated needle particles and larger particles. The HA-CuOxCl formulation (D) exhibits elongated and planar particles. The HA-CuHyOx formulation (E) reports particle morphologies similar to the HA-CuTBS formulation: particles with a needle shape (attributable to HA) and particles with a rounded form attributable to Cu(II) compound.

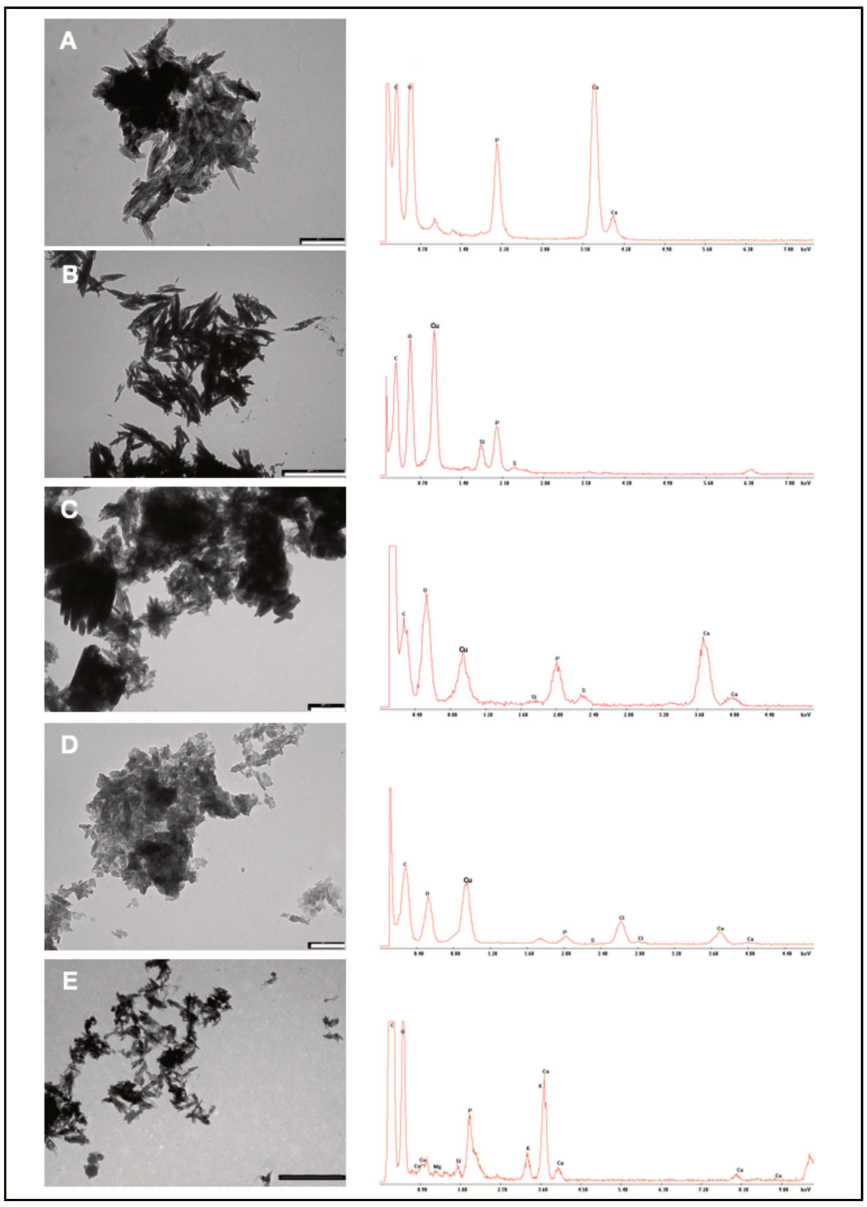


Figure 4. Transmission Electron Microscope images of the detected particles in: formulation HA-CuSPHy (A), formulation HA-CuTBS (B), with X-Ray spectra of elements. Showing the different shape and composition of the particles. The metering bar corresponds to 1000 nm (A) and 200 nm (B). The HA-CuSPHy formulation (A) shows single and non aggregated particles (as in the case of HA particles): from microanalysis, the typical calcium phosphate ratio of HA is not longer visible. In the HA-CuTBS formulation (B) particles with two different morphologies are spotted: both HA and CuTBS are readily distinguishable by the microanalysis spectra, revealing a Ca:P ratio similar to that of HA.

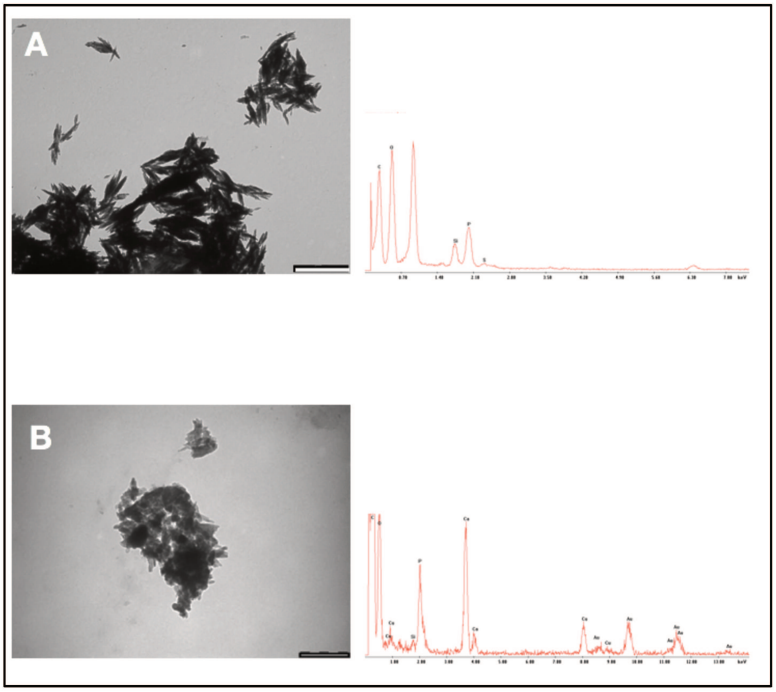
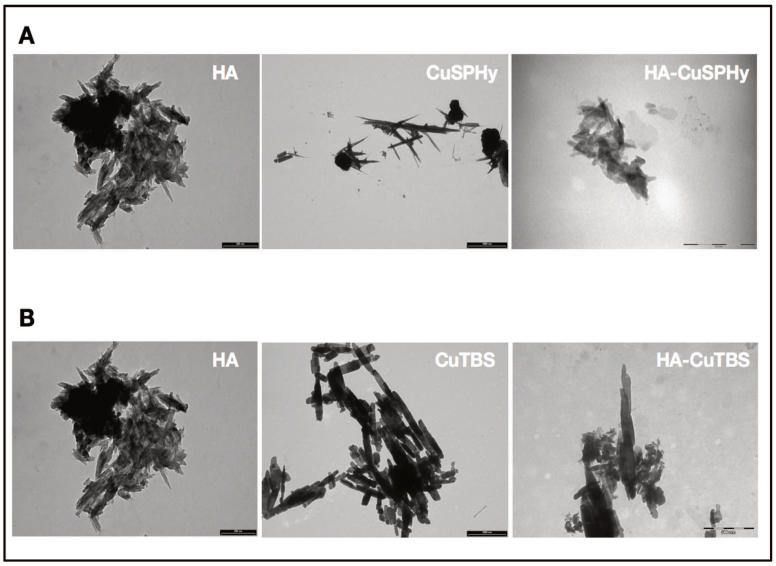


Figure 5. Transmission Electron Microscope images of the detected particles in: formulation HA-CuSPHy (A), formulation HA-CuTBS (B). Showing specific morphological differences. A uniform morphology distribution was observed in the formulation based on HA-CuSPHy (A), while in the HA-CuTBS formulation (B), both the morphology of CuTBS and of HA particles are recognized.

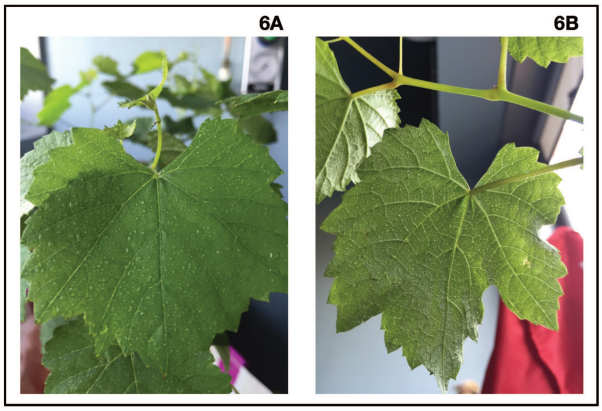


In the functionalization of HA with CuOxCl, the particles exhibit a morphology slightly different from that of the A-needle, but always elongated and planar, similar to that of the CuOxCl particles forming aggregates and, in these images, cover the HA particles separately (3D).
Even in this case, microanalysis shows how calcium and phosphorus can be detected and their ratio maintained despite the signals being crushed by the presence of the chlorine and copper signal.
The TEM observation of the HA-CuHyOx formulation (2E), also shows what is already reported in Fig. 2C: two different morphologies of particles of which one have a needle shape due to HA and one have a rounded form due to CuHyOx particles. The microanalysis carried out on this sample shows how calcium and phosphorus are present and how they are present in the characteristic ratio of HA confirming that the structure and composition of HA remain unaltered since CuHyOx also has a basic pH.

4.3. Application and characterisation *in planta*

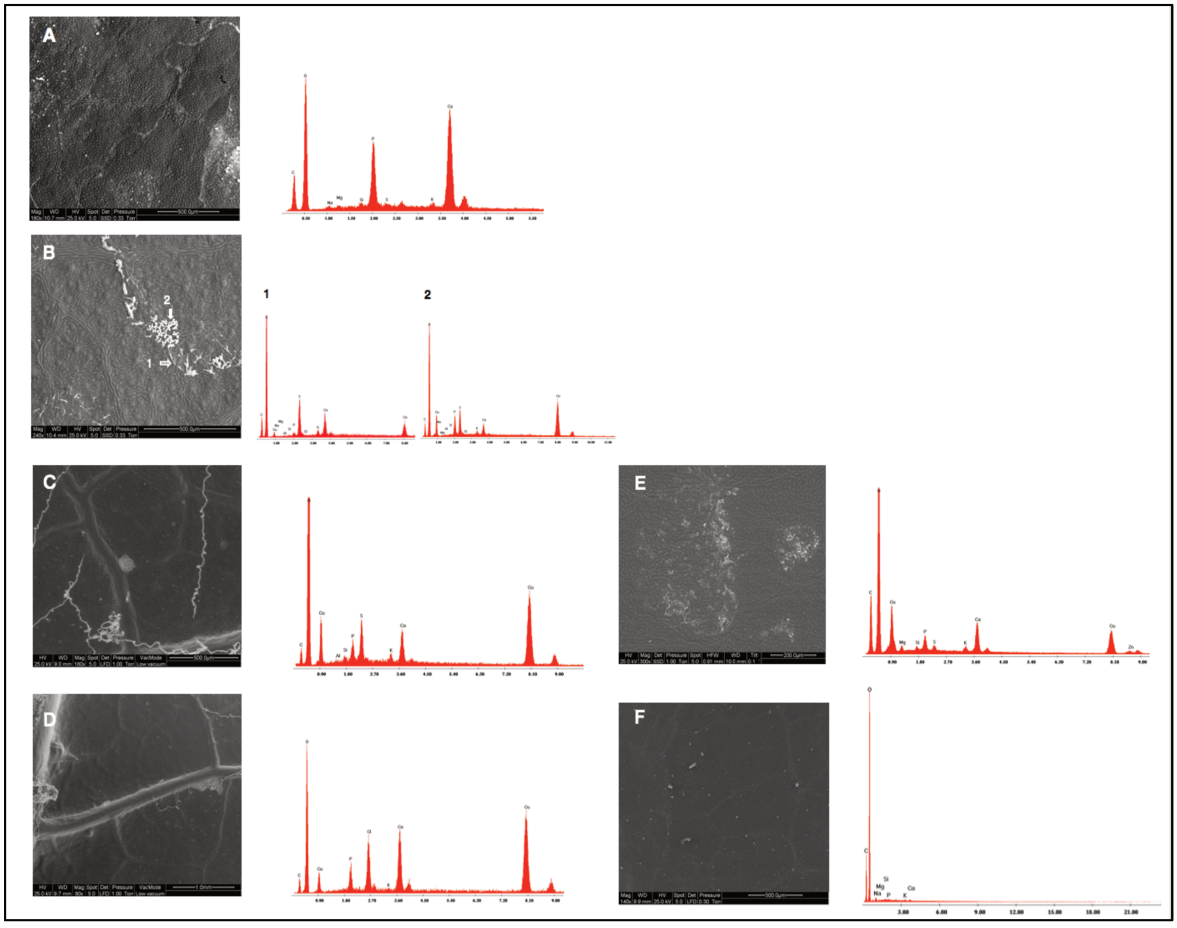
4.3.1. Treatments. Fig. 6 shows the adaxial (6A) and the abaxial (6B) surface of leaves treated with formulations based on HA and copper (II) compounds. In the present study, a solid phase, represented by HA, was suspended in certain formulations. Nevertheless, this did not affect the sample nebulisation on the leaves. A consistent phytotoxic effect was seen on leaves treated with CuSPHy, while the formulation based on HA-CuSPHy did not produce any phytotoxic effect.

Figure 6. Adaxial (A) and abaxial (B) leaf surface of potted plants (*Vitis vinifera*) after the treatments.



4.3.2. Environmental Scanning Electron Microscopy. The *in-planta* studies showed a relevant particle aggregation. Distribution of the various formulations on the leaf surfaces of treated potted vines were studied 24 hours post treatment.

Figure 7. Environmental Scanning Electron Microscope images paired to the related X-ray spectra of elements and related to the formulations applied on the leaf surface of *V. vinifera*: pure suspension of HA (7A), formulation HA-CuSPHy (7B), formulation HA-CuTBS (7C), formulation HA-CuOxCl (7D) and formulation HA-CuHyOx (7E). The leaf sample treated with distilled water as control, is shown in 7F. A uniform morphology distribution was observed in the formulation based on HA-CuSPHy (A), while in the HA-CuTBS formulation (B), both the morphology of CuTBS and of HA particles are recognized.

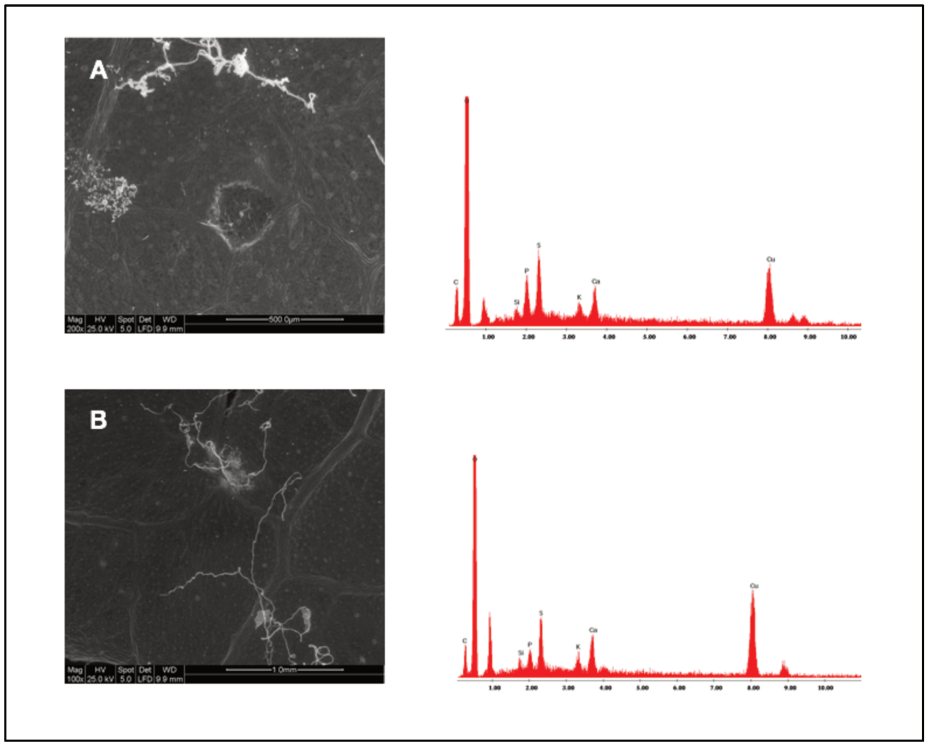


Particles based on pure HA were easily recognized by the EDS analysis, which revealed the respective Ca/P ratio of HA i.e. 1.67. These particles were not evenly distributed over the leaf surface but were aggregated in spotted areas (7A). A similar observation was seen for the particles based on HA-CuSPHy, which appear with a non-homogeneous distribution on the leaf surface that tend to be concentrated in one point (7B). It should also be noted that HA-CuSPHy particles have homogeneous morphology that tends to be distributed in an uneven manner. The formulation based on HA-CuTBS has clearly a more homogeneous distribution of particles

on the leaf than the HA-CuSPHy treatment (7C). The same observation was seen with HA-CuOxCl and HA-CuHyOx in Fig. 7D and Fig. 7E, in which at the latter formulation is distributed less uniformly than the other one derived from insoluble copper compound. In the leaf samples treated with distilled water a particulate distributed on the leaf surface was detected. Elemental analysis of the particulates did not reveal any signal corresponding to copper, calcium or phosphorous, indicating that the particulate was not attributable to the applied substances (7F).

A further comparison (Fig. 8) of leaves treated with formulations of HA-CuSPHy (8A) and HA-CuTBS (8B) highlights the different aggregation and composition of the particles. The first sample shows needle-like particle of Ca-S and the second one spherical particles of P-Cu with different X-ray spectra of elements within them.

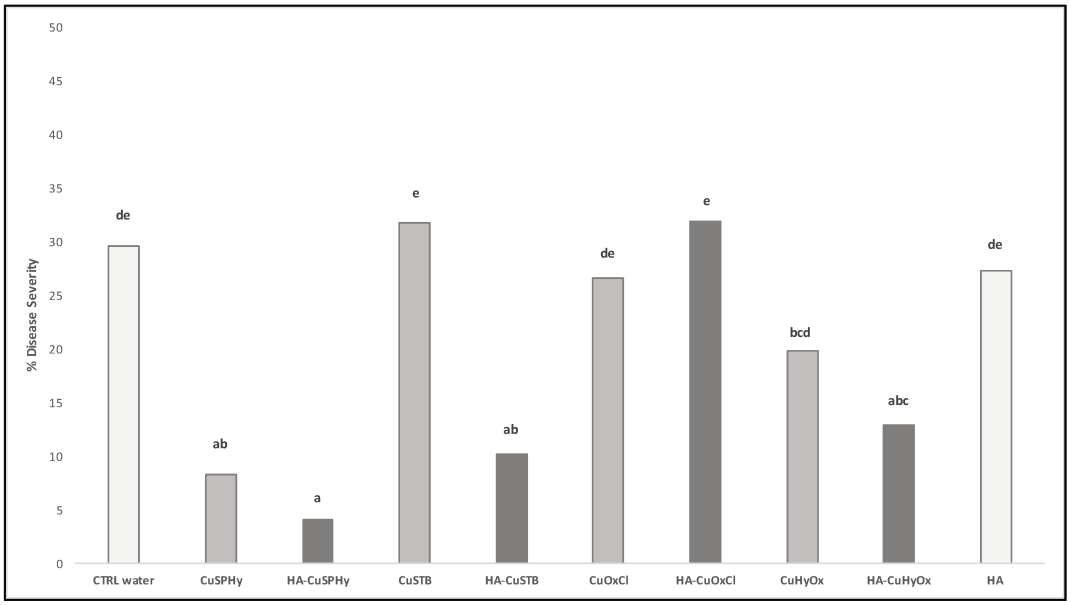
Figure 8. Environmental Scanning Electron Microscope images and X-ray spectra of elements recovered in, showing the different aggregation and composition of the particles of two copper compounds formulated with HA: formulation HA-CuSPHy (8A) and formulation HA-CuTBS (8B). The first treatment (A) shows needle-like particles of Ca-S and the second one (B) reveals spherical particles of P-Cu with different X-ray spectra of elements.



4.3.3. Disease severity on the treated leaves. Formulations based on HA and copper(II) compound were applied *in planta* to verify the potential protective effect against the infection of *P. viticola*. Disease severity 7 days post treatment and inoculation is shown in Fig. 9. Under

greenhouse conditions, the pathogen inoculation on the control plants, i.ee treated with distilled water, showed a high infection rate. Treatment based on HA did not show any fungitoxic effect and disease severity was the same as in the control plants. Treatments based on the pure copper(II) compounds showed a significant variability in the present plant protection test. The lowest disease severity resulted from the treatment based on CuSPHy, which also showed a high phytotoxic effect. The three others pure copper(II) compounds did not have any significant effects on *P. viticola* under the present experimental conditions. The formulations based on HA and the copper(II) compounds have showed interesting results on pathogen control. Especially for the HA-CuSPHy and HA-CuTBS, the formulation with HA significantly improved the protective activity of both copper(II) compounds. The same observation was seen with HA-CuHyOx, but with a lower protective activity. An opposite result was seen in the treatment based on HA-CuOxCl in which the formulation with HA gave significantly lower disease control compared to CuOxCl.

Figure 9. Disease severity (%) detected under greenhouse conditions, 7 dpi, in leaves of potted plants (*Vitis vinifera*) treated with: water (control), CuSPHy, HA-CuSPHy, CuTBS, HA-CuTBS, CuOxCl, HA-CuOxCl, CuHyOx, HA-CuHyOx, HA.



5. Discussion

The present investigation was focused on the aggregation *in planta* of the HA nanostructured particles, functionalised with several copper(II) compounds, in order to verify the micrometric aggregation of the applied substances. The results show that the pH of each copper(II) compound influences the final pH of the formulations with HA (Table 2). As described in the literature, pH plays an important role in the growth of HA nanocrystals, which increase from pH higher than 4.00 [94, 107]. The pure HA applied in the present study, showed a pH of 12.78, which changed after the formulations with each copper(II) compound. Considering CuSPHy, the only soluble copper(II) compound, the pH variation was particularly strong and after formulation with HA the pH was 2.24. Based on this data, it was expected to detect a partial or total dissolution of the HA particles in such a formulation. The insoluble copper(II) compounds showed a lower impact on the final pH of the formulations with HA and values were approximately around 11.00. In those formulations, it was expected to observe a more stable structuration of the particles based on HA.

Besides HA particle stability and growth, pH obviously has a further impact on Cu(II) ion exchange with HA. As a strong ion exchanger, Ca(II) on the surface of HA can exchange divalent heavy metals [108, 109]. In a recent study, performed on a composite based on HA partially substituted in magnesium, it was observed that adsorption strongly depends on the solution pH and increased with increasing pH. In particular, the Cu(II) adsorption clearly increased from 19.4% to 97.6% when the pH of the solution was increased from 1.0 to 5.9 and then slightly decreased to 95.8% at pH 6.9. This phenomenon was associated with the possible reactions behind the Cu(II) exchange from the solution [110]. This observation suggests a chemical-physical study on the potential mechanisms and kinetics of the Cu(II) ions exchange between HA and the copper(II) compounds applicable in agriculture.

The XRD results support the above observations. The typical diffraction pattern was detected for the pure HA. Despite the over concentration of the copper(II) compounds, which in almost all the formulations covers the HA diffraction pattern, it was possible to discriminate the presence or not of the HA particles in the analysed samples. As for the pH, XRD patterns of the formulations differed according to the hydrogen ion activity of the pure copper(II) compounds. The diffraction pattern of HA-CuSPHy differed from that of the pure compounds, showing diffraction maxima related to $CaSO_4$ (gypsum), which indicates the possible dissolution of the HA and the consequent recombination between calcium and sulfate. This observation was not reported by the diffraction pattern of the formulations based on insoluble copper(II)

compounds. In those cases, the formulation based on HA-CuTBS revealed a diffraction pattern attributable, in part, to the presence of HA in the analysed sample. Furthermore, considering the broadening of the diffraction maxima corresponding to HA, which indicate the degree of crystallinity, it was relatively higher in the HA-CuTBS sample.

Concerning the dimensions of the studied particles, the DLS results demonstrate the interesting configuration in micro clusters of the applied nanostructured HA. This evidence, according to the patented method of synthesis [105], tends to exclude the presence of single nanoparticles, thereby reducing the environmental risks associated with applications of nano-sized materials [111]. Despite the lack in the literature of the physical and chemical characterisation of the copper(II) compounds applied in agriculture, the DLS findings may give a real indication on the average size of the Cu(II) based particles used as pesticides of between 1 and 100 µm. Excluding the result related to the HA-CuSPHy sample, which is probably referred to particles mainly based on $CaSO_4$, an interesting size distribution is reported by the samples based on HA-CuTBS and HA-CuHyOX, in which the high percentage of particles between 0.5 and 1 µm may represent a positive condition to optimize the application and distribution *in planta*. In fact, particle dimension has potentially a direct impact on the droplet size generated during the spraying application of the pesticide, which represents a crucial factor for the efficiency of a treatment [112].

According to Mavrocordatos *et al.*, microscopy methods are all single particle methods, this mean that data does not arise from an ensemble of particles such as is the case with light scattering. This enables information to be collected on each particle free from interferences from other particles or background solutes [113]. In the present study, TEM images (Fig. 5) were fundamental to understand particle aggregation rather than particle size, in combination with the elemental composition of the particle by the energy dispersive X-ray spectrometer, taking into consideration that the measurement uncertainty of EDX is generally 20% [114].

Besides the well-known aggregation of HA in micro clusters comprised of 100 nm sized nanocrystals [115], the TEM observations revealed two main type of shapes on the studied particles. The XRD and DLS data related to the HA-CuSPHy sample were confirmed by the detection of needle-like particles that differ partially from the typical shape of the HA particle and totally from the shape of the other Cu(II) based particles, which show a more rounded form. Elemental analysis also revealed a very low presence of calcium in the particle detected on the HA-CuSPHy sample, ratifying the conclusions of the XRD results. Therefore, despite the notable form and dimension of such particles, homogenous aggregates, more similar to the typical aggregates of the pure HA, were observed in the sample based on HA-CuTBS. In this

case, the elemental analysis of the aggregates indicated the presence of Cu(II) associated to particles based on calcium and phosphorous (HA). In particular, comparing both samples (Fig. 4), a higher delivery effect is expected by the particles of the HA-CuSPHy sample and related to the needle form of the aggregates, meaning also a high specific surface able to release potentially Cu(II) ions. On the other hand, the higher particle density in the aggregates of the HA-CuTBS sample may represent a crucial aspect on the stability and persistence of the treatment *in planta*.

After the treatments *in planta*, a relevant phytotoxic effect was detected on both the abaxial and adaxial leaf surfaces treated with sample based on CuSPHy. This effect has been reported in the literature but does not occur with plant protection products because to be authorised as a fungicide in agriculture (with the common name of Bordeaux mixture) CuSPHy has to be neutralised by calcium hydroxide [116, 117]. In the present study, Bordeaux mixture was not examined as it is not a pure copper(II) compound and its potential exchange in Cu(II) ions is conditioned by the neutralisation process. Considering that the treatment based on HA-CuSPHy did not produce any phytotoxic effect on treated leaves, it seems to have a similar effect on CuSPHy as neutralisation with calcium hydroxide. This possibility is supported by the XRD results (Fig. 1), which show the differences between the diffraction patterns of the CuSPHy sample and the formulation HA-CuSPHy. Although, the pH of both applied samples was quite similar (Table 4), the non-toxicity of the HA-CuSPHy based treatment was probably linked to the rearrangement and interaction of the Cu(II) ions with the detected particles based on phosphorous (Fig. 3).

ESEM was useful technique to study particle aggregation *in planta*, especially to evaluate the efficiency of treatments in protecting the plant against the pathogens. In this respect, application of HA is based specifically on its property to generate nano-structured biologically active coatings [94]. The leaf sample treated with HA shows such particles aggregated on the leaf surface. The most similar configuration was observed on leaves treated with HA-CuSPHy, confirming the findings of TEM. The treatments based on the insoluble copper(II) compound and HA, especially the HA-CuTBS, appear to be more homogenously distributed in smaller aggregates, indicating a different particle aggregation compared to the HA clusters.

The efficiency of a such distribution is reflected in the disease severity detected 7 days post treatment and 6 days after the ESEM study on treated leaves. Under greenhouse conditions and high inoculation pressure, the treatment based on HA-CuSPHy provided very efficient control of *P. viticola*, that differed significantly from the other formulations It has to be specified that the positive result of the treatment based on CuSPHy was expected considering the high

solubility of Cu(II) ions. However, this treatment is not satisfactory due to the high phytotoxicity recorded and is not truly representative of CuSPhy, which is widely used in plant protection as Bordeaux mixture. Likewise, the low efficiency reported by the treatments based on CuOxCl and CuHyOx is probably related to the application in the present study of the pure technical compound without any co-formulate that is used in the commercial products.

A further comment has to be added on the treatment based on HA alone, which did not show any toxic effect on the plant or on the pathogen. The potential toxicity of HA is not clearly described in literature, and it has been extensively studied and applied for many years in the medical field for class I, II and IIa devices. Nevertheless, a promising result on the grapevine pathogen control was revealed by the HA-CuSPHy sample and also the HA-CuTBS, which is supported by the previous observations on the particle aggregation, stability and distribution. This observation, although it was not specifically the object of the present study, suggests the need for further investigation of the delivery properties of nanostructured HA to enhance the distribution, persistence and optimal release of Cu(II) ions *in planta*. This is even more remarkable for the potential reduction of the amount of active substances that need to be applied in plant protection, which is one of the main goals for sustainable disease management. In this respect the efficacy of reduced dosage of copper against *P. viticola* was recently investigated on commercial formulations applied in organic viticulture [28].

6. Conclusions

The present investigation showed interesting features of the HA particles functionalised with copper(II) compounds. Applications of HA for drug delivery has previously been studied mainly in the medical field [101, 102]. The release properties of HA related to the delivery of metal ions has been extensively described by many authors [73, 95–97]. However, such a delivery model has not been reported in literature for the treatments of plants to control fungal pathogens, including fungal diseases of *V. vinifera*.

In this paper X-Ray analysis showed a different interaction between HA and the copper(II) compound, possibly based on the solubility and pH of the different formulations. The DLS analysis showed a granular distribution ranging globally out of the nanometer range. In this respect, further observations with the TEM and ESEM microscopes showed, in all formulations, large aggregates partially nanostructured, which were recognised as aggregates and not clusters, and thus stable in their micrometric dimensions. The same observations have not been reported for any nanoparticles. The detected particles, based on calcium, phosphorous and copper, did not show any phytotoxic effect after their application to leaves of *V. vinifera*. In particular, the formulations based on HA and CuSPHy revealed the positive role played by HA to neutralise the Cu(II) ions released by CuSPHy, which are responsible for the high phytotoxicity when applied without any coformulation. The same formulations showed promising results on controlling the fungal pathogen, confirming the potential role of HA as an innovative delivery system of Cu(II) ions. Nevertheless, new methodologies of characterisation should apply to investigate in greater detail the interaction between HA and various ions. In particular, the homologation or not of such formulations to the nanomaterials class needs to be followed by specific studies regarding the potential toxicological effects on the applied tissues at the nanometric scale.

In conclusion, the present study indicates the possibility to improve the biological activity of a bioactive substance by modifying its structure through a specific and achievable formulation with a biocompatible material. In this context, the development of functional models might encourage further interesting applications on agricultural tools based also on organic and inorganic compounds.

Chapter 2

In vitro and *in vivo* evaluation of the biological activity of hydroxyapatite functionalized with copper(II) compounds on the grapevine fungal pathogens control

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Battiston E., Antonielli L., Di Marco S., Fontaine F. and Mugnai L. (2018). Innovative delivery of copper(II) ions by a nano-structured hydroxyapatite: potential application *in planta* to enhance the sustainable control of *Plasmopara viticola*.

1. Abstract

Grapevine (*Vitis vinifera* L.) is one of the fruit crops requiring the highest fungicide impact for diseases control. *Plasmopara viticola* is probably the most serious grapevine disease able to cause consistent yield losses. Over the years, several active ingredients were developed to control the potential disease damage. The systematic use of organic fungicides has induced the emergence of resistant strains of *P. viticola.* Only a copper-based treatment regime avoids this, thanks to the non-specific mode of action of copper-containing fungicides. In organic viticulture, the protection against downy mildew is essentially ensured by the use of cupric fungicides, the use of which in agriculture is expected to be further restricted by the European countries, because of its critical ecotoxicological and phytotoxicological profile. The research on innovative forms of copper for downy mildew control appeared the most promising approach as well as the optimization of the distribution and persistence of copper-based pesticides. The present research investigates the delivery properties of biomimetic synthetic hydroxyapatite (HA) to enhance the biological activity of Cu(II) ions. To this aim, four Cu(II) compounds (CuSPHy, CuTBS, CuHyOx and CuOxCl) were formulated with the innovative HA component and applied in a preliminary *in vitro* antifungal assay against *Botrytis cinerea*, a common grapevine pathogen suitable for *in vitro* activity tests, and finally in the *in-planta* efficacy assays against *P. viticola* under greenhouse conditions. The *in vitro* results highlighted a different grade of inhibition by each Cu(II) compound according to their applied dosage and indicating also the delivery role potentially played by the HA, especially on the insoluble copper salts. Under greenhouse conditions, further findings on the biological activity of the applied formulations were gained, especially on the efficacy of variable percentages of HA into the formulations, on the influence of dose variation of the formulation and the treatment efficiency and persistence under rain–washing effect. In conclusion, the present study revealed promising findings on the formulation based on the HA particles and the soluble copper salt (CuSPHy), which resulted highly efficient in reducing both the disease severity and incidence in all the experimental conditions, suggesting in the meantime a deeper investigation of functional models and the co-formulation process on the insoluble Cu(II) compounds.

2. Introduction

Grapevine (*Vitis vinifera* L.) is the most widely cultivated and economically important fruit crop [118]. Grapevine, grown both for table grape and wine production, is also one of crops requiring the highest fungicide impact for diseases control [3]. The high amount of fungicides used is especially linked to the susceptibility of most of the *Vitis vinifera* cultivars to downy and powdery mildews in climatic conditions favourable to these diseases [2]. Downy mildew, caused by the fungal pathogen *Plasmopara viticola* (Berk. & Curt.) Berl. & de Toni, is probably the most serious grapevine disease, especially in grape growing regions with relatively cool and wet spring season [119]. In optimal weather conditions (wet, moderate temperature) for the pathogen and with no protective treatment, downy mildew can cause huge losses [6]. For this reason, in such climates, disease control becomes crucial to avoid consistent yield losses.

Chemical control is the most effective measure currently used to control downy mildew [123]. In the last decades, fungicides based on different chemical families (strobilurin, cyazofamid, carbamate, phenylamid, dithiocarbammate, phthalimide or cupric compounds) have been developed and applied by winegrowers according to a calendar schedule as protection against the disease [124].

Over the years, the massive use of synthetic fungicides has induced the emergence of resistant strains of *P. viticola* [125], especially in single site fungicides due to their specific mode of action. Recently, resistant strains were also detected in an experiment under controlled conditions, after the application of a mixture based on multisite synthetic fungicides [126]. Resistance to copper-based fungicides has never been reported in *P. viticola* thanks to the non-specific mode of action of copper-containing fungicides.

Copper was the first fungicide used for control of Oomycete pathogens [21]. The wide activity spectrum of copper is based on the complexes that copper forms with membrane enzymes, showing sulphydryl-, hydroxyl-, amino-, or carboxyl-groups, which lead to enzyme inactivation [123]. For efficient disease control, copper needs to be applied before zoospores germinate. Since the discovery of the efficacy of copper against downy mildew by Millet in the nineteenth century [20], fungicides based on different cupric salts were applied over the years in preventive control strategies and applied according to the meteorological conditions favorable for infection [26].

In organic viticulture, protection against downy mildew still relies on the use of copper-based fungicides [18], according to specific regulations that limit the amount of Cu applied [127]. In European countries, including Spain, Italy and France, the use of copper for protecting crops is

allowed up to 6 kg ha^{-1} per year or 30 kg ha^{-1} per 5 years, established in the Regulation (EC) 354/2014 [128]. Nevertheless, in some other countries (e.g. Netherlands, Denmark), the use of copper in agriculture is forbidden, and in other countries there are additional restrictions (e.g. 3 kg ha^{-1} per year in Germany). In this legislative frame, EU is discussing further quantitative limitations for the future [28].

The ecotoxicological profile of copper is well known and described in the literature [129]. Among the side effects of copper, accumulation in agricultural soils is considered the most controversial. Being a heavy metal, copper is not degraded in soil and the long-term use of cupric fungicides in organic agriculture is responsible for extensive copper accumulation in soils [130]. In addition, the persistent application of copper for downy mildew control often results in phytotoxicity such as burning of young shoots and leaves [131]. Nevertheless, the use of copper is still tolerated, especially in organic viticulture, considering its exclusive property as wide-spectrum fungicide and the lack of efficient alternative treatments for controlling grapevine downy mildew.

In view of the legislative restrictions, recent studies have been aimed at reducing the dosage of copper in commercial formulations for control of *P. viticola* in organic viticulture revealing a potential for efficient control at 200 to 400 g Cu ha^{-1} per application [28]. The impact of the climate change on disease management has to be considered as a further complication in the control and management of downy mildew. Salinari *et al.* predicted the need for at least two more fungicide sprays to control *P. viticola* epidemics under the most negative climate scenario [132].

Research oriented towards development of innovative forms of copper for downy mildew control has appeared to be one of the most promising new approaches. In a comparative trial performed in a greenhouse and vineyard, two novel copper-based formulations showed interesting results. The first, based on copper gluconate, was particularly effective in the vineyard and the second one, based on copper peptidate, provided high levels of disease control in both conditions but induced signs of phytotoxicity in treated plants [24].

The possibility to optimize the distribution and persistence *in planta* of copper-based pesticides, has also been considered by many studies in nanotechnology. In particular, the development of slow-release systems for pesticides has opened new perspectives to reduce the amount of the active substance applied leading to efficient plant protection and disease control [77, 78]. Promising results were achieved by the engineered nanoparticles (NPs), some formulated with copper (Cu NPs) [85]. Nonetheless, numerous authors have reported the need

for further evaluations on such NPs about the cytotoxicity and genotoxicity within the plant tissues [88, 89].

The present research arises from successful biomedical applications of a nanostructured and biocompatible material. In that respect the biomimetic synthetic hydroxyapatite (HA) has been studied extensively due its unique drug delivery properties active on metal ions and on both organic and inorganic compounds [98, 94]. The aggregation between the clusters of a particular nanostructured HA and four Cu(II) compounds was previously investigated by Battiston *et al.* (accepted), revealing the possibility to improve the distribution of copper on the leaves by modifying its structure through a specific and achievable formulation with a biocompatible material.

The aim of the present study was to assay the activity, under controlled conditions, of formulations based on HA particles and four different Cu(II) compounds against *P. viticola*. More specifically, the work was focused (i) on the antifungal activity of each Cu(II) compound formulated with HA, tested on reference fungal pathogens *in vitro* and *in vivo*; (ii) on the efficacy of variable percentages of HA in the formulations; (iii) on the influence of dosage variation of the treatments (at lower Cu(II) percentage than the dosages recommended for cupric fungicides); and (iv) evaluation of the rain–washing effect on the protective treatments.

3. Materials and methods

3.1. Functionalisation of hydroxyapatite with Cu(II)

A water suspension containing 30% (w w^{-1}) of nanostructured hydroxyapatite (Ndg Natural Development Group Srl, Italy) was obtained according to a patented process of synthesis [105]. Four Cu(II) compounds (Cnr-Ibimet, Bologna, Italy) were prepared to functionalize HA: copper sulfate pentahydrate (CuSPHy), tribasic copper sulfate (CuTBS), copper oxychloride (CuOxCl) and copper hydroxide (CuHyOx). Functionalization of the products was carried out by maintaining the HA slurry with the respective Cu(II) compound and water complex. The various Cu(II) compounds were pre-solubilized or suspended in water and then added to the HA slurry, keeping in slow agitation for 4 h. Formulations were prepared based on two approaches for two different efficacy assays: (i) varying both the HA and Cu(II) % w w^{-1} (Table 1) and (ii) varying the HA % w w^{-1} and maintaining a predefined Cu(II) concentration (Table 2). The formulations were analysed by Inductively Coupled Plasma Optical Emission Spectrometry (Arcos-Spectro, Ametek, Kleve, Germany) to verify the copper concentration.

Table 1. Samples based on HA and Cu(II) compounds and related formulations prepared according to the variation of both the HA and Cu(II) % w w^{-1}; pH was measured at 20°C on samples after dilution with distilled water (pH 7.50); Efficacy Assay 1 (EA1).

Samples	HA % w w^{-1}	Cu(II) compound % w w^{-1}	Cu(II) % w w^{-1}	pH
HA-3	3	0.0	0.0	7.88
HA-6	6	0.0	0.0	10.35
CuSPHy-0	0	21.0	5.2	5.57
CuSPHy-3	3	21.0	5.2	5.70
CuSPHy-6	6	16.0	4.0	6.05
CuTBS-0	0	9.6	5.2	7.52
CuTBS-3	3	9.6	5.2	7.79
CuTBS-6	6	7.4	4.0	7.35
CuOxCl-0	0	9.5	5.2	7.61
CuOxCl-3	3	9.5	5.2	7.80
CuOxCl-6	6	7.0	3.8	7.31
CuHyOx-0	0	8.5	5.2	7.65
CuHyOx-3	3	8.5	5.2	7.80
CuHyOx-6	6	6.5	4.4	7.19

3.2. Copper doses

In the samples (Tables 1 and 2), the Cu(II) concentration was established at 5.25 ± 0.04% w w^{-1} for the formulations up to 5.4% w w^{-1} of HA and 4.15 ± 0.30% w w^{-1} for the formulations with 6% w w^{-1} of HA, due to the Cu(II) compound stability in formulation with HA.

The amount of product applied (or treatment dose) for each sample was varied so that the final amount of Cu(II) applied *in planta* in each replicate was standardized among all treatments.

Table 2. Samples based on HA and two Cu(II) compounds (CuSPHy, CuTBS) and related formulations prepared variating the HA (% w w^{-1}) and maintaining a predefined Cu(II) concentration (5.2% w w^{-1}); Efficacy Assay 2 (EA2) and 3 (EA3).

	Samples	HA % w w^{-1}	Cu(II) compound % w w^{-1}	Cu(II) % w w^{-1}
EA2	CuSPHy-0.6	0.6	21.0	5.2
	CuSPHy-1.2	1.2	21.0	5.2
	CuSPHy-2.4	2.4	21.0	5.2
	CuSPHy-3.6	3.6	21.0	5.2
	CuSPHy-5.4	5.4	21.0	5.2
EA3	CuTBS-0.6	0.6	9.6	5.2
	CuTBS-1.2	1.2	9.6	5.2
	CuTBS-2.4	2.4	9.6	5.2
	CuTBS-3.6	3.6	9.6	5.2
	CuTBS-5.4	5.4	9.6	5.2

According to a previous study, which analysed the effective amount of Cu(II) on treated leaves in downy mildew control assays [28], samples were applied *in planta* by spraying solutions/suspensions with the following empirically derived concentrations of Cu(II): 0.01, 0.025, 0.05, 0.075% w w^{-1} (Table 3). Such concentrations were also adopted according to the aim of each assay. Otherwise, considering the irrelevant antimicrobial activity of the pure HA described in literature [133, 98], in the preliminary *in vitro* antifungal assay the samples (Table 1) were applied at higher concentrations than *in planta*. More specifically, the solid growth media was dosed with Cu(II) at concentrations of 0.05, 0.1, 0.2% w w^{-1}.

Table 3. Cu(II) concentrations applied *in planta* by the experimental treatments per each Efficacy Assays.

Efficacy Assay	Cu(II) % w w^{-1}
EA1	0.025
EA2	0.050 - 0.025 - 0.010
EA3	0.050 - 0.025 - 0.010
EA4	0.075

3.3. Preliminary *in vitro* antifungal assay

For a preliminarily understanding of the putative antifungal activity of HA formulated with each Cu(II) compound, the samples (Table 1) were applied *in vitro* for the mycelial growth inhibition (GI) test [134].

A strain of *Botrytis cinerea* Pers. was chosen as representative grapevine pathogen, often tested for *in vitro* antifungal assays alongside *in vivo* control assays on *P. viticola* [136]. Malt extract agar (MEA), composed of malt extract 20 g L^{-1} di (Liofilchem Srl, Italy), agar 15 g L^{-1} (Liofilchem Srl, Italy) and distilled water, was amended with three different dosages (Table 1) of three Cu(II) concentrations (0.05, 0.1, 0.2% w w^{-1}) combined with the HA concentration related to the sample. Petri dishes (diameter 9 cm), each containing 15 mL of medium, were inoculated by transferring 0.7 mm diam. mycelial discs taken from the periphery of 5–7 days-old cultures of *B. cinerea* and placed upside down at the centre of the plate. Treated and untreated control plates were incubated at 25°C in darkness with three replicates per treatment. Control plates were plain MEA. Growth inhibition was calculated daily until the 5th day post inoculation as follows: GI = [(DC–DO)/DC] X 100, where, DC is the diameter of mycelial growth in control plates and DO is the diameter of mycelial growth in treated plates.

3.4. *In planta* antifungal assay

Experiments in controlled conditions were performed in an experimental greenhouse at Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy). Four consecutive efficacy assays (EA) against *P. viticola* were carried out to evaluate: (EA1) the performance of each Cu(II) compound formulated with HA; (EA2–EA3) the efficacy of various concentrations of HA into the formulations based on the two most effective Cu(II) compounds and applied *in planta* at various dosages; (EA4) the efficacy and stability of the most effective formulations under a rain-washing effect. The same protocol was applied for all the EA, applying a rain fastness test in EA4.

3.4.1. Plant material. 1-year-old grapevine plants (*Vitis vinifera* L, cv Chardonnay grafted on Kober 5BB rootstock) were grown in individual 2.5-liter pots of peat-rich, pre-fertilized (NPK: 14:16:18) standard soil (Topfsubstrat D450; Stender, Schermbeck, Germany) under natural light (15 h each day) at temperatures ranging from 18–28°C. Six potted vines with at least 1–2 shoots and 8–10 developed leaves were selected as replicates for each treatment.

3.4.2. Preventive treatments and inoculation. Distilled water was used as the control treatment, alongside the standard treatments based on cupric or organic commercial fungicides (Table 4).

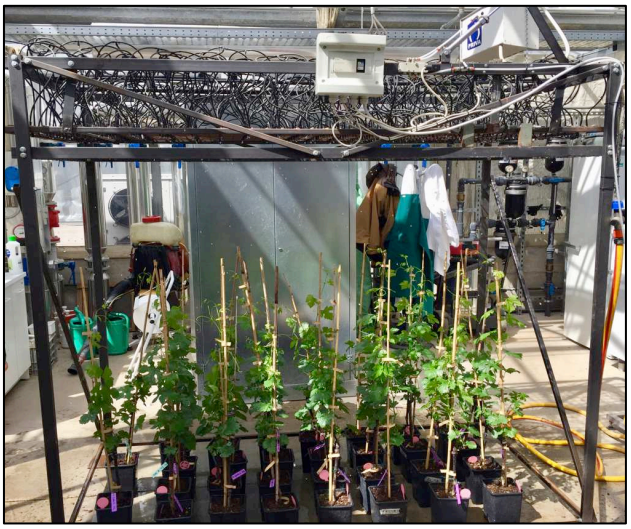
Table 4. Reference treatments results on the mean disease severity (%) and mean disease incidence (%) detected in potted grapevines (cv. Chardonnay) in each Efficacy Assays (EA).

		Treatment	a.i % w w^{-1}	Dosage % w w^{-1}	Disease Severity (%) ± SE	Disease Incidence (%) ± SE
EA1		Water (positive control)	--	--	35.4 ± 10.6	96.4 ± 3.6
		Quantum® (Adama)	Dimetomorf 50	0.05	5.5 ± 1.0	72.6 ± 6.6
		Coprantol WG® (Syngenta)	CuHyOx 42.37	0.20	6.6 ± 1.3	66.6 ± 7.2
EA2		Water (positive control)	--	--	60.9 ± 7.4	100.0 ± 0.0
		Quantum® (Adama)	Dimetomorf 50	0.05	2.4 ± 0.5	20.0 ± 8.4
		Bordo Flow® (Manica)	CuSPHy 49.6	0.40	4.6 ± 0.6	63.1 ± 10.4
EA3		Water (positive control)	--	--	38.7 ± 11.7	86.2 ± 8.5
		Quantum® (Adama)	Dimetomorf 50	0.05	1.7 ± 0.4	28.0 ± 5.5
		Tri Base® (Nufarm)	CuTBS 28	0.33	6.5 ± 2.0	61.3 ± 10.8
EA4	0 mm rain	Water (positive control)	--	--	55.7 ± 7.9	100.0 ± 0.0
		Quantum® (Adama)	Dimetomorf 50	0.05	2.8 ± 0.8	44.4 ± 10.3
		Bordo Flow® (Manica)	CuSPHy 49.6	0.60	1.2 ± 0.5	20.0 ± 7.0
		Tri Base® (Nufarm)	CuTBS 28	0.49	2.4 ± 0.3	40.8 ± 3.4
	50 mm rain	Water (positive control)	--	--	54.9 ± 2.9	100.0 ± 0.0
		Quantum® (Adama)	Dimetomorf 50	0.05	5.6 ± 0.7	63.5 ± 9.6
		Bordo Flow® (Manica)	CuSPHy 49.6	0.60	5.3 ± 1.9	65.9 ± 11.7
		Tri Base® (Nufarm)	CuTBS 28	0.49	12.6 ± 0.6	97.8 ± 1.3

3.4.3. In laboratory conditions (temperature 20 ± 1°C, relative humidity 65 ± 5%, artificial light), reference fungicides were diluted according to the recommended dosage, while each experimental sample were dissolved or suspended in a specific volume of distilled water according to the Cu(II) concentration investigated *in planta* in each EA. The pH of the diluted samples and distilled water (Table 1) was measured with a pH meter (pH 538; WTW GmbH, Weilheim, Germany). Approximately 30 mL of each solution or suspension were applied to the plants with a garden spray pump (Sprayer 0.6 L, Leroy Merlin, Italy) taking care to cover homogenously both the adaxial and abaxial leaf surface. Before inoculation, plants were left to dry at room temperature. Inoculum of *P. viticola* was prepared immediately before inoculation by washing grapevine leaves that had freshly sporulating lesions with cold (4°C) distilled water. The concentration of the inoculum suspension was adjusted to 5 x 10^5 sporangia mL^{-1} based on previous sporangia count with a haemocytometer. Six h post treatment, the suspension was sprayed on potted vines taking care to cover the abaxial surface of leaves, except six control plants that were not inoculated as control. Plants were transferred to a dark growth chamber for 16 h, at 20 ± 1°C, relative humidity (RH) of 80 to 99% and then incubated in the greenhouse for 7 days (incubation period), at 25 ± 1°C, RH 60% and natural light. After the incubation period, plants were placed again in a dark growth chamber for 16 h, at 20 ± 1°C and RH 80 to 99% to promote *P. viticola* sporulation.

3.4.4. Rain fastness test. In the fourth Efficacy Assay (EA4), rain fastness was simulated 4 h after the treatments. Potted vines were subjected to 0 or 30 mm of simulated rain (30 mm/h) applied by means of 14 sprinklers positioned 2.2 m above the soil (Fig. 1), protected from wind, and regulated to produce drops similar in size to raindrops (0.3 to 2.5 mm). After the simulated rainfall, plants were left to dry 4 h and then inoculated with the pathogen as previously described.

Fig. 1. Rain fastness simulator (FEM, San Michele all'Adige, Italy) equipped of 14 sprinklers positioned 2.2 m above the soil and regulated to produce drops similar in size to raindrops (0.3–2.5 mm).



3.4.5. Assessment of disease severity and incidence. The treatment activity was evaluated based on two parameters: disease incidence (the number, expressed as percentage, of leaves with oil spot symptoms and/or visible sporulation) and disease severity (percentage of leaf area covered by sporulating lesions). According to the EPPO standard scale [137], both parameters were visually estimated using continuous value of percentage.

3.4.6. Environmental Scanning Electron Microscopy (ESEM). In the first Efficacy Assay (EA1), the distribution of the treatments on the plant foliage and the structural features of the particles suspended in the tested formulations (based on HA 3%), were assessed by direct analyses *in planta* with an Environmental Scanning Electron Microscope (Fei Quanta 200 ESEM, FEI Corporation, Eindhoven, The Netherlands), operating in low-vacuum mode, at 25 kV, without pre-treatment of the samples. X-ray (EDS) microanalysis by an Energy-Dispersive System, coupled to the ESEM (EDAX, software EDAX Genesis, AMETEK, Mahwah, NJ, USA) was used to investigate the composition of the particles and the distribution of the related elements

on the leaf surface, that are (namely) Ca, P, Cu, S, Cl. Observations were performed on the leaves of treated potted vines, 8 days post treatment. Foliar samples were collected from each replicate of all the investigated conditions by counting from the basal part of the primary shoot and sampling the fourth leaf. Leaves were cut into small portions (10 mm x 10 mm) and transferred to a conductive adhesive carbon mounted on aluminium stubs.

3.5. Statistical analysis

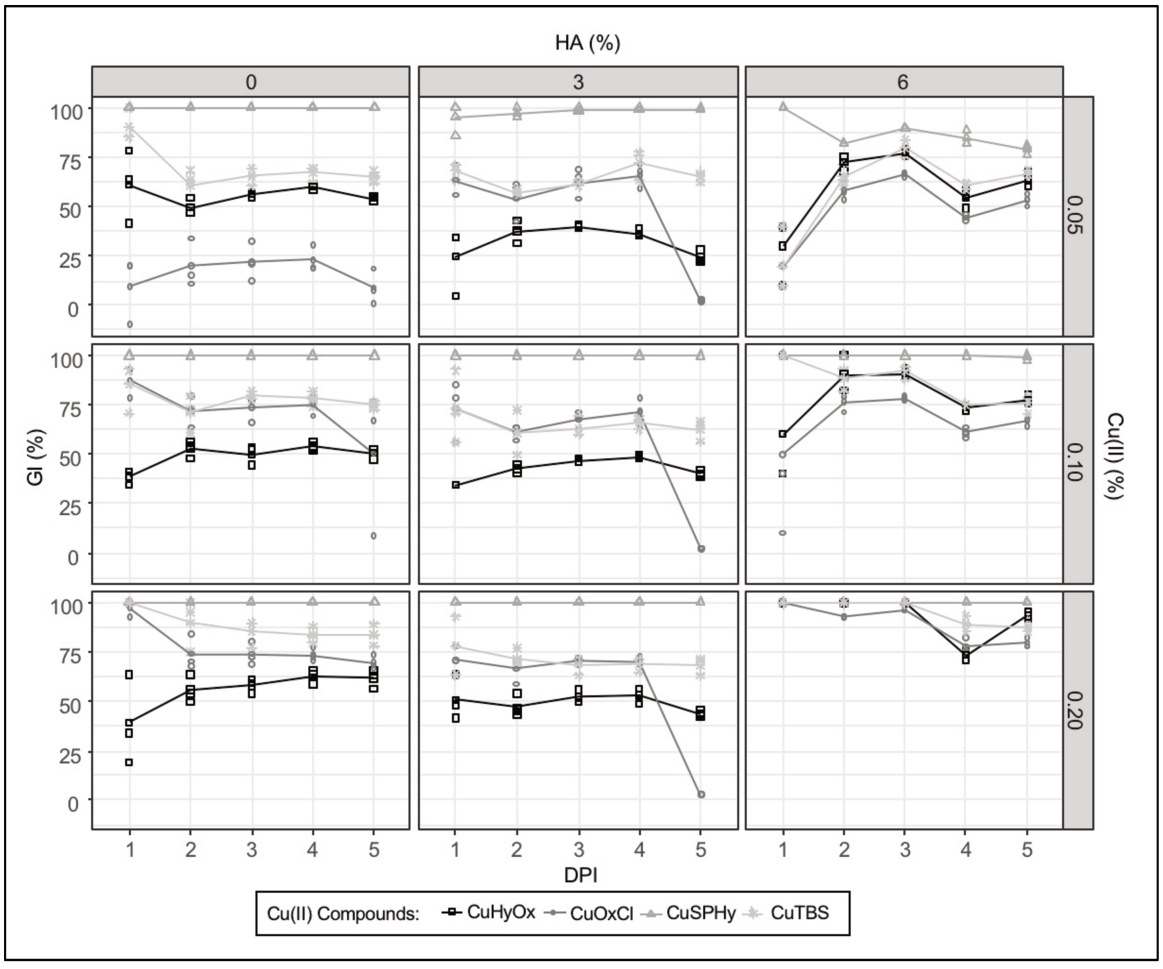
Statistical analysis was performed using R Statistical Software. In the *in vitro* assay, the measurements were replicated in three independent experiments. ANOVA was performed to study the significance of differences ($P \leq 0.05$) between mean values of GI %. Experiments performed under greenhouse conditions were designed as randomized complete blocks. Data of the disease severity and incidence on leaves were analyzed with ANOVA as indicator of the disease development and the efficiency of the treatments. When differences were found ($P \leq 0.05$), Tukey's HSD test based on least square means (lsmeans R package) was applied to study differences between the various levels of the factor [135]. The contrasts between control treatments (commercial fungicides and water), tested in different assays, was performed separately from the comparisons of the experimental treatments in order to not affect the data interpretation as the firsts do not express the factors and/or the factor levels of the seconds. Nevertheless, the results concerning commercial fungicides were plotted (ggplot2 R package) to evaluate the effects of these products on severity of downy mildew infection and determine the relationship between the amount of the active ingredient applied and the efficacy of the treatment.

4. Results

4.1. Preliminary *in vitro* antifungal assay

The biological activity on the mycelial growth of *B. cinerea* of three concentrations of formulations based on Cu(II) compounds and HA are shown in Fig. 2.

Fig. 2. Effects on mycelial growth (%) of *B. cinerea* of different Cu(II) concentrations from four Cu(II) compounds formulated with different HA concentrations. Petri dishes were incubated at the temperature of 25°C for 5 days in darkness.



All the Cu(II) compounds were found to inhibit the growth of *B. cinerea* in different percentage. CuSPHy was the most effective for inhibiting mycelial growth in the time and at all applied concentrations of Cu(II) (0.05, 0.1, 0.2% w w^{-1}). Mycelial growth of *B. cinerea* was inhibited by CuTBS at nearly 65% by the lower amount and 87% by the higher amount of Cu(II). CuHyOx showed the lowest activity against the pathogen growth at 0.05 and 0.1% of Cu(II), without increasing the inhibition at the highest % of Cu(II), while CuOxCl responded to the amount

effect as the lowest concentration of Cu(II) showed the lowest pathogen inhibition, which significantly increased at 0.2% of Cu(II), respectively from 10% to 70%. The factor HA played a variable effect on the Cu(II) compounds, depending on its concentration. At 3% of HA, CuSPHy was still the most effective on the full control of the *B. cinerea* growth, even reducing the amount of Cu(II). At the lowest concentration of Cu(II), the same level of HA had an opposite effect on CuHyOx and CuOxCl, reducing the inhibition activity on the first and increasing the growth inhibition % on the second. CuTBS showed a similar inhibition of the correspondent pure Cu(II) compound, while the performance of CuHyOx was reduced by the 3% of HA, especially at the higher amount of Cu(II). Different results were reported by the 6% of HA. Such a level reduced the inhibitory activity of the lowest concentration of CuSPHy, while on the other Cu(II) compounds increased the GI%, especially on the CuOxCl, and CuHyOx at 0.2% of Cu(II). The statistical analysis showed that GI index was different ($P \leq 0.05$) for the Cu(II) compounds, the applied Cu(II) amounts and the % of HA.

4.2. *In planta* efficacy assay

4.2.1. Effect of Cu(II) compounds formulated with HA. The greenhouse trial EA1 investigated the efficacy of treatments based on four Cu(II) compounds formulated with two percentages of HA in the control of the grapevine pathogen *P. viticola*. Results on disease severity are shown in Fig. 3. Data of treatments based on (i) water as positive control, (ii) organic fungicide, (iii) cupric fungicide and (iv) HA 3% are presented separately (Fig. 3A) and compared to the results of the experimental treatments (Fig. 3B). The positive control showed a variable disease severity, with a mean of 35%, with max value nearly 75%, significantly different from both fungicides, which have reported a good disease control. Treatment based on 3% of HA did not show any effect against *P. viticola* revealing a disease severity distribution similar to the positive control. No foliar symptoms related to *P. viticola* infection developed on the non-inoculated potted vine controls. The effects due to the factors Cu(II) compound and HA were both found to be significantly different ($P \leq 0.05$). In Table 5, the contrasts (Tukey's HSD test) within the factor «Cu(II) compound» and the factor «HA %» are reported for the disease severity and incidence. Considering the pure Cu(II) compounds, the lowest disease severity was reported by CuSPHy, showing 5% of mean disease severity, significantly different from the other Cu(II) compounds. The applied Cu(II) concentration (0.025% w w^{-1}) was not effective for the other pure compounds. Concerning the formulations based on 3% of HA, a high significant reduction in disease severity was revealed on CuTBS, showed also at 6% of HA by a slighter reduction. The

CuOxCl seems to respond positively to the formulation with HA, showing a significant difference in disease severity at 6% of HA but not corresponding to an acceptable disease control. The apparent efficiency of the formulation based on CuHyOx with 3% of HA was not significant and was not enforced at 6% of HA. Concerning the disease incidence, the most interesting results was found on CuSPHy at 3% of HA, showing 52% of symptomatic leaves, followed by CuTBS at 3 and 6% of HA with approximately 70% in disease incidence, while the rest of the experimental treatments were over 80% and the positive control at 100% of incidence (data not plotted). A consistent phytotoxic effect was reported on all the leaves treated with CuSPHy and it was not observed on the same compound at 3% and 6% of HA. No phytotoxic signs were reported by the control, the commercial and the other experimental treatments.

Fig. 3. Results on disease severity (%) detected in potted grapevines (cv. Chardonnay) treated with water (positive control), organic fungicide (Quantum®, Adama), cupric fungicide (Coprantol WG®, Syngenta), HA 3% and compared to the experimental treatments. A, Commercial fungicides and the positive control are presented and analyzed separately from the experimental treatments in order to not affect the data interpretation. B, experimental treatments.

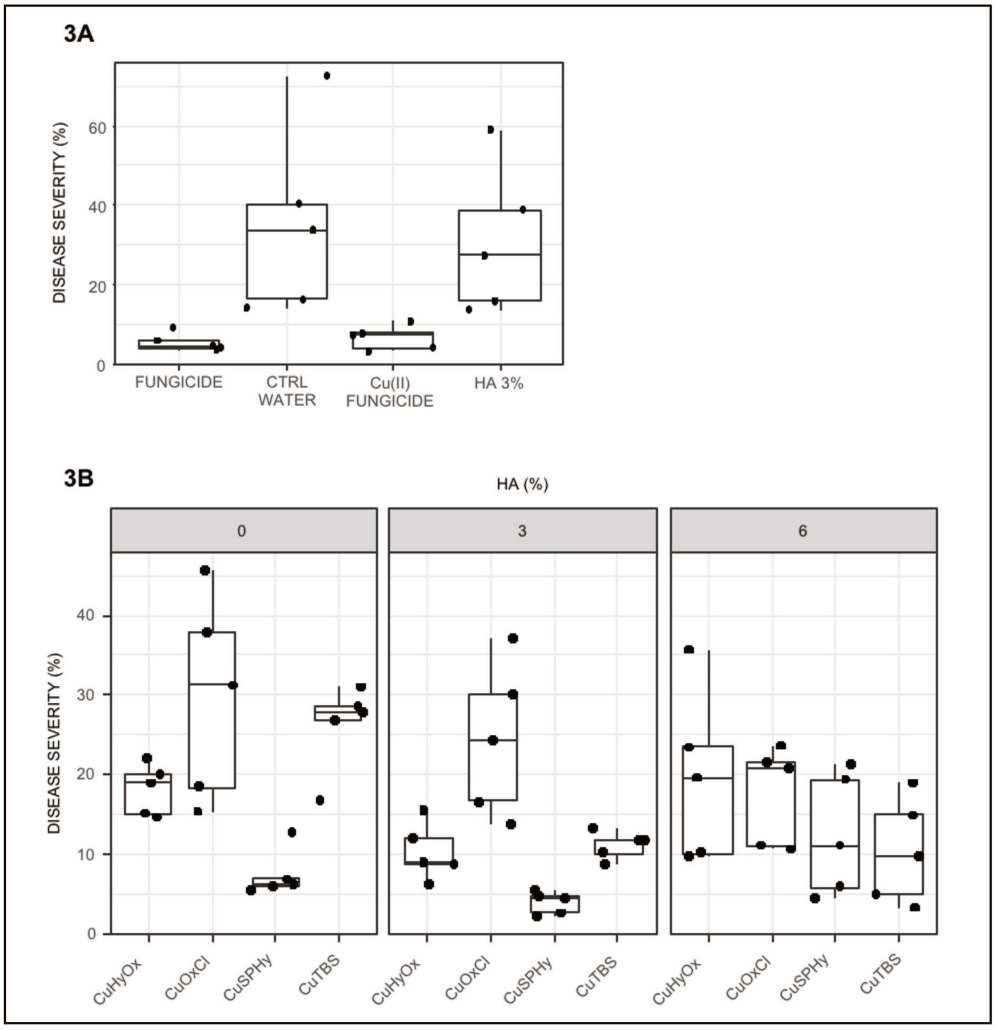


Table 5. Contrasts in pairs (Tukey's HSD test) within the factor «copper compound» and the factor «HA %» showing the significant differences on disease severity and incidence; Efficacy Assay 1.

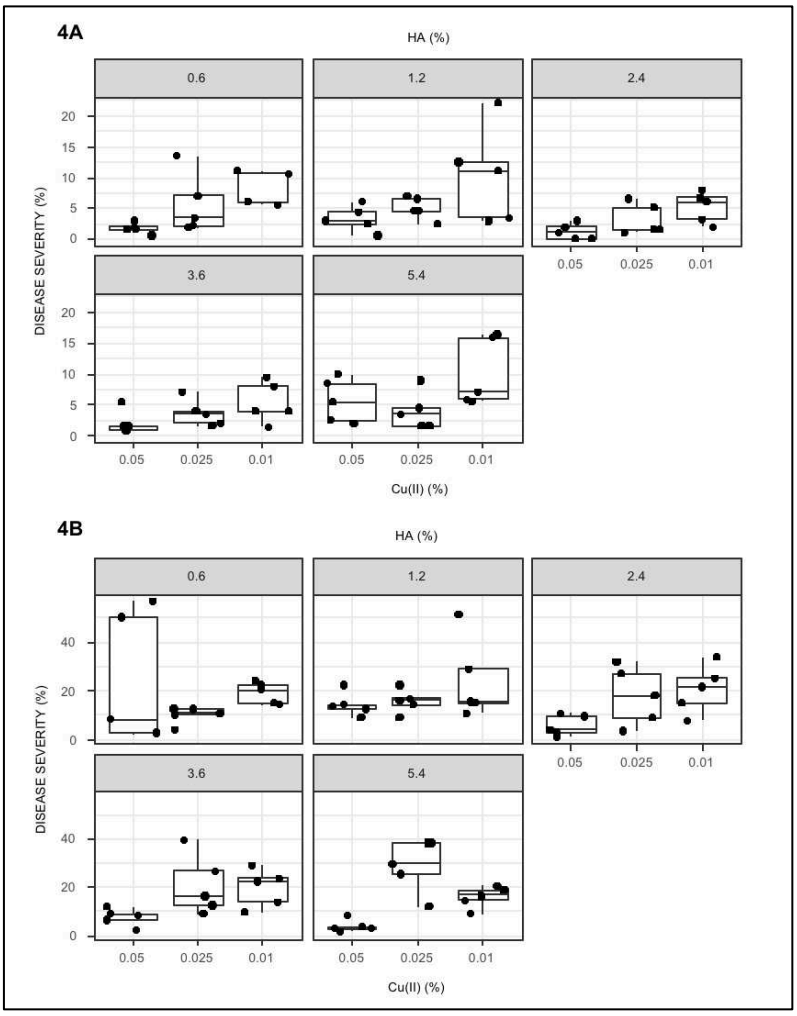
Disease Incidence			Disease Severity											
			CuHyOx			CuOxCl			CuSPHy			CuTBS		
			0	3	6	0	3	6	0	3	6	0	3	6
CuHyOx	0			n.s.	n.s.	*	--	--	*	--	--	n.s.	--	--
	3		n.s.		*	--	*	--	--	n.s.	--	--	n.s.	--
	6		n.s.	n.s.		--	--	n.s.	--	--	n.s.	--	--	*
CuOxCl	0		n.s.	--	--		n.s.	*	n.s.	--	--	*	--	--
	3		--	n.s.	--	n.s.		n.s.	--	*	--	--	*	--
	6		--	--	n.s.	n.s.	n.s.		--	--	n.s.	--	--	n.s.
CuSPHy	0		n.s.	--	--	n.s.	--	--		n.s.	n.s.	*	--	--
	3		--	*	--	--	*	--	*		n.s.	--	n.s.	--
	6		--	--	n.s.	--	--	n.s.	n.s.	*		--	--	n.s.
CuTBS	0		n.s.	--	--	n.s.	--	--	n.s.	--	--		*	*
	3		--	n.s.	--	--	*	--	--	*	--	n.s.		n.s.
	6		--	--	n.s.	--	--	n.s.	--	--	n.s.	n.s.	n.s.	

* = significant difference at $P \leq 0.05$, n.s. = not significant difference at $P \leq 0.05$; Tukey's HSD test.

4.2.2. Effect of HA concentration and applied amount of Cu(II). The variability of the factor «HA %» into the formulation and the effect of the factor «dosage % w w^{-1}» was studied in greenhouse trials EA2 and EA3, by applying treatments based respectively on CuSPHy and CuTBS, which were the two most effective Cu(II) compounds in EA1. In Table 4 results of the control and the comparison treatments are shown. No symptoms were detected in the negative control. Symptoms were most severe and diffused in EA2 (respectively 60.9% and 100%) than in EA3 (respectively 38.7% and 86.2%), and in both experiments the differences were significant. The treatment based on Dimetomorf was highly efficient in reducing both the disease severity and incidence. Figure 4 shows the disease severity (%) detected in EA2 (Fig. 4A) and in EA3 (Fig. 4B). Considering the formulations based on CuSPHy (Fig. 4A) in comparison to the CuSPHy fungicide (Table 4), good disease control was reported by all the HA % and at Cu(II) 0.05%, revealing a higher variability in disease severity at HA 5.4% and the lowest mean severity between HA 2.4–3.6%. At lower levels of the factor «dosage % w w^{-1}», the same formulations revealed a higher variability in disease severity, which is close to the commercial fungicide (4.6%) at Cu(II) 0.025% while at Cu(II) 0.01% shows higher mean values. Despite the performance in controlling the disease, a high phytotoxic effect was detected on the grapevine leaves treated by the CuSPHy formulations at HA 0.6% and less at 1.2%, with major intensity at the higher dosage. Overall, the CuSPHy treatments showing the lower disease severity and the most stable results, even at lower Cu(II) %, were based on HA 2.4% and 3.6%. In the parallel

experiment EA3, the CuTBS fungicide (Table 4) was effective in controlling *P. viticola*, even considering the lower disease severity and incidence reported by the positive control compared to those in EA2. Most of the observations showed by the CuTBS based treatments (Fig. 4B) concerned values in disease severity over than 10%, without corresponding to an acceptable disease control. For CuTBS formulations applied at Cu(II) 0.05%, the factor «HA %» (from 0.6% to 5.4%) revealed an increasing reduction in disease severity variability, showing a good disease control over the 2.4% of HA. A reverse response was given by the same treatments applied at Cu(II) 0.025%, while such tendencies were not confirmed by the lowest dosage in Cu(II), showing disease severities nearly 20% for all HA %. No signs of phytotoxicity were detected on the leaves treated with CuTBS based formulations at all Cu(II) %. In both experiments EA2 and EA3, the factor «dosage % w w^{-1}» was found significant ($P \leq 0.05$) while the factor «HA %» was found significant just in EA2.

Fig. 4. Results on disease severity (%) detected in potted grapevines (cv. Chardonnay). **A**, results of CuSPHy based treatments. **B**, results of CuTBS based treatments. Both copper(II) compounds were formulated with five different percentages of HA and applied *in planta* in three different dosages corresponding to decreasing amounts of Cu(II).



Comparisons of means by Tukey's HSD test within the factor «HA %» and the factor «dosage % w w^{-1}» (a = 0.05, b = 0.025, c = 0.01% w w^{-1}) are presented in Table 6, highlighting the significant differences on disease severity in both trials. Unlike EA1, few contrasts in EA2 and EA3 were significant on both Cu(II) compounds and within both factors. However, based on the disease severity results, the most effective formulations were found at HA 2.4–3.6% for CuSPHy and at HA 3.6–5.4% for CuTBS, applied at Cu(II) 0.5%. The same evidences, in terms of tendency and significance, were revealed by the disease incidence detected on both trials (data not plotted).

Table 6. Contrasts in pairs (Tukey's HSD test) within the factor the factor «HA %» and the factor «dosage % w w^{-1}» (a = 0.05, b = 0.025, c = 0.01% w w^{-1}) showing the significant differences on disease severity; Efficacy Assay 2 and 3.

			CuSPHy													
			0.6			1.2			2.4			3.6			5.4		
			a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
CuTBS	0.6	a		n.s.	*	n.s.	--	--	n.s.	--	--	n.s.	--	--	n.s.	--	--
		b	*		n.s.	--	n.s.	--	--	n.s.	--	--	n.s.	--	--	n.s.	--
		c	n.s.	n.s.		--	--	n.s.	--	--	n.s.	--	--	n.s.	--	--	n.s.
	1.2	a	n.s.	--	--		n.s.	*	n.s.	--	--	n.s.	--	--	n.s.	--	--
		b		n.s.	--	n.s.		n.s.	--	n.s.	--	--	n.s.	--	--	n.s.	--
		c	--	--	n.s.	n.s.	n.s.		--	--	*	--	--	*	--	--	n.s.
	2.4	a	*	--	--	n.s.	--	--		n.s.	n.s.	n.s.	--	--	*	--	--
		b	--	n.s.	--	--	n.s.	--	n.s.		n.s.	--	n.s.	--	--	n.s.	--
		c	--	--	n.s.	--	--	n.s.	*	n.s.		--	--	n.s.	--	--	*
	3.6	a	*	--	--	n.s.	--	--	n.s.	--	--		n.s.	n.s.	n.s.	--	--
		b	--	n.s.	--	--	n.s.	--	--	n.s.	--	n.s.		n.s.	--	n.s.	--
		c	--	--	n.s.	--	--	n.s.	--	--	n.s.	n.s.	n.s.		--	--	*
	5.4	a	*	--	--	n.s.	--	--	n.s.	--	--	n.s.	--	--		n.s.	*
		b	--	*	--	--	n.s.	--	--	n.s.	--	--	n.s.	--	*		*
		c	--	--	n.s.	--	--	n.s.	--	--	n.s.	--	--	n.s.	n.s.	n.s.	

* = significant difference at $P \leq 0.05$, n.s. = not significant difference at $P \leq 0.05$; Tukey's HSD test.

4.2.3. Treatments efficacy and stability under a rain–washing effect. Considering the previous results, treatments based on CuSPHy and CuTBS, formulated at HA 3%, were applied in greenhouse rain fastness test (EA4) to study their efficacy and stability in comparison to the pure copper(II) compounds and the reference treatments (Table 4). The negative control plants were not affected by the pathogen. No significant differences were detected in both positive controls, potted vines washed by the simulated rain and unwashed, which showed 100% of incidence and nearly 55% of severity.

4.2.4. In Fig. 5, results of the rain fastness test are shown: commercial fungicides and the positive control (Fig. 5A) were analyzed separately from the experimental treatments (Fig. 5B) in order to not affect the data interpretation.

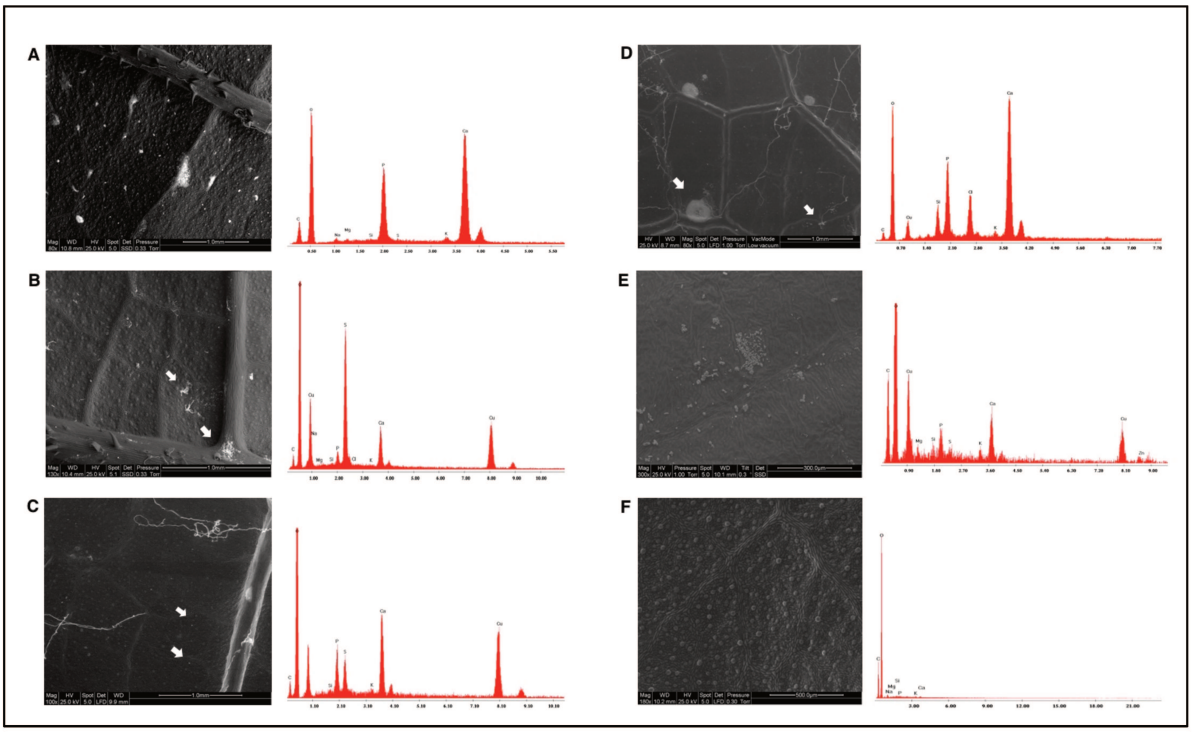
Fig. 5. Results of the rain fastness test on disease severity (%) and disease incidence (%) detected on leaves of potted grapevine (cv. Chardonnay) under greenhouse conditions. Plants were treated and exposed to 0 or 50 mm of simulated rain before to be inoculated with *Plasmopara viticola* (5 x 10^5 sporangia mL^{-1}). **A**, Commercial fungicides (Quantum®, Adama; Bordo Flow®, Manica; Tri Base®, Nufarm) and the positive control are presented and analyzed separately from the experimental treatments in order to not affect the data interpretation. **B**, experimental treatments.

The rain-washing effect was quite imperceptible on leaves treated with organic fungicide, being highly effective in both conditions. CuSPHy fungicide showed the best performance in reducing the disease incidence and severity, as it shown a good disease control even after the simulated rain. CuTBS fungicide lost efficiency in the simulated rain condition, showing approximately the same diffusion in downy mildew infection as the positive control (Fig. 5A). Treatment based on CuSPHy revealed the highest control activity reducing thus severity and incidence, and the result variability in both conditions (Fig. 5B). However, the treatment resulted in severe and

diffused symptoms of phytotoxicity. Otherwise, CuSPHy formulated with HA did not show any phytotoxic sign and despite the non-significant difference from the results reported by the same pure compound, all the disease severity % recorded were less than 5% in both conditions (Fig. 5B). Treatment based on CuTBS was significantly less effective than CuSPHy. The differences in disease severity and incidence were much higher in the simulated rain test. In such condition, the formulation based on CuTBS and HA has not shown a significant reduction in severity and incidence of *P. viticola* in comparison to the CuTBS based treatment, revealing a similar protection to the CuTBS fungicide. Pairwise comparisons by Tukey's HSD test within the factor «Cu(II) compound» and the factor «rain–washing effect», were highly significant for both factors.

Environmental Scanning Electron Microscopy (ESEM). The *in-planta* ESEM observations showed a relevant particle aggregation (Fig. 6). Distribution of the particles on the leaf surfaces of treated potted vines were studied 8 days post treatment (EA1).

Fig. 6. Environmental Scanning Electron Microscope images paired to the related X-ray spectra of elements and related to the formulations applied on the leaf surface of *V. vinifera*. A, pure suspension of HA (bar = 1 mm). B, formulation HA-CuSPHy (bar = 1 mm). C, formulation HA-CuTBS (bar = 1 mm). D, formulation HA-CuOxCl (bar = 1 mm). E, formulation HA-CuHyOx (bar = 0.3 mm). F, distilled water in control leaf sample (bar = 0.5 mm).



Particles based on pure HA revealed the respective Ca/P ratio of HA i.e. 1.67. These particles were aggregated in spotted areas and not regularly distributed over the leaf surface (Fig. 6A). A similar observation was noted on the CuSPHy-HA based particles, which were distributed on the leaf surface tending to be concentrated in one point (Fig. 6B). The HA-CuTBS based treatment showed a more homogeneous and spread distribution of particles on the leaf than the HA-CuSPHy treatment (Fig. 6C). The same observation was seen for HA-CuOxCl and HA-CuHyOx based treatments, in Fig. 6D and Fig. 6E, highlighting in the first case, the joint presence of particles and round patchy clusters of the same and in the second one the higher density of micrometric aggregates. In the leaf samples treated with distilled water (positive control) a particulate distributed on the leaf surface was detected. Elemental analysis of the particulates did not reveal any signal corresponding to copper, calcium or phosphorous, indicating that the particulate was not attributable to the applied substances (Fig. 6F).

5. Discussion

The principle aim of the present research was to evaluate the application *in planta* of an innovative tool to enhance the biological activity of Cu(II) ions for the sustainable control of the grapevine pathogen *P. viticola*.

The research highlighted the delivery properties of biomimetic synthetic hydroxyapatite (HA) which was shown to enhance the biological activity of Cu(II) ions. Clearly promising results were shown applying HA in the final *in planta* efficacy assays against *P. viticola* under greenhouse conditions, especially on the soluble copper(II) salt (CuSPHy), reducing both the disease severity and incidence in all the experimental conditions. The treatment also showed a high efficiency and persistence under rain–washing effect.

Despite the higher practical significance and efficiency of the *in-planta* assays in screening fungicides [138], significant and remarkable findings on the antifungal activity of the investigated substances were revealed by the preliminary *in vitro* assay. Being susceptible to broad-spectrum treatments based on copper [139], *Botrytis cinerea* was also studied based on previous laboratory trials performed to evaluate mycelial growth inhibition by innovative antimicrobial particles [140]. In general, it was highlighted a different grade of inhibition by each Cu(II) compound, according to their applied dosage. As expected, CuSPHy was highly efficient in inhibiting mycelial growth of *B. cinerea* at all the applied concentrations and thanks to the high solubility of this copper(II) salt, which dissolves in solution the highest concentration of Cu(II). The lower antifungal activity reported by the other copper(II) salts, such as CuTBS, CuOxCl and CuHyOx, is a result of their lower solubility, as such compounds tend to remain in suspension, releasing slowly and in certain conditions the Cu(II) ions [20]. The non-significant antimicrobial activity reported in the literature for particles based on HA [98, 133] could explain the contradictory role shown by the HA formulation on each copper(II) compounds. On CuSPHy, the most effective copper(II) salt, the highest concentration of HA (6%) was found to reduce significantly the inhibitory activity of CuSPHy when applied at the lowest concentration of Cu(II) (0.05%). This was supposed to be related to the excess of HA, with no antimicrobial activity, compared to the Cu(II) ions in solution. In the same condition, the opposite evidence was reported on CuHyOx. In general, this effect was shown on all the Cu(II) compounds applied at Cu(II) 0.2%, in which the HA 6% enhanced significantly the inhibition of *B. cinerea* in the time. These observations have suggested to investigate *in planta* the delivery role potentially played by the HA in relation to the functional copper(II) salt and to the concentration ratio between HA and Cu(II) ions applied into the formulation.

Under greenhouse conditions, promising findings on the biological activity of formulations based on Cu(II) and nanostructured particles of HA were gained by the present experiments. The formulations previously applied *in vitro*, were sprayed in the trial EA1 onto the foliage of potted vines at the same Cu(II) ions concentration (0.025%) of the cupric fungicide in comparison, according to an early greenhouse efficacy assays, which defined such amount as the minimal quantity of copper ions able to prevent acceptably downy mildew [24]. In this condition, CuSPHy formulated with 3% of HA reduced significantly both disease incidence and severity while with a higher HA content (6%), the HA-CuSPHy formulation lost protective activity against *P. viticola*, confirming the tendency detected on the growth inhibition of *B. cinerea* by the same formulations. The non-significant biological activity shown by the same concentration of HA confirms that the high efficiency of the HA-CuSPHy formulation is surely not a consequence of the fungitoxic activity of the HA based particles. On the other hand, a very good disease control was also achieved by the treatment based on the pure CuSPHy, but this resulted in high phytotoxicity on the treated leaves. As described by Gessler *et al.* (2011), CuSPHy is the main constituent of Bordeaux mixture, which is a cupric fungicide developed by neutralising and fixing the highly soluble and phytotoxic Cu(II) ions of this copper salt [20]. It is known in the literature for being the most ancient and efficient fungicide applied for the control of *P. viticola* [141]. Among the non-soluble Cu(II) compounds, in the EA1 there was no evidence of significant effect on controlling the disease in comparison to the cupric fungicide (based on CuHyOx). However, on the experimental treatments, the formulation with HA revealed the same tendency shown *in vitro* such as the reduced variability of the results (CuOxCl) and the general increase in treatment efficiency (CuTBS), except for CuHyOx that showed the lowest disease severity at HA 3%. Likewise, the low efficiency reported by the treatments based on the pure non-soluble Cu(II) compounds is certainly related to the application of the pure technical compounds without any co-formulate that is used in the commercial products.

Electron microscopy was useful for studying the distribution of particles and aggregation *in planta*, as the application of HA arises on its property of generating nanostructured biologically active coatings [94]. Such aggregated particles were detected on the leaf samples treated with HA. A similar configuration was observed on leaves treated with HA-CuSPHy. While treatments based on the insoluble Cu(II) compounds and HA appear distributed in smaller particle aggregations, showing different aggregates compared to the HA clusters. The diverse particle conformation and distribution reflect the different results shown by the greenhouse trial EA1. This aspect highlights the importance of spreading and delivering of Cu(II) ions on the leaf surface with respect to the control of the early fungal infection and their agents, the *P. viticola*

zoospores [121, 122]. Furthermore, the variable response of treatments in EA1, may be explained by the effect of the irregular aggregation of particles and therefore their irregular size, which have a direct impact on the droplet size generated during the spraying application of the protective substance, thus influencing significantly the treatment efficiency (Ferguson 2016).

The formulation HA-CuSPHy was further investigated as a potential innovative cupric fungicide as compared to the formulation HA-CuTBS, based on the insoluble Cu(II) compound. In both formulations the efficacy of various concentrations of HA was studied in relation to Cu(II) concentrations. In general, the results (EA2) confirmed that the most effective CuSPHy formulation (containing Cu(II) 5.2%) is based on HA varying between 2.4% and 3.6%, as seen in the greenhouse trial EA1. This encouraging result was shown by the over dosage (Cu(II) 0.05%) and unexpectedly, at under dosage treatment ((Cu(II) 0.01%). This latter evidence opens interesting perspectives in comparison to early studies aimed at investigating the efficacy of reduced copper dosage against *P. viticola* [24, 28, 142, 143] and considering probable future restrictions on the use of copper in agriculture. Otherwise, the findings collected on the formulations based on CuTBS (EA3) indicate a dissimilar activity of the factor «HA %» on the insoluble copper salt. In fact, despite a generally lower efficacy of CuTBS formulations than the ones based on CuSPHy, the less variables result in disease severity were reported at the highest % of HA and at the over and under dosage of Cu(II) ions. This observation suggests a deeper investigation on the methods and materials applicable for the optimal co-formulation between the suspension of HA nanostructured particles and the suspensions of the insoluble Cu(II) compound, such as CuTBS.

In greenhouse experiments carried out under rain–washing effect, it was possible to evaluate the stability and persistence on grapevine leaves of the formulations based on HA-CuSPHy and HA-CuTBS. In contrast to the study by Dagostin *et al.* (2010), the cupric treatments were applied at higher dosage of Cu(II) ions (0.075%) and a higher amount of simulated rain (50 mm) received by the potted vines to highlight in this condition the potential performance of the less efficient formulation based on CuTBS [120]. However, despite the high solubility in water of CuSPHy, the formulations based on this salt strongly reduced the disease severity and incidence in both conditions (0 and 50 mm). Excluding the positive control showed by the pure CuSPHy treatment because of the severe and diffused phytotoxicity on the treated leaves, it was confirmed the remarkable control achieved by the HA-CuSPHy without any side effect on the plants, therefore indicating a significant interaction between the Cu(II) ions and the HA nanostructured particles. The significant less control activity under rain–washing effect by the

HA-CuTBS treatment was further correlated to a non-optimal co-formulation method considering the similar tendency reported also by the pure CuTBS treatment and in comparison, to the lower disease severity showed by the CuTBS fungicide. In this respect the relevance of the formulation process on the cupric fungicide efficacy was already commented in literature, especially under environmental conditions such as weather conditions and the plant growth, able to influence strongly the product efficiency [24].

5.1. Conclusions

The present study indicates the possibility to enhance the biological activity *in vitro* and *in planta* of Cu(II) ions by modifying their distribution, persistence and delivery on the leaf surface, through a formulation with nanostructured particles of hydroxyapatite. This forms a biocompatible material able to generate biologically active coatings on the treated surface. In this context, a deeper study of functional models might encourage research into other interesting applications on agricultural tools based also on organic and inorganic compounds, applicable for sustainable plant protection purposes.

Chapter 3

Distribution and efficiency of hydroxyapatite functionalized with two copper(II) compounds in grapevine woody tissues colonized by a vascular fungal pathogen and characterization of the defense responses activated by the plant

In preparation for Pesticide Biochemistry and Physiology

Battiston E., Compant S., Antonielli L., Simoni A., Di Marco S., Mugnai L., Rabenoelina F., Clément C. and Fontaine F. (2018). *In planta* activity of copper(II) based formulations to control the colonisation of the esca-associated fungus *Phaeoacremonium minimum*

1. Abstract

Grapevine Trunk Diseases (GTDs) are becoming a serious and increasing menace in vineyards worldwide. The need for innovative disease management strategies is still persisting since the pesticides applied to control some of the GTDs have been banned and so far, no effective treatments were developed. In this context, the application of imaging methods, already applied to study plant-microbe interactions, could represent a crucial tool to understand the effect of experimental treatments on GTDs related pathogens and on the colonized tissues.

In the present investigation, trials were carried out to assess the efficacy of treatments based on two copper(II) compounds released by a delivery system based on the biomimetic inorganic hydroxyapatite and applied to protect propagating material from *Phaeoacremonium minimum*, a fungal pathogen associated to GTDs. Treatments were applied during the re-hydration process of rootstocks (*Vitis Berlandieri* x *Vitis riparia* cv. K5BB) and scions (*Vitis vinifera* L, cv. Chardonnay). After callusing, grafted vines were grown under greenhouse conditions. The vines were inoculated with an agar plug containing the pathogen (gfp7 transformed or wild type) by drilling a hole in the rootstock. Fifteen weeks post-inoculation, plants were harvested and woody tissues were observed using a Confocal Laser Scanning Microscopy (CSLM). After the observations, copper was quantified in the same plant material by the ICP-OES instrument. Finally, the grapevine defense responses were studied in treated leaves of the same plants, by analyzing the expression of genes related to the GTDs.

Results clearly showed the correlation between the pathogen colonization (CLSM images) and the distribution and persistence of copper(II) (ICP-OES data) over time and in different tissues. Furthermore, these parameters were found to be highly variable, with consequences on the pathogen colonization. In the same time, the transcriptomic analysis revealed the potential role of the innovative formulations in stimulating the plant defense responses.

In conclusion, the research highlighted the relevance of studying the plant-pathogen interaction through imaging methods. In this perspective, especially for the management of GTDs, experimental control strategies should be also related to the distribution and persistence of the applied substance and its potential biological role, as fungicide and plant biostimulant.

2. Introduction

During the last twenty years, Grapevine Trunk Diseases (GTDs) have become serious and destructive diseases of vineyards worldwide. Since the pesticides traditionally applied to control GTDs have been banned, no new effective treatments were developed. The research for sustainable alternatives to control GTDs is consequently required [38].

Based on the need for innovative disease management strategies, a coordinated research approach was promoted in Europe trough the COST Action FA1303 on the «sustainable control of Grapevine Trunk Diseases», which for four years was aimed to develop a network of European expertise to improve understanding of GTDs by acquiring knowledge on occurrence of pathogens, vine-pathogen interaction, ecology of wood-inhabiting microorganisms, and to develop new management protocols and biocontrol approaches [144].

Among the experimental strategies still in assessment, the control of the GTDs related pathogens colonising the wood, certainly represents an ambitious purpose. More specifically, the application of imaging methods, already applied to study the plant-microbe interaction [145], could represent a crucial tool to understand the effect of the experimental treatments on the pathogens and on the tissues colonised by them.

In this framework, a recent investigation conducted by Pierron *et al.* [146] has confirmed the opportunity to study the colonisation of GTDs related pathogens in grapevines, using fungal transformation with a vital marker, appreciating the fungal structures in the host plant: specifically, the authors have studied the *Phaeoacremonium minimum::gfp7* colonisation in grapevine tissues, by the application of Confocal Laser Scanning Microscopy (CSLM). In this study, *P. minimum* colonisation was assessed six and twelve weeks post-inoculation in two different types of tissues: in the node and the internode of one-year-old rooted cuttings of cv. Cabernet sauvignon. These processes of colonisation were compared with the colonisation by the wild-type strain using a non-specific lectin probe Alexa Fluor 488-WGA. The approach was able to demonstrate that *P. minimum* colonisation can vary according to the type of tissues and the type of spread using pith, bark and fibres. Woody tissues can respond to the injury and to the presence of this fungus, and xylem fibres play a key role in the early colonisation of the internode by *P. minimum* before the fungus can colonise xylem vessels.

Previously, the grapevine infection by *P. minimum* has been investigated using Scanning Electron Microscopy (SEM) on single-bud cuttings of cv. Cabernet sauvignon [147]. The authors have observed the penetration pathway and colonisation degree in cortex, epidermis and the pith, considering also both phloem and xylem vessels. Electron microscopy analysis was also

applied in another study, confirming that *P. minimum* and *Phaeomoniella chlamydospora* are vascular pathogens colonising wood fibres, xylem vessels as well as pith, and revealing the development of ultrastructural features in xylem vessels of grapevines affected by esca disease complex (GTDs) [148].

Among the GTDs related pathogens, a further application of fungal transformation to study the fungal colonisation in plant was performed transforming *P. chlamydospora* (isolate CBS 229.95) using a pCT74 construct which contained the genetic markers for synthetic green fluorescent protein (sGFP). Wood colonization was estimated through epifluorescence microscopy and was affected by incubation temperature. The use of sGFP-transformed *P. chlamydospora* helped to clarify different aspects associated with the location of this pathogen in grapevine tissue, before disease symptom expression [6]. As well as, other studies have proved the use of marker genes (e.g. sGFP) to study the colonisation of GTDs associated fungi [149, 150, 151].

The study approach based on the application of imaging methods can help a better understanding of the grapevine structural response to fungi involved in GTDs. Gómez *et al.* [152] have performed observations with SEM to examine the grapevine responses to fungal attack, including morphological and physiochemical defence mechanisms in the vascular system to reduce fungal infections. The study showed the formation of tyloses inside xylem vessels of diseased grapevines, which seem to be the first plant response to constrain fungi in the xylem vessels, while highlight the complementary action of the phenolic compounds to inhibit the fungi growth and colonisation.

Based on this background, a study was established to evaluate the application of a specific nanostructured inorganic hydroxyapatite (HA) as potential innovative delivery system of copper(II) ions [105], acting as amplifier of the copper antifungal activity for the control of GTDs related pathogens.

Preliminary assays on grapevine protection against *Plasmopara viticola* have revealed promising results on the application of nanostructured HA functionalised with several copper(II) compounds (Battiston *et al.* accepted). The results demonstrate the role of a such smart material as innovative delivery system but not yet as a carrier of bioactive substances. To this aim, a crucial study consists to observe, applying the modern techniques of electron and fluorescent microscopy, the vascular tissues of treated potted vines, affected by a pathogen related to GTDs, in order to understand the role of the HA delivery system on optimising the distribution and the releasing of copper(II) ions, amplifying their potential GTDs control in the infected grapevine tissues.

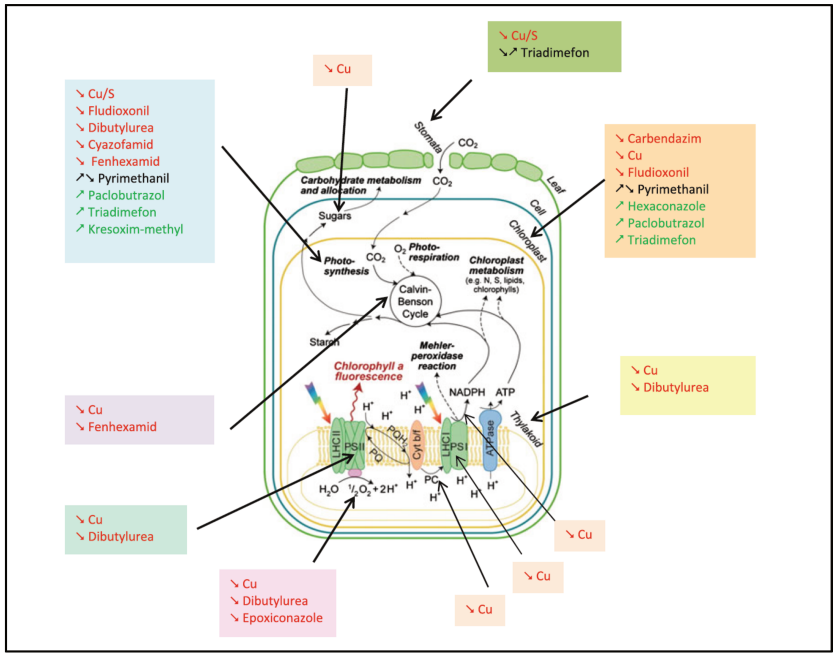
Considering the HA chemical and structural nature, the modern imaging methods usually applied to study the interaction between endophytes and *Vitis vinifera* L. appear to be useful to evaluate the antifungal activity of HA functionalised with Cu(II) compounds against the pathogen colonisation in the grapevine wood. There is not yet, however, a study concerning the application of imaging methods to evaluate the colonisation control of pathogens related to GTDs in the grapevine wood, but such a study would enable a better understanding of the role of traditional and innovative bioactive substances to protect the niches colonised by *P. minimum* in the grapevine vascular tissues.

In the meantime, a further investigation concerning the expression of grapevine defence-related genes by the experimental treatments based on HA and Cu(II) compounds was performed in the same plant material. As widely known and described in literature, grapevine can develop enhanced resistance to pathogen infections following treatments with certain organic or inorganic, natural synthetized substances, which can also act as resistance inducers and stimulate grapevine resistance to the pathogens. Grapevine defense responses occurred to respond to the fungi attack, including those related to GTDs. More specifically, the defense reactions in a such plant-pathogen remain still to clarify. Overexpression of defense-related genes (such as PAL, STS, CHIT4C and GST1) in leaves before and after apoplexy-esca symptoms development has been described by Letousey *et al.* [153]. A later study, revealed an early and stronger defense response regarding the induction of PAL and STS genes in less vulnerable cultivars when compare to the most susceptible Cabernet sauvignon, after elicitation with *P. chlamydospora* [154].

Based on these evidences, it is supposed that an efficient tool for protecting the grapevine plants against GTDs is potentially represented by the application of elicitors able to activate the defense mechanisms.

In this sense, the effect of copper(II) on photosynthesis in crop plants were extensively described by Petit *et al.* [155], highlighting its impact on the constitution of proteins photosynthetic proteins (plastocyanin) and respiratory proteins (cytochrome oxidase), the inhibition of photosynthesis, the stomatal closure, the inhibition of Rubisco, the decreased of chlorophyll, the modification on chloroplast ultrastructure, the PSII inactivation, the PSI inactivation, the inhibition of other sites of electron transport chain (Fig. 1). A similar biological activity has never been investigated for HA and not even in the biomedical fields, where such material was essentially applied for drug delivery purposes [94].

Figure 1. Schematic representation showing the interactions of the main processes in C3 photosynthesis in higher plants and impacts of fungicides [155].



Nevertheless, being based on calcium phosphate organized in a bio-reactive structure due to the high specific surface able to exchange a high rate of ions, it expected a potential impact on the plant physiology and more specifically, on the growth and even on phytotoxicity [87].

For these reasons, the grapevine defence responses were studied in treated leaves by analysing the expression of genes, which were selected among those described in literature as related to the GTDs and the treatments based on copper(II).

Such a study has completely matched the main purposes of the COST Action FA1303, concerning the disease management, such as the focus on: (i) a solution that will be friendly for the environment and no risk for the human life; (ii) the establishment of nursery and vineyard field experiments to evaluate the efficacy of biological or chemical products; (iii) different protocols for the prevention and the reduction of the impact of GTDs pre-existing infections in the field and in the nursery.

3. Materials and methods

3.1. Preliminary *in vitro* antifungal assay

In order to understand preliminarily the putative antifungal activity of HA formulated with two copper(II) compounds, sulphate pentahydrate (CuSPHy) and tribasic sulphate (CuTBS), the samples (Table 1) were applied *in vitro* for the mycelial growth inhibition (GI) test [134]. A strain of *P. aleophilum* CBS 100398, previously transformed with plasmid pCBCT in *P. minimum*::gfp7, was chosen as pathogen target related to GTDs. The Malt Extract Agar (MEA) medium, composed of malt extract 20 g L^{-1} di (Liofilchem Srl, Italy), agar 15 g L^{-1} (Liofilchem Srl, Italy) and distilled water, was autoclaved for 15 min at 120 °C and under sterile conditions was fractioned and added of three different dosages per each sample (Table 1) in order to investigate the antifungal activity of three Cu(II) concentrations (0.05, 0.1, 0.2% w w^{-1}) combined with the HA concentration related to the sample. 15 ml of such media were poured in Petri dishes (diameter 9 cm) and left to solidify. Mycelial discs of *P. minimum*::gfp7 were transferred from a colony to Petri dishes containing MEA, and grown for 28-30 days at 22°C. Six replicates of the pure culture were stocked and maintained at 4°C. Afterwards, each Petri dish was inoculated by transferring mycelial discs of 0.7 cm in diameter, removed from the periphery of 28–30 days-old cultures of *P. minimum*::gfp7 and placed upside down on the plate center. The procedure was done per each different sample and concentration, counting three replicates per condition. Treated and untreated control plates were incubated at 25°C in darkness. The growth inhibition was calculated daily until the 28th day post inoculation as follows: GI = [(DC–DO)/DC] X 100, where, DC is the diameter of mycelial growth in control plates and DO is the diameter of mycelial growth in treated plates.

Table 1. Samples based on the pure compounds or on the formulations, applied for hydrating and foliar treatments.

Samples	HA % w w^{-1}	Cu(II) compound % w w^{-1}	Cu(II) % w w^{-1}
HA	3	0.00	0.00
CuSPHy	0	21.00	5.25
CuSPHy+HA	3	16.00	4.00
CuTBS	0	9.60	5.22
CuTBS+HA	3	7.36	4.00

3.2. Plant material and nursery trials

During hydration of propagating material, just before grafting, experimental treatments (sample concentration = 2% w v^{-1}) were performed comparing the water treatment to 2 copper(II) compounds sulphate pentahydrate (CuSPHy) and tribasic sulphate (CuTBS) pure or in formulation with hydroxyapatite, by hydrating the rootstocks (*Vitis Berlandieri* x *Vitis riparia* cv. K5BB) and scions (*Vitis vinifera* L, cv. Chardonnay) in several water batches containing (n = 6 conditions). After callusing, grafted vines (n = 210) were planted in plastic pots and grown in greenhouse conditions in the nursery. Plants were inoculated when at least six leaves were fully developed. Inoculation was performed with hyphae of *P. minimum*::gfp7 (n = 15 per condition) from five PDA plates or with the wild-type strain of *P. minimum* (n = 30 control plants) grown on PDA, by drilling the rootstock approximately 5 cm under the grafting point and inserting a PDA plug. The same number of plants (n = 15 per condition) were not inoculated. On 18 control plants (n = 3 per condition) inoculated with wild-type strain of *P. minimum,* the same treatments applied during hydration were subsequently applied to the foliage by spraying such formulations (sample concentration = 2% w v^{-1}) according to the following timing: first treatment 7 weeks post inoculation, repeated every 2 weeks per three further times. Foliar samples for transcriptomic analysis were collected 8 h and 24 h after the last foliar application and stored at -80°C. Plants were finally harvested fifteen weeks post-inoculation and stored at 4°C until the microscopy investigation.

3.3. Confocal Laser Scanning Microscopy

The approach described by Pierron *et al.* [146], was applied. Plants inoculated with *P. minimum*::gfp7 and *P. minimum* wild-type were cut with secateurs, transversally (3 cm above and 3 cm below the inoculation site) and then longitudinally. All the data result from observations (n = 4) of rootstocks (n = 6) of each treatment (*P. minimum*::gfp7) and of control plants (*P. minimum*::gfp7 and wild-type strain of *P. minimum*), at fifteen weeks post-inoculation.

All observations of pure hyphae or treated plants were carried out using a confocal microscope (Olympus Fluoview FV1000 with multi-line laser FV5-LAMAR-2 and HeNe(G)laser FV10-LAHEG230-2, Japan). Observations with the confocal microscope were done at objective of 10x and between 20 and 40 X, Y, Z pictures containing 20 to 70 scans were separately taken at 405, 488, 594 nm wavelengths in blue/green/orange-red channels respectively, with the same settings each time.

Imaris software was used at the confocal microscope to visualise 3D reconstructions. X, Y, Z pictures from different channels were then merged (RGB for red, green and blue merging) using the image J software 1.47v.

3.4. Inductively Coupled Plasma Optical Emission Spectrometry

The rootstock samples analyzed at the CLSM (n = 6 per each treatment) were finally processed to quantify the copper content in the wood. Such samples consisted on the plant material treated during hydration, then grafted, callused, inoculated with *P. minimum*::gfp7 and grown under greenhouse conditions. The samples were sectioned, separating accurately the following three tissues: bark, vascular tissues and pith.

The organic material was completely lyophilized for 48 h (Lyovapor L-300, Buchi, Swiss) and pulverized with a mixer mill (Retsch MM 400, Retsch, Germany). Afterwards, 0.5 g of each sample were added of 10 ml of nitric acid concentrated and digested in a microwave oven (CEM Mars 5, NC, US) according to a maintenance program at 175°C for 20 minutes (US EPA 3050). Then samples were brought to volume with bidistilled water and then filtered to 0.2 µm with PTFE filters and diluted 1:20 with bidistilled water. After digestion, the qualitative and quantitative determination of the elements extracted in solution was performed ICP-OES instrument (Arcos-Spectro, AMETEK, Kleve, Germany).

The same procedure was performed on the rootstock cuttings (n = 6 per each treatment), sampled immediately after the hydrating treatment and stored at 4°C, in order to allow the estimation of the copper amount applied by the treatments during hydration, its distribution in the tissues of the wood and its stability on the time.

3.5. Transcriptomic analysis

Expression analysis was performed on leaf samples studying a set of genes selected according to a literature research [153, 154, 155, 157–75] related to GTDs and copper(II) (Table 2). The protocols for RNA Extraction and the Real-Time RT-PCR Analysis of Gene Expression were applied according to the methodology published by Spagnolo *et al.* [156].

PlantRNA Purification Reagent (Thermo Fischer Scientific Inc., Waltham, MA, USA) was used to extract total RNA from 50 mg of powdered green stem tissues and was DNase treated. The quality of RNA was checked by agarose gel electrophoresis, and the quantity was determined by measuring the absorbance at 260 nm. Reverse transcription was performed on 150 ng of total RNA using the Verso cDNA synthesis kit (Thermo Fischer Scientific Inc.). Real-time PCR was

performed with Absolute Blue QPCR SYBR Green (Thermo Fischer Scientific Inc.) using a CFX96 thermocycler system (Bio-Rad, Hercules, CA, USA). The thermal profile was: 15 s at 95°C (denaturation) and 1 min at 60°C (annealing/extension) for 40 cycles. Melting curve assays were performed from 65–95°C at 0.5°C s^{-1}. Melting peaks were visualized to check the specificity of each amplification. Results are expressed as the values of relative expression ($\Delta\Delta C_t$) and correspond to the mean from three independent experiments. The genes analyzed were considered significantly up- or down-regulated when changes in their expression were >2-fold or <0.5-fold, respectively. aspect

Table 2. Target genes selected according to a literature research [12, 13, 14, 19–38].

Abbreviation	Gene
EF1	EF1-elongation factor
60SRP	60S ribosomal protein L18
PAL	phenylalanine ammonia-lyase
POX	peroxidase
CHIT4C	chitinase
PPO	polyphenol oxidase
GLU	b-1,3-glucanase
LOX	lipoxygenase
PR-1	PR protein
PR-6	PR protein
PR-10	PR protein
PGIP	polygalacturonase-inhibiting proteins
STS	stilbene synthase
IFRL4	isoflavone reductase-like protein 4
PSBP1	PSBP subunit of PS II

3.6. Statistical analysis

Statistical analysis was performed using R Statistical Software. In the *in vitro* assay, the measurements were replicated in three independent experiments. ANOVA was performed to study the significance of differences ($P \leq 0.05$) between mean values of GI %. Experiments performed under greenhouse conditions were designed as randomized complete blocks.

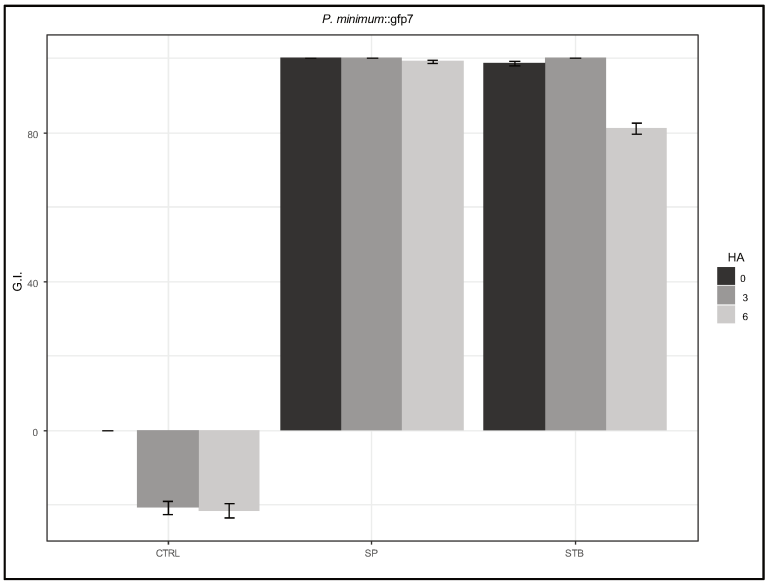
Data of the elements quantification (ICP-OES) have been logarithmically transformed in order to present all the elements regardless of the order of magnitude. On the same data, the CCA (Constrained Correspondence Analysis), was performed to highlight the distribution of each element in the tissue.

4. Results and discussion

4.1. Preliminary in vitro antifungal assay

The GI % of *P. minimum*::gfp7 mycelia was calculated 28 days post inoculation. The samples were applied in the solid medium so that it was drugged with the following concentrations of Cu(II): 0.05, 0.1, 0.2% w w^{-1}. Observing the curve of GI (Fig. 2), both copper(II) compounds were effectives. Remarkable the response of HA that did not show any fungi-toxic effect but an initial stimulant effect on the mycelial growth was observed. And interesting also to see that this effect has not reduce the efficacy of CuSPHy, confirming its positive interaction with HA, which has been less reported on the CuSTB.

Figure 2. *In vitro* mycelial growth inhibition % of *P. minimum*::gfp7 on treated media. Bar plots represents the mean values of GI % at three tested amounts, detected after 28 dpi.

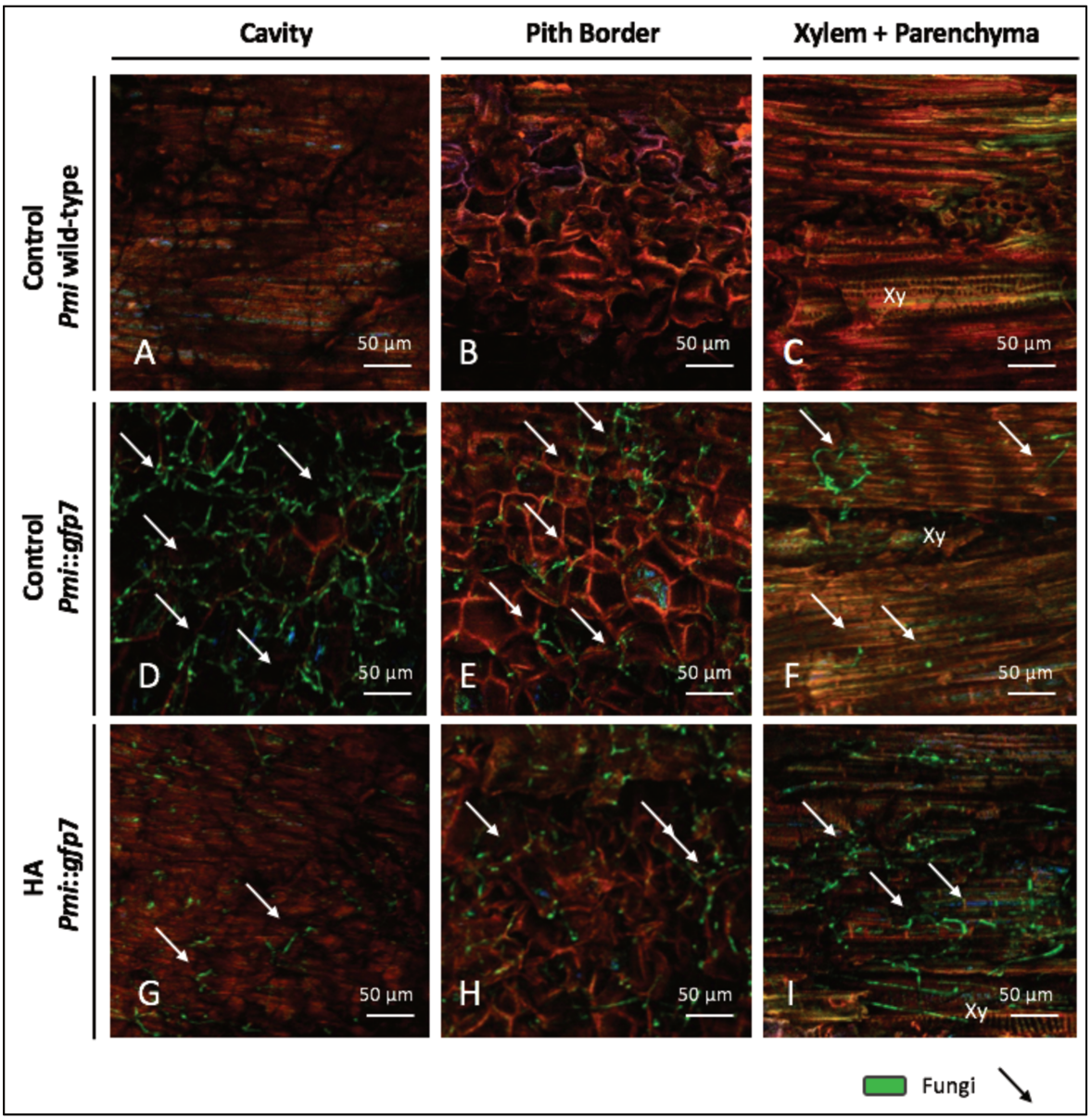


4.2. CLSM images and ICP-OES data

CLSM images are presented according to (i) the type of *P. minimum*, wild-type strain and *P. minimum*::gfp7, (ii) the part of the inoculation site, cavity, pith border and xylem with parenchyma, (iii) and the treatment, control, HA, CuSPHy, CuTBS, CuSPHy+HA and CuTBS+HA. (Fig. 3 and 4). No signal was detected in control plants inoculated with *P. minimum* wild-type strain (Fig. 3A, 3B and 3C). Inoculation of *P. minimum::gfp7* was successful in control plants (Fig. 3D, 3E and 3F) and led to more successful colonization of hyphae in the cavity site compared

to other tissues (Fig. 3D). Hyphae of *P. minimum::gfp7* were detected in plant material treated with HA (Fig. 3G, 3H and 3I) especially in the xylem and within the parenchyma (Fig 3I).
GFP signal was also detected in plant material of each treatment, showing clearly a different intensity and distribution of hyphae between the treatments and within the part of the inoculation site (Fig 4).

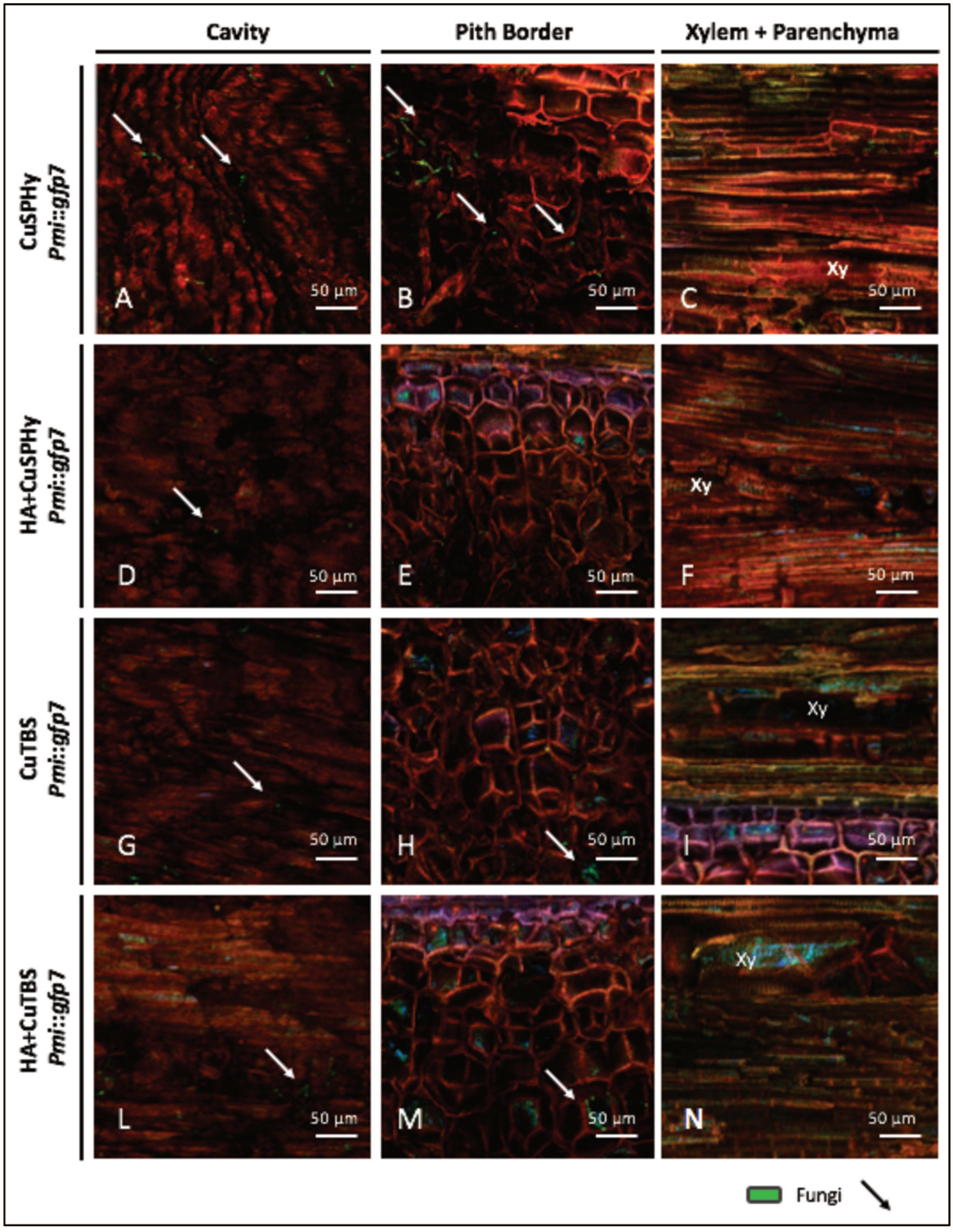
Figure 3. Confocal Laser Scanning Microscopy images (1).



Concerning the copper-based treatments, a very low green signal was detected in the inoculation point in all treatments. In the pith, only the treatment based on HA+CuSPHy has not shown the presence of the pathogen, while the pathogen was not detected in the xylem vessels, in all the treatments.

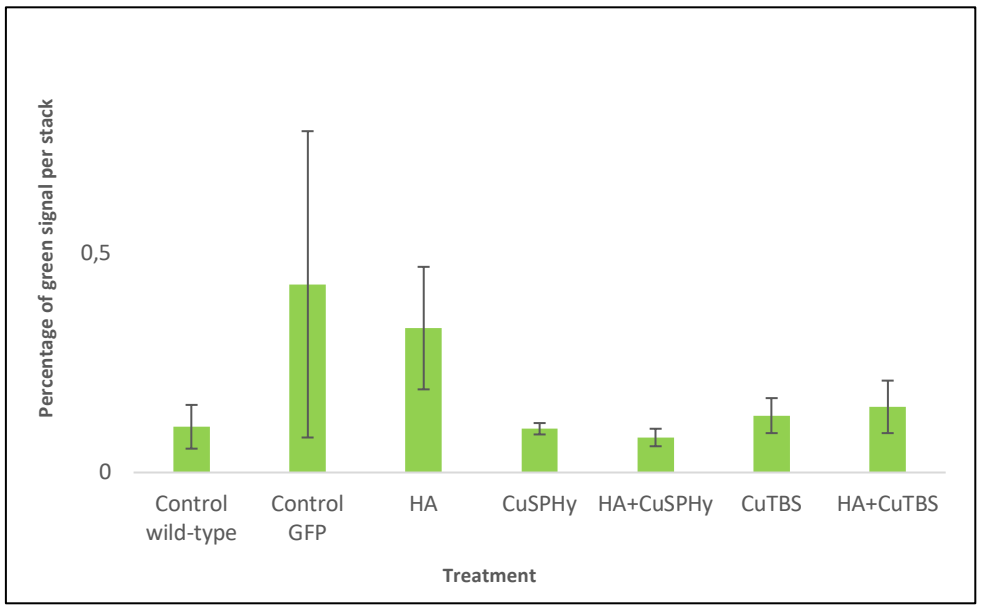
In the present investigation, the imaging methods applied seemed to reveal distinctly the fungal presence and colonization within and around the inoculation site in the rootstocks, treated with experimental substances and un treated.

Figure 4. Confocal Laser Scanning Microscopy images (2).



Concerning the efficacy of the treatments on controlling the fungal colonization, the area of *P. minimum::gfp7* colonization, was estimated by processing the data with the Image J software 1.47v. The graphic in Fig. 5 shows the green signal rate summarized per each treatment: the wild type is considered the negative control reference, confirming the positive role played by the copper treatments on controlling the pathogen colonization, in the vascular tissues and partially in the pith.

Figure 5. Presence of green signal per Z project of pictures from CSLM.



Further signs concerning potential correlation between the pathogen colonization showed by both the CLSM images and the copper distribution and persistence in the same tissues were revealed by the ICP-OES data (Fig. 6 and 7). The copper quantification is presented according to the plant tissues (pith, vascular tissues, bark), the detection time (post-treatment, post-harvest) and the treatments and results are expressed in ppb. The bark was considering just to appreciate the dynamic of the treatment in the plant surface, showed by a significant difference in function of the compound but also an unexpected persistence. In the vascular tissues and pith, the scale changes considerably: in the pith, there is a very low copper accumulation significant only for CuSPHy, explaining so the results reported by the CLSM images. In the vascular tissues, it was not possible to make a conclusion, because of an expected high copper content in the control maybe due to a contamination.

Figure 6. Copper quantification in the tissues of rootstock cuttings based on the logarithmic transformation of the data. The evidence is given on the abundance curves of the elements in the three different tissues (bark, wood and pith) arranged in columns, while the factors formulation and HA are arranged in line.

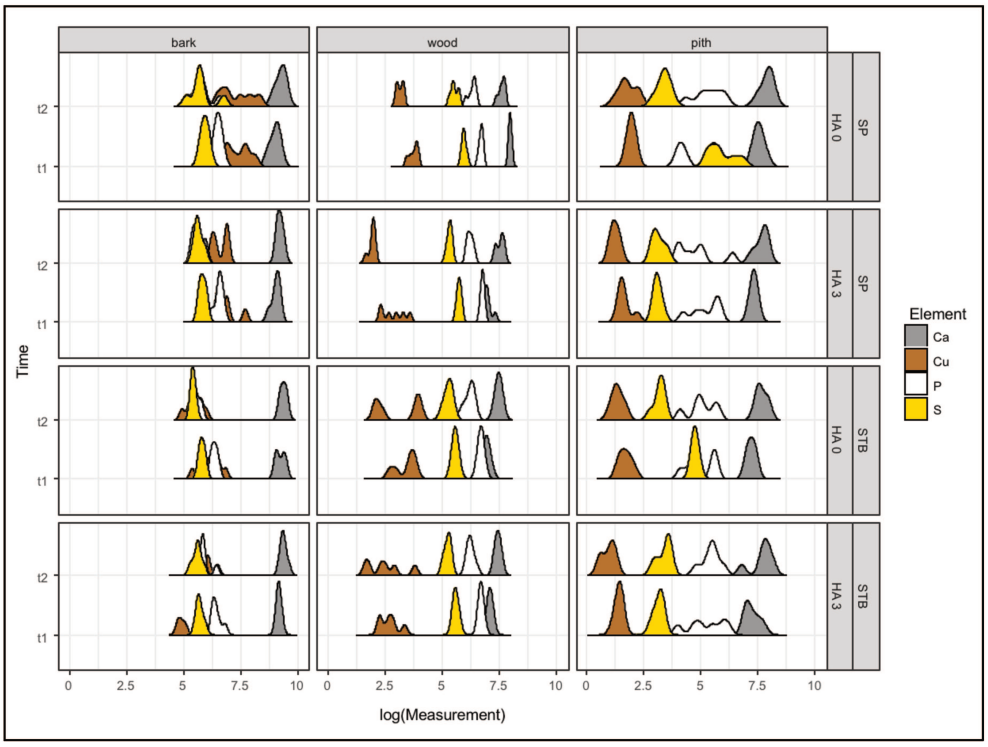
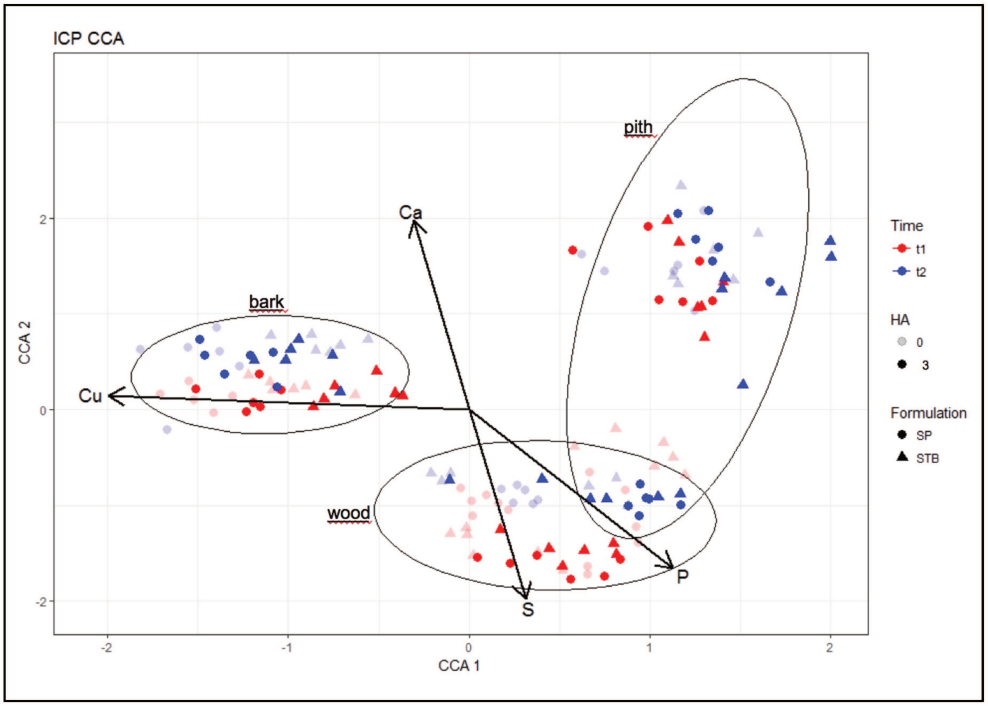


Figure 7. Copper quantification (mg kg^{-1}) in the tissues of rootstock cuttings based on the Constrained Correspondence Analysis (CCA). Formulations are represented in different geometric shapes, the factor time in different colors and the factor HA in different intensity of color. The main factor in grouping the samples is the type of tissue, represented by ellipses. A separate statistic was made for the element vectors.

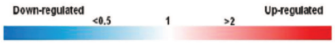


4.3. Relative gene expression

Analyzing the relative expression values at 8 h post treatment (Fig. 8), a different response was revealed depending on the copper compound, especially for the formulations based on HA. Despite the significant results reported by the pure CuSPHy, it was responsible of consistent phytotoxic effect already known in literature and related the high compound solubility and the very low pH. In fact, such compound has to be neutralized with calcium hydroxide to be applied then for crop protection with the common name of Bordeaux mixture.

Figure 8. Relative Gene Expression 8hpt.

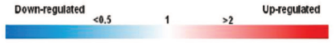
	CTRL Pmi	HA	HA+CuTBS	HA+CuSPHy	CuTBS	CuSPHy	CTRL NI
CHIT4C	1.82	0.83	0.72	1.42	0.33	2.83	1.36
GLU	3.81	1.02	6.98	2.31	1.18	6.81	1.40
IFRL4	2.92	0.88	0.82	0.64	0.76	0.37	1.06
LHCA3	0.42	0.81	0.62	0.57	0.84	0.30	1.17
LOX	1.90	0.82	0.89	0.50	0.95	1.24	1.05
PAL	2.86	0.93	2.77	1.35	0.86	6.11	1.06
PGIP	2.13	0.94	2.73	0.68	1.09	1.56	1.10
POX	0.48	1.07	0.74	1.17	0.96	1.17	0.76
PPO	1.56	1.28	0.68	0.44	0.59	1.18	1.07
PR1	1.96	0.65	2.50	0.95	0.68	1.59	1.17
PR10	1.01	0.53	0.46	3.90	0.75	18.73	1.38
PR6	2.20	3.80	2.13	10.26	0.95	15.29	1.53
PSBP1	0.60	0.71	0.72	0.65	0.79	0.37	1.20
STS	2.11	0.72	1.98	1.10	0.65	4.73	1.14



Regarding the relative expression values at 24 h post treatment (Fig. 9), a higher up regulation was reported for both copper(II) compounds. The pure CuSPHy was not satisfactory due to the high phytotoxicity detected, while the high values reported by the CuTBS indicate a high energetic cost for the plant metabolism. So, the results showed by the treatments based on the copper(II) compounds and HA, especially at 8 h post treatment, suggests further investigation considering different conditions, pathogens and methods of application.

Figure 9. Relative Gene Expression 24hpt.

	CTRL Pmi	HA	HA+CuTBS	HA+CuSPHy	CuTBS	CuSPHy	CTRL NI
CHIT4C	1.93	0.91	0.39	2.43	6.69	28.53	1.07
GLU	0.94	0.66	0.91	1.99	26.74	51.25	1.00
IFRL4	0.50	0.53	0.53	1.46	2.49	64.15	1.02
LHCA3	0.95	1.12	1.18	0.96	0.38	0.29	1.02
LOX	0.77	0.66	0.80	1.08	2.41	35.07	1.00
PAL	0.60	0.69	0.74	1.32	17.84	42.89	1.04
PGIP	0.66	0.70	0.81	1.12	2.54	48.21	1.01
POX	1.00	1.27	1.11	1.65	x	x	1.18
PPO	0.73	0.78	0.72	1.41	20.64	44.99	1.07
PR1	0.61	1.11	1.71	5.45	26.54	55.78	1.89
PR10	6.84	2.02	1.08	9.14	2.63	39.61	1.50
PR6	0.71	0.68	0.64	18.55	19.22	143.35	1.22
PSBP1	0.92	1.03	0.96	0.95	0.85	0.75	1.04
STS	0.61	0.82	0.80	2.00	21.39	50.35	1.14



5. Conclusions

The results of this study clearly show the relevance of studying the plant-pathogen interaction through imaging methods especially in the condition of an experimental nursery control on propagating material. Moreover, the efficiency of a control strategy should be also related to the distribution and persistence of the applied substance over time and in different tissues. In this sense, the present experience revealed how these parameters can be highly variable, with consequences on the pathogen colonization.

The parallel investigation based on the transcriptomic analysis, allowed to appreciate the potential role of innovative formulations based on HA and copper(II) compounds in stimulating the plant defense responses. Such treatments could be applied as biostimulant tool during the callusing process and/or the growth in the nursery field. The fungicide activity of copper could contribute to the control of GTD related pathogens and at the same time to ungal pathogens. Once again copper shows its potential role, especially for the disease management in organic agriculture, but the question still concerns the copper use in long term: on one side, the consistent reduction of the basis amount applied would be a first positive answer to the question but other side, the potential bio stimulation would represent an ambiguous energetic cost for the plants.

Conclusions and Perspectives

Plant protection is more and more oriented to encourage the development of sustainable and innovative low impact approaches. This is even more challenging in the control of still poorly understood diseases that can severely affect the crop. This is the case of the grapevine trunk diseases (GTDs) that are becoming a serious problem in viticulture.

In this context, the present research was developed as multidisciplinary study aimed to respond to crucial goals: the considerable reduction in the amount of the active substances usually employed in grapevine protection, the optimization of the biological activity of the same substances, even in controlling vascular pathogens related to complex diseases.

The research has led to several promising findings, which support the potential application of a specific hydroxyapatite (HA) in plant protection to enhance the efficiency of copper(II) compounds, in the perspective of an innovative and sustainable approach for the control of fungal diseases in *Vitis vinifera* L.

The HA functionalized with copper(II) compounds was fully characterized and the biological stability was established in performing applications to leaf tissues of *V. vinifera* for plant protection purposes. The morphology, structure and composition of HA functionalized with different copper(II) compounds and sprayed on grapevine leaves was detected and finally, a different and specific delivery model was developed for each copper(II) salt: the models are based on the high pH difference between the copper(II) compounds. Based on the results achieved, the micrometric aggregation and shape of HA was described in relation to its stability when formulated with Cu(II) ions. A particular affinity was highlighted for the formulations based on copper sulfate pentahydrate and copper tribasic sulfate. Electron microscopy on the substances and *in planta* revealed a different delivery model for copper sulfate pentahydrate and copper tribasic sulfate and the non-homologation of their aggregates to the nanostructures. This was confirmed also by the greenhouse test on *Plasmopara viticola*, in which the pure HA showed no toxic effect to the plant and to the pathogen, while the formulations based on copper sulfate pentahydrate and copper tribasic sulfate have shown a higher pathogen control, if compared to the pure copper compounds.

Based on this first approach, the biological activity of HA functionalized with copper(II) compounds was further studied *in vitro* and *in vivo*, on the control of grapevine fungal pathogens. Results collected evaluating the disease severity and incidence on treated potted vines, inoculated with *P. viticola*, showed the HA effect with a trend significantly different for each copper(II) compound. Considering these promising results, furthers trials were performed assessing the stability of the treatments to the rainfall washing-off effect, gaining positive

results on the efficiency and persistence of the experimental treatments in comparison with commercial fungicides.

The distribution and efficiency of HA functionalized with copper(II) compounds, in the grapevine lignified tissues colonized by vascular pathogens, was investigated by studying the plant-pathogen interaction through imaging methods in an experimental frame on propagating material. In this regard, the study revealed how these parameters can be highly variable, with consequences on the pathogen colonization. The collected data revealed a different concentration, distribution and persistence of copper on the treated plant material analyzed after the treatment and after harvest. Differences are related also to the copper compound applied, confirming the delivery models previously hypothesized.

A parallel investigation based on the transcriptomic analysis, allowed an appreciation of the potential role of innovative formulations based on HA and copper(II) compounds in stimulating the plant defense responses. Such treatments could be applied as a biostimulant tool during the callusing process and/or the growth in the nursery field. The fungicidal activity of copper could contribute to the control of GTDs related pathogens and at the same time to other fungal pathogens. Once again copper shows its potential role, especially for the disease management in organic agriculture, but the question still concerns the long-term use of copper: on one side, the consistent reduction of the base amount applied would be a first positive answer to the question but on the other side, the potential bio stimulation would represent an ambiguous energetic cost for the plants.

About the perspectives opened by the application of the HA for plant protection purposes, more extensive and significant considerations will be done based on the results collected from the parallel assays in vineyards and nursery, which are still in progress due to the necessity to observe several biological cycles.

The present study confirms the great potential of nanotechnological materials in agriculture, which are already playing a key role in the controlled release of agrochemicals. In particular, nanotechnology is innovating the formulation of fertilizers, pesticides, veterinary medicines, and developing smart materials able to enhance disease management and plant protection by the improvement of the effectiveness, bio availability of active substances, and their controlled release.

Despite the potential positive impact in various fields, several nanomaterials present properties that classify them as potentially hazardous. In particular, great attention is presently addressed to the hypothetical risks of phytoxic effects, to the human and environmental exposure to nanomaterial residues in crops and soil, including the possible bioaccumulation from the

environment to the food chain. For these reasons the application of nanostructured materials must be critically evaluated and regulated to guarantee safe use in agriculture. Due to the fast growth of synthetic nanomaterials and their presence on some markets, a legislative frame is being developed in Europe by using the findings of the current projects in toxicity testing, material characterization and environmental exposure.

Nevertheless, the HA applied in the present research was already widely assessed in the biomedical field and promoted thanks to the high biocompatibility with the natural calcium phosphates, excluding in this sense any potential risks related to human exposure. In the same way, no phytotoxic effects were reported by the study on the plant tissues treated with the pure HA. While further investigations are suggested for what concerns the stability of the HA nanostructured aggregates and the persistence of the biological substances released by them.

At the same time, the present study highlights once again the role played by copper(II) compounds in the management of grapevine fungal diseases, especially the control of downy mildew. Nowadays, copper remains a substance that is difficult to substitute for the control of *P. viticola* in organic vineyards and is still widely used in the so-called conventional plant protection, especially for post-flowering treatments. The European Union has included copper in the list of products "candidates for substitution" and its authorization expires in 2019. An extension, probably of 5 years, will be given, but the current proposal concerns its reduction from 6 to 4 kg per ha per year, which is creating a serious alarm between the European producers, especially those who operate in an organic regime.

In this perspective, the present study demonstrates the possibility to reduce the amount of copper applied, by enhancing its efficiency through the functionalization of HA nanostructured aggregates with copper(II) ions. It is a crucial goal on disease management that is based on a better distribution, a higher persistence and a smart delivery of the biological substance on the treated plant tissues.

The same insights suggest to perform further studies for the control of complex diseases, such as the Esca disease complex, in order to develop a curative long-term effective treatment to control the disease with a minimum amount of fungicide. This will include completing the work on the defense response started in the vine by foliar applications, which will complete the picture on the potential activity of this product, and a final evaluation on its potential in the field in reducing Esca complex symptoms.

In conclusion, combining the results of the physical, chemical, microscopical, biological and molecular approaches, the research investigated a potential alternative way to enhance the protection of grapevine against two important fungal diseases.

Résumé substantiel en Française

1. Introduction générale

La vigne (*Vitis vinifera* L.) est l'une des cultures les plus importantes au monde, en ce qui concerne la production de vin et de raisin de table [1]. Malheureusement, la plupart des cépages utilisés pour la vinification sont très sensibles à plusieurs agents pathogènes [2]. Au cours du siècle dernier, la gestion chimique des maladies était la mesure la plus efficace utilisée dans le monde pour contrôler les agents pathogènes, en particulier dans la viticulture moderne. Considérant que les infections fongiques sont principalement responsables des dégâts à la qualité du raisin et des pertes de rendement, les pesticides les plus utilisés en viticulture sont les fongicides [9].

Depuis leur dissémination dans le continent américain au 19ème siècle, *Plasmopara viticola* (agent du mildiou) et *Erysiphe necator* (agent de l'oïdium) sont les maladies fongiques les plus importantes de la vigne. Avec *Botrytis cinerea* (agent de la pourriture grise), ces trois maladies sont responsables du plus grand nombre de traitements dans les vignobles [3].

Cette protection de la vigne est d'autant plus difficile dans la lutte contre des maladies encore très peu connues et responsables de pertes de production, de la mort des vignes et d'un déclin général de la vigne. Parmi ces maladies qui affecte gravement la productivité du vignoble à travers le monde, nous pouvons citer les Maladies du Bois de la vigne (MDB). Elles deviennent un problème sérieux en viticulture, tant en Italie qu'en France et dans le monde. Les MDB sont connues et bien décrites dans la littérature pour les cultures arboricoles. Leur propagation actuelle et l'augmentation de leur sévérité dans le monde [37, 38] sont peut-être la conséquence des changements climatiques et indubitablement liées aux pratiques modernes de la viticulture intensive [39].

Les MDB affectent la souche et les cordons de la vigne et apparaissent comme un complexe de plusieurs maladies causées par des agents pathogènes associés à un symptôme spécifique, comme les Botryosphárioses causées par *Botryosphaeriaceae* ou le dépérissement d'Eutypa causé par les pathogènes fongiques *Diatrypaceae* et le pied noir, maladie causée par les espèces *Dactylonectria* et *Ilyonectria*. Parmi les MDB, la forme la plus fréquente et la plus dommageable en Europe est le complexe de l'esca associé à des agents pathogènes fongiques vasculaires, aux agents responsables de chancres et de dépérissement ainsi qu'à leurs interactions. Vu cette complexité, un aspect crucial pour le développement des MDB est l'interaction entre ces différents pathogènes et l'état physiologique de la plante [40].

De nos jours, la protection de la vigne est de plus en plus orientée pour encourager le développement d'approches durables et innovantes à faible impact pour contrôler en

particulier les pathogènes fongiques. Les problèmes liés à l'impact environnemental et à la toxicologie des anciens fongicides chimiques de synthèse et à la demande croissante de produits exempt de tout résidu ont encouragé la recherche de nouveaux outils pour la protection des cultures. Les entreprises agrochimiques développent de nouvelles formulations à base de substances bioactives ayant un impact moindre sur la santé humaine et sur l'environnement, et un nouveau mécanisme d'action pour réduire l'augmentation alarmante des souches fongiques résistantes aux fongicides. De plus, la recherche d'alternatives aux fongicides organiques synthétiques est de plus en plus orientée vers l'étude et l'application de principes actifs à base de microorganismes et de plantes [3].

Les connaissances sur l'activité biologique de nombreuses molécules naturelles ont considérablement augmenté au cours des dernières décennies et les recherches sur l'application de ces substances à des fins de protection des plantes sont en augmentation. Les mélanges de substances naturelles provenant de diverses parties des plantes, principalement des graines, mais aussi des fruits et différents tissus, sont communément reconnus comme stimulateurs de défense ou éliciteurs, parmi lesquels les huiles végétales essentielles sont fortement étudiées. Plusieurs aspects critiques limitent toujours l'application des Stimulateur de Défense Naturelle (SDN) les stratégies de protection des plantes à grande échelle, d'autres études et des formulations innovantes sont donc nécessaires pour améliorer l'efficacité du contrôle des pathogènes et réduire le coût élevé des produits commerciaux.

Dans ce contexte, le cuivre est l'élément le plus ancien et le plus commun utilisé pour lutter contre le mildiou dans la viticulture. Outre son effet direct sur l'agent pathogène, le niveau accru d'indicateurs de défense des plantes tels que les peroxydases, les phénols, le resvératrol et les anthocyanes a été démontré sur des plants de vigne traités avec des composés à base de cuivre (II). De nos jours, le cuivre reste le fongicide le plus efficace en viticulture biologique puisque c'est un composé non synthétique avec un large spectre d'activité. D'autre part, son utilisation à long terme est responsable d'une accumulation de cuivre dans les écosystèmes agricoles qui cause de graves problèmes environnementaux. En conséquence, la réglementation européenne limite l'utilisation du cuivre à un maximum de 6 kg Cu ha^{-1} par an ou un maximum de 30 kg Cu ha^{-1} par 5 ans [24].

En ce qui concerne le contrôle durable des MDB, diverses activités de recherche ont été promues au niveau international, permettant une meilleure compréhension de ces maladies, de leur étiologie, de leur biologie et de leur épidémiologie, mais de nombreuses questions restent ouvertes sur la gestion des maladies [43]. Ceci est lié à plusieurs aspects critiques, comme une compréhension encore insuffisante du rôle des agents impliqués, ainsi que des

mécanismes aboutissant à l'expression des symptômes foliaires, en particulier dans le cas de l'expression de la forme lente de l'esca (GLDS, Grapevine Leaf Stripe Disease).

Un premier aspect critique est lié à l'absence de produits à faible toxicité mais de bonne efficacité pour le contrôle de ces maladies. L'arsénite de sodium était considéré comme le seul agent chimique efficace sur GLSD ("esca"), agissant notamment par son accumulation dans les tissus traités [44]. Quoi qu'il en soit, en raison de sa forte toxicité, l'arsénite de sodium n'est plus autorisé en viticulture [45]. Une réduction prometteuse - quoique faible - de l'incidence de cette maladie a été récemment obtenue dans des essais sur le terrain avec des préparations de cuivre qui pourraient pénétrer partiellement dans le bois [46], altérant probablement la croissance et le métabolisme des pathogènes fongiques impliqués. Cette découverte a ouvert un nouveau champ de recherche sur l'application des produits de pénétration du bois, plutôt que systémique. Jusqu'à présent, le fosétyl-aluminium, un fongicide utilisé pour contrôler *P. viticola*, est le seul produit systémique à avoir réduit efficacement l'incidence du GLSD; son activité est apparue liée à sa capacité à activer les réponses de défense de la vigne [47].

Un deuxième aspect critique est lié à la protection inefficace des plaies de taille, un point majeur de pénétration puis de colonisation de la vigne pour tous les agents fongiques des MDB. Les produits disponibles dans le commerce étaient efficaces *in vitro* [48], mais ils n'étaient pas en mesure d'assurer une protection persistante par rapport à la sensibilité prolongée des plaies de taille [49]. Actuellement, l'application d'agents de lutte biologique appartenant principalement à l'espèce *Trichoderma* spp a été étudiée. Les résultats obtenus après plusieurs années d'expériences ont révélé des effets positifs sur l'expression des symptômes du GLSD. L'agent de lutte biologique colonise la plaie de taille, ce qui permet de limiter l'infection par des agents associés aux MDB et une interaction persistent avec la plante [50].

Un troisième aspect critique, après les difficultés de contrôler ces MDB, et en particulier le GLSD, est que les pathogènes sont localisés dans les tissus ligneux. Les causes de l'expression des symptômes dans les feuilles sont probablement liées à des réactions de défense activées dans les feuilles en lien avec la production de toxines fongiques dans le tronc et transportées par le tissu vasculaire, sous l'influence d'autres - pas bien déterminés - cofacteurs environnementaux. De ce point de vue, certains résultats positifs ont été obtenus avec des applications des engrais foliaires, dont l'efficacité pourrait être fortement améliorée par une meilleure distribution et pénétration du produit [51]. Ainsi, un groupe de recherche de l'Université de Poitiers (France) a récemment étudié deux types de composés ayant des modes d'action différents et complémentaires. Une première étude a été réalisée en utilisant des molécules existantes modifiées pour obtenir des produits capables de se déplacer (molécules

ambimobiles), après une application foliaire, dans le tronc de la vigne où les agents pathogènes sont localisés. Une deuxième étude a associé ces substances mobiles à des microorganismes bénéfiques capables de stimuler les défenses de la plante. [52].

Le contrôle des MDB représente un grand défi pour les viticulteurs, les pépiniéristes, les techniciens et les scientifiques principalement en raison de leur complexité par rapport aux autres maladies de la vigne comme le mildiou et l'oïdium, par exemple. Le contrôle des MDB est un exemple clair de la nécessité d'une approche de gestion intégrée. En effet, les risques d'infection élevés dans les pépinières et les vignobles ainsi que l'absence de traitements curatifs ont renforcé l'idée d'une stratégie transversale de contrôle des MDB dans le secteur vitivinicole [3, 53].

Malgré le manque d'outils ou des protocoles développés avec une efficacité spécifique contre les agents pathogènes des MDB, la gestion méticuleuse des plantes mères est la première étape pour prévenir les infections dans les matériels de multiplication de la vigne au stade de la pépinière. Les pratiques les plus efficaces en pépinière sont l'application de bonnes mesures d'hygiène et la prévention de la contamination des plants à chaque fois que le matériel de multiplication est blessé ou coupé. La reconnaissance de l'importance critique de ces deux facteurs soulève la question d'une évolution possible de l'ensemble du processus de production en pépinière [54, 55, 56].

Les outils de contrôle durable, déjà développés pour les applications en pépinière, incluent principalement l'utilisation d'agents de biocontrôle, en particulier de plusieurs espèces de *Trichoderma*, qui se sont avérées efficaces sur plusieurs des principaux pathogènes liés aux MDB [57, 58].

Le traitement à l'eau chaude, de mieux en mieux caractérisé, est fréquemment utilisé pour obtenir des plants commerciaux dans de bonnes conditions sanitaires. Un tel traitement sanitaire est généralement effectué à 50°C pendant 30 min, mais il est stressant pour la plante [56] et des résultats encore variables en termes d'efficacité et de qualité de la plante ont été rapportés [36]. Néanmoins, d'autres expérimentations sont nécessaires pour valider l'applicabilité et l'efficacité des outils de contrôle innovants et à faible impact tels qu'une gamme de produits de désinfection [54] et l'utilisation d'eau ozonée [58].

Parmi les approches visant à réduire les symptômes liés aux MDB dans le vignoble, les traitements foliaires à base de minéraux et d'extraits d'algues [59] ou d'inducteurs de défense comme le fosétyl-Al [44] ont montré une efficacité sur les facteurs nutritionnels et de défense associée à l'esca (GLSD). Cependant, de telles applications devraient être approfondies pour

mieux vérifier l'efficacité à long terme sur la réduction de la mortalité et la constance productive dans les vignobles touchés par les MDB.

De nouveaux outils curatifs potentiels sont plus orientés sur le contrôle de l'activité des pathogènes du tronc colonisant le bois et leurs facteurs de virulence. Récemment, des formulations à base de cuivre et de co-formulants ont été proposées pour être appliquées directement sur le bois afin de simuler le même mécanisme probablement activé par pulvérisation hivernale de l'arsénite cancérigène de sodium, qui n'est plus applicable [46]. Sur les plantes touchées par des symptômes sévères, il est également suggéré d'appliquer des mesures de chirurgie du tronc, consistant en le renouvellement du tronc, le regreffage et le curetage, qui ont conduit à l'élimination significative du bois affecté. C'est une technique invasive mais fréquemment utilisée pour prolonger la productivité du vignoble [60, 61].

Une approche préventive dans le vignoble est également fortement recommandée. Ainsi il est conseillé d'enlever et brûler les branches, les vignes mortes / mourantes, les résidus de taille, des pratiques essentielles pour limiter la propagation de l'inoculum des pathogènes MDB. Il est également important de limiter et de protéger les plaies de taille et d'éliminer la partie morte du tronc ou des bras voire, si nécessaire, de remplacer complètement la plante symptomatique [62]. De plus, des pratiques de gestion des maladies basées sur l'amélioration des sols et la résistance des plantes à l'infection devraient être étudiées afin de maintenir la vigne dans son meilleur état, prête à réagir à l'infection et à limiter le développement des symptômes [63].

Cependant, la protection des plaies de taille (basée principalement sur l'application des formulations à base de souches de *Trichoderma* spp.) contre l'infection apparaît actuellement comme la mesure préventive la plus prometteuse pour contrôler l'expression des MDB [29, 57]. D'autres essais sont en cours avec des produits de protection des plaies basés sur la mise en place de barrières physiques, ce qui peut représenter des outils innovants pour l'avenir [64].

La stratégie de lutte intégrée contre les MDB devrait prendre en compte tous les facteurs potentiellement responsables des dysfonctionnements du transport de l'eau et de la sève, ainsi que la résilience des plantes, y compris la taille, la nutrition, la protection et la gestion des sols. Par exemple, les deux premières méthodes peuvent créer des niches d'infection pour les pathogènes, mais aussi perturber le flux et l'équilibre de l'eau dans la vigne [65]. Il a été observé qu'une carence en eau a un effet primaire mais non exclusif sur les facteurs de virulence des pathogènes (phytotoxines), conduisant également à une réduction de l'activité photosynthétique [40] et dans les conditions les plus extrêmes, au dépérissement jusqu'à la mort de la vigne.

Dans la perspective de contrôler les MDB, une vision holistique du problème pourrait être la clé pour définir des stratégies gagnantes pour ce contrôle, dont le modèle pourrait également être appliqué au contrôle d'autres maladies complexes des plantes [53]. Dans ce contexte, le développement des nouveaux outils de détection et de suivi de l'état sanitaire de la vigne constitue un véritable défi, notamment dans les vignobles fortement touchés par les MDB.

Parmi les MDB, l'une des plus répandus dans les vignobles européens est l'esca (GLDS) [66], dont les symptômes incluent une nécrose internervaire foliaire typique, donnant aux feuilles une apparence typique de rayures tigrées qui peut ne pas apparaître à chaque phase de croissance. Cette discontinuité des symptômes foliaires sur chaque vigne rend presque impossible la détermination de l'incidence réelle de l''esca au vignoble à un moment donné, car de nombreuses vignes infectées peuvent ne pas présenter de symptômes chaque année [67]. Plusieurs auteurs ont clairement démontré une altération non négligeable des fonctions photosynthétiques ainsi qu'une stimulation des réponses de défense chez les vignes atteintes plusieurs jours avant l'apparition des premiers symptômes visibles sur les feuilles [68, 69].

Di Gennaro *et al.* (2016) ont proposé une approche innovatrice pour la surveillance de l'esca (GLSD), fondée sur la corrélation entre les images de l'indice de végétation par différence normalisée (NDVI - Normalized Difference Vegetation Index) acquises par une plate-forme UAV (Unmanned Aerial Vehicle) et le comptage des symptômes par observation au vignoble. La méthodologie innovante visait principalement à effectuer une analyse quantitative et qualitative de la propagation des symptômes, puis à réaliser un modèle prédictif de l'apparition des symptômes de l'esca. Cette étude intéressante a montré de nouvelles possibilités d'utilisation des technologies de télédétection pour la viticulture de précision. Les caractéristiques des UAV, telles que le faible coût, la capacité de donner en temps opportun des images à haute résolution, fournissent une approche qui peut être facilement appliquée pour la planification des stratégies d'enquête et de lutte contre les maladies dans les vignobles. Ces méthodes sont susceptibles d'être utiles, à la fois pour la recherche et pour des applications pratiques en viticulture.

La détection précoce pourrait également aider dans le choix des itinéraires de traitements sur le terrain économiquement rentables, ou l'application de méthodes de lutte spécifiques sur des vignes ciblées avant qu'elles ne montrent les symptômes. Les applications d'un tel outil prédictif pourraient ouvrir de nouvelles perspectives dans la connaissance et le contrôle de ces MDB, dans le cadre du développement croissant des approches de l'agriculture de précision en viticulture [70].

La présente recherche découle des perspectives ouvertes par des applications précédentes de cristaux inorganiques basés sur une forme spécifique du phosphate de calcium biogénique, l'hydroxyapatite (HA), qui a été largement étudiée et appliquée dans de nombreux domaines [71, 72, 73].

Sur la base de ces applications HA effectuées dans le domaine biomédical, ce matériau performant peut potentiellement agir comme un système de transporteur de substances actives, y compris des ions métalliques, tels que le cuivre(II), et des composés organiques. Une des principales investigations a été réalisée en médecine, pour remplacer la chimiothérapie dans le traitement du cancer des os, grâce à l'efficacité de HA avec une molécule anticancéreuse [74, 75]. Une seconde application efficace a été le développement d'un dentifrice à base du même matériau, devenu efficace avec des ions zinc, pour remplacer le fluor, le SLS, le dioxyde de titane et les parabènes [76].

Dans ce contexte, ce travail vise à évaluer, à travers une approche multidisciplinaire, le développement et l'application de HA pour améliorer l'activité biologique de substances actives pour contrôler des maladies fongiques chez *V. vinifera*.

Ce but répond à des objectifs cruciaux : (i) réduire significativement la quantité de fongicides chimiques habituellement utilisés pour la protection de la vigne, (ii) optimiser la distribution, la pénétration et la persistance de la substance active dans les tissus végétaux, (iii) utiliser cette technologie pour le contrôle des agents pathogènes dont ceux impliqués dans les maladies vasculaires et complexes.

Les activités expérimentales et les résultats associés sont présentés et discutés en fonction des actions suivantes: (i) caractérisation et stabilité de l'HA fonctionnalisée avec des composés de cuivre (II) appliqués sur *V. vinifera* à des fins de protection contre des maladies fongiques ; (ii) évaluation *in vitro* et *in vivo* de l'activité biologique de l'HA fonctionnalisée avec des composés de cuivre(II) sur le contrôle des pathogènes fongiques de la vigne ; (iii) évaluation de la distribution et de l'efficacité de l'HA fonctionnalisée avec deux composés de cuivre(II) dans des tissus ligneux de la vigne colonisés par un pathogène vasculaire et caractérisation des réponses de défense activées par la plante.

2. Résumés des chapitres

2.1. Chapitre 1 – Caractérisation et stabilité de l'hydroxyapatite fonctionnalisée par des composés de cuivre(II), appliquée aux tissus foliaires de *Vitis vinifera* L. pour la protection de la plante.

Article scientifique publié dans Pest Management Science

Battiston E., Salvatici M.C., Lavacchi A., Gatti A., Di Marco S. and Mugnai L. (2018). Functionalisation of a nano-structured hydroxyapatite with copper(II) compounds as pesticide: *in situ* TEM and ESEM observations of treated *Vitis vinifera* L. leaves.

Au fil des années, la recherche en nanotechnologie a conduit au développement de matériaux intelligents révolutionnaires. Dans l'agriculture, l'approche nanotechnologique est souvent appliquée pour améliorer la productivité des plantes, la qualité des cultures et la gestion des maladies, à travers des matériaux nanométriques. La mise au point de systèmes à libération lente pour les pesticides ouvre de nouvelles perspectives pour réduire la quantité de substances actives appliquée, permettant une protection efficace des plantes et une lutte contre des maladies.

En considérant la phytotoxicité possible de ces matériaux, e cas d'étude a concerné une hydroxyapatite synthétique (HA) biomimétique, déjà étudiée de manière extensive en raison de sa bioactivité et de sa biocompatibilité unique.

Le but de la présente recherche était d'étudier un système de libération de médicament biocompatible applicable à la protection des plantes pour améliorer la distribution et potentiellement l'activité antimicrobienne des substances bioactives courantes. A cette fin, HA a déjà été développé pour des applications dans l'agriculture et le cuivre(II), largement appliqué pour protéger la vigne (*Vitis vinifera* L.) contre le pathogène fongique *Plasmopara viticola*, ont été choisis comme cas d'étude.

P. viticola est certainement responsable de l'une des maladies les plus nocives, causant le plus grand nombre de traitements dans les vignobles [3]. Depuis la découverte de l'activité antifongique du cuivre, les fongicides cupriques ont été largement utilisés pour lutter contre ce pathogène et aujourd'hui encore, le cuivre est le plus efficace. Cependant, parmi les effets secondaires liés à l'utilisation du cuivre, l'accumulation importante dans les sols de ce métal est responsable de graves problèmes environnementaux [27], résultant de la mise en œuvre des lois qui limitent son utilisation. Pour cette raison, l'efficacité des doses réduites de cuivre, réalisable en optimisant son activité biologique et sa persistance dans les planta, est prise en considération, afin d'adapter son utilisation aux restrictions législative [24, 28].

L'HA utilisée a été synthétisée par une méthode spécifique qui permet l'agrégation des particules nanostructurées en agrégats micrométriques, réduisant ainsi la présence de nanoparticules uniques [105].

2.1.1 Fonctionnalisation et caractérisation de l'HA avec le Cu(II)

Selon les applications précédentes concernant la fonctionnalisation de l'HA avec des ions métalliques, quatre composés différents à base de cuivre(II), tels que le sulfate de cuivre pentahydraté (CuSPHy), le sulfate de cuivre tribasique (CuTBS), l'oxychlorure de cuivre (CuOxCl) et l'hydroxyde de cuivre (CuHyOx), ont été choisis. L'étude était axée sur l'agrégation des particules de HA dans les plantes, en termes de forme et de dimension, afin de vérifier l'agrégation micrométrique de l'HA.

Par microscopie électronique, le même aspect a été vérifié sur les formulations obtenues en fonctionnalisant l'HA avec des ions cuivre(II) et sur des feuilles de *Vitis vinifera* infectées par le pathogène fongique, *P. viticola*, et traitées par pulvérisation avec les mêmes formulations sur les surfaces abaxial et les surfaces.

Les résultats montrent que le pH de chaque composé de cuivre(II) influence le pH final des formulations avec HA. Comme décrit dans la littérature, le pH joue un rôle important dans la croissance des nanocristaux HA, qui augmentent à partir d'un pH supérieur à 4,00.[18,38] L'HA pure appliquée dans cette étude, a montré un pH de 12,78. Ce paramètre a subi une variation après formulation de l'HA avec chaque sel cuivre(II) (Table 2).

En considérant CuSPHy, le seul composé soluble de cuivre(II), la variation du pH était particulièrement forte et après formulation avec l'HA, le pH était de 2,24. Sur la base de ces données, il était prévu de détecter une dissolution partielle ou totale des particules de HA dans une telle formulation. Les composés de cuivre(II) insolubles ont eu un impact moindre sur le pH final des formulations avec HA et les valeurs étaient approximativement de 11,00. Dans ces formulations, il était attendu d'observer une structuration plus stable des particules à base de HA.

Outre la stabilité et la croissance des particules d'HA, le pH a évidemment un impact supplémentaire sur l'échange d'ions Cu(II) avec HA. En tant qu'échangeur fort d'ions, Ca(II) à la surface de l'HA peut échanger des métaux lourds divalents [108, 109]. Dans une étude récente, réalisée sur un composite à base de l'HA partiellement substituée en magnésium, on a observé que l'adsorption dépend fortement du pH de la solution et elle est augmentée avec l'augmentation du pH. En particulier, l'adsorption de Cu(II) a clairement augmenté de 19,4% à 97,6% lorsque le pH de la solution a été augmenté de 1,0 à 5,9 puis légèrement diminué à

95,8% pour un pH de 6,9. Ce phénomène était associé aux réactions possibles de l'échange Cu(II) de la solution [110]. Cette observation suggère une étude physico-chimique des mécanismes et cinétiques potentiels de l'échange d'ions Cu(II) entre HA et les sels cuivre(II).

Les résultats de XRD (X-Ray Difraction) confortent les observations ci-dessus (Fig. 1). Le diagramme de diffraction typique a été détecté pour l'HA pure. Malgré la concentration excessive des composés de cuivre(II), qui dans la majorité des formulations couvre le diagramme de diffraction HA, il était possible de discriminer la présence ou non des particules de HA dans les échantillons analysés. En ce qui concerne le pH, les diagrammes DRX des formulations diffèrent en fonction de l'activité des ions hydrogène des composés de cuivre(II) purs.

Concernant les dimensions des particules étudiées, les résultats de DLS (Dynamic Laser Scattering) démontrent la configuration intéressante dans les micro-agrégats de l'HA nanostructurée appliquée (Fig. 2). Malgré l'absence dans la littérature de caractérisation physique et chimique des composés de cuivre(II) appliqués en agriculture, les résultats DLS peuvent donner une indication réelle sur la taille moyenne des particules à base de Cu (II) utilisées comme pesticides entre 1 et 100 µm. En excluant le résultat de l'échantillon HA-CuSPHy, qui est probablement lié à des particules principalement à base de CaSO4, une distribution de taille intéressante est décrite par les échantillons HA-CuTBS et HA-CuHyOX, pour lesquels le pourcentage élevé de particules entre 0,5 et 1 µm peut représenter une condition positive pour optimiser l'application et la distribution dans la plante. En fait, la dimension des particules a potentiellement un impact direct sur la taille des gouttelettes générées lors de l'application du pesticide par pulvérisation, ce qui représente un facteur crucial pour l'efficacité d'un traitement [112].

Dans la présente étude, les images TEM (Transmission Electron Microscopy) étaient fondamentales pour comprendre l'agrégation des particules plutôt que leur taille, en combinaison avec la composition élémentaire de la particule spectromètre à rayons X dispersif (EDX), en considérant que l'incertitude de mesure d'EDX est généralement de 20% [114].

En plus de l'agrégation bien connue de l'HA dans des micro-agrégats composés de nanocristaux de taille 100 nm [115], les observations TEM ont révélé deux types principaux de forme sur les particules étudiées (Fig. 3). Les données XRD et DLS associées à l'échantillon HA-CuSPHy ont été confirmées par la détection de particules ressemblant à des aiguilles qui diffèrent partiellement de la forme typique de la particule HA et totalement de la forme des autres particules à base de Cu(II).

L'analyse élémentaire a également révélé une très faible présence de calcium dans la particule détectée sur l'échantillon HA-CuSPHy, ce qui valide les conclusions des résultats par XRD.
Par conséquent, en dépit de la forme et de la dimension de ces particules, des agrégats homogènes, plus similaires aux agrégats typiques du HA pur, ont été observés dans l'échantillon à base de HA-CuTBS. Dans ce cas, l'analyse élémentaire des agrégats a indiqué la présence de Cu(II) associé à des particules à base de calcium et de phosphore (HA).
En particulier, en comparant les deux échantillons (Fig. 4, 5), un effet de distribution plus élevé est attendu par les particules de l'échantillon HA-CuSPHy et il est lié à la forme d'aiguille des agrégats, signifiant également une surface spécifique élevée capable de libérer des ions potentiellement Cu(II). D'autre part, la densité plus élevée des particules dans les agrégats de l'échantillon HA-CuTBS peut représenter un aspect crucial sur la stabilité et la persistance du traitement dans la plante.

2.1.2 Application et caractérisation *in planta*

Après les traitements sur les feuilles, un effet phytotoxique a été détecté à la fois sur les surfaces des feuilles abaxiales et adaxiales traitées avec le produit à base de CuSPHy. Cet effet a été décrit dans la littérature mais ne concerne pas les produits phytopharmaceutiques car pour qu'il soit autorisé en tant que fongicide en agriculture (avec le nom commun de bouillie bordelaise) CuSPHy doit être neutralisé par l'hydroxyde de calcium [116, 117].
Dans notre étude, la bouillie bordelaise n'a pas été testée car ce n'est pas un composé de cuivre(II) pur et son échange de potentiel dans les ions Cu(II) est conditionné par le processus de neutralisation. Considérant que le traitement à base de HA-CuSPHy n'a pas produit d'effet phytotoxique sur les feuilles traitées, il semble avoir un effet similaire à la neutralisation par l'hydroxyde de calcium sur CuSPHy. Cette possibilité est renforcée par les résultats de XRD, qui montrent les différences entre les diagrammes de diffraction de l'échantillon CuSPHy et la formulation HA-CuSPHy. Bien que le pH des deux échantillons appliqués était assez similaire, la non-toxicité du traitement à base de HA-CuSPHy était probablement liée au réarrangement et à l'interaction des ions Cu(II) avec les particules détectées à base de phosphore.
ESEM (Environmental Scanning Electron Microscope) était une technique utile pour étudier l'agrégation des particules dans les plantes, en particulier pour évaluer l'efficacité des traitements dans la protection de la plante contre les pathogènes (Fig. 7, 8). À cet égard, l'application de HA est basée spécifiquement sur sa propriété de générer des revêtements biologiquement actifs nanostructurés.[18] L'échantillon de feuille traité avec HA montre de telles

particules agrégées sur la surface de la feuille. La configuration la plus similaire a été observée sur des feuilles traitées avec HA-CuSPHy, confirmant les résultats au TEM.

Un commentaire supplémentaire doit être ajouté sur le traitement basé sur HA seul, qui n'a montré aucun effet toxique sur la plante ou sur le pathogène. La toxicité potentielle de l'AH n'est pas clairement décrite dans la littérature, et elle a fait l'objet de nombreuses études et applications pendant plusieurs années dans le domaine médical pour les dispositifs de classe I, II et IIa.

Néanmoins, un résultat prometteur sur le contrôle pathogène de la vigne a été révélé par l'échantillon HA-CuSPHy et aussi par le HA-CuTBS, ce qui est confirmé par les observations précédentes sur l'agrégation, la stabilité et la distribution des particules (Fig. 9). Cette observation, bien que n'étant pas spécifiquement l'objet de cete étude, suggère la nécessité d'une étude plus approfondie des propriétés de l'HA nanostructuré pour améliorer la distribution, la persistance et la libération optimale des ions Cu(II) dans les plantes. Ceci est encore plus remarquable pour la réduction potentielle de la quantité de substances actives qui doivent être appliquées dans la protection des plantes, ce qui est l'un des principaux objectifs de la gestion durable des maladies. A cet égard, l'efficacité du dosage réduit du cuivre contre *P. viticola* a été récemment étudiée sur des formulations commerciales appliquées en viticulture biologique.

En conclusion, le présent travail indique la possibilité d'améliorer l'activité biologique d'une substance bioactive en modifiant sa structure à travers une formulation spécifique et réalisable avec un matériau biocompatible.

2.2. Chapitre 2 – Évaluation *in vitro* et *in vivo* de l'activité biologique de l'hydroxyapatite fonctionnalisée par des composés de cuivre(II) pour le contrôle des pathogènes fongiques de la vigne.

Article scientifique soumis à Phytopathology

Battiston E., Antonielli L., Di Marco S., Fontaine F. and Mugnai L. (2018). Innovative delivery of copper(II) ions by a nano-structured hydroxyapatite: potential application *in planta* to enhance the sustainable control of *Plasmopara viticola*.

La vigne (*Vitis vinifera* L.) est l'une des cultures fruitières utilisant le plus de fongicides pour contrôler des maladies*. Plasmopara viticola* est probablement la maladie la plus grave de la vigne capable de causer des pertes de rendement constantes. Au fil des années, plusieurs ingrédients actifs ont été développés pour contrôler les dommages potentiels de cette maladie. L'utilisation systématique de fongicides organiques a provoqué l'émergence de souches résistantes de *P. viticola*. Seul un régime de traitements à base de cuivre permet d'éviter cela, grâce au mode d'action non spécifique des fongicides contenant du cuivre. En viticulture biologique, la protection contre le mildiou est essentiellement assurée par l'utilisation de fongicides cupriques, dont l'utilisation en agriculture devrait être davantage restreinte par les pays européens, en raison de son profil écotoxicologique et phytotoxicologique critique. La recherche sur les formes innovantes de cuivre pour lutter contre le mildiou est apparue comme l'approche la plus prometteuse, ainsi que l'optimisation de la distribution et de la persistance des pesticides à base de cuivre.

La présente recherche étudie les propriétés de transporteurs (« drug delivery ») de l'hydroxyapatite synthétique (HA) biomimétique pour améliorer l'activité biologique des ions Cu(II). A cet effet, quatre composés du cuivre(II) (CuSPHy, CuTBS, CuHyOx et CuOxCl) ont été formulés avec le composant HA innovant et appliqués dans un essai préliminaire antifongique *in vitro* contre *Botrytis cinerea*, un autre pathogène commun de la vigne. Dans un second temps, des essais d'efficacité *in planta* contre *P. viticola* en serre ont été réalisés.

La recherche a mis en évidence les propriétés de transporteur de l'hydroxyapatite synthétique biomimétique (HA) qui a été étudié pour améliorer l'activité biologique des ions Cu(II). Des résultats clairement prometteurs ont été montrés en appliquant l'HA à des essais d'efficacité sur la vigne contre *P. viticola* en serre, en particulier avec le cuivre soluble (CuSPHy), réduisant à la fois la sévérité et l'incidence de la maladie dans toutes les conditions expérimentales. Le traitement a également montré une efficacité et une persistance élevées sous l'effet de la pluie. Cependant, des résultats significatifs et remarquables sur l'activité antifongique des substances étudiées ont été révélés par le test *in vitro* préliminaire.

Étant sensible aux traitements à large spectre basés sur le cuivre [139], *Botrytis cinerea* a également été étudié sur la base d'essais en laboratoire antérieurs réalisés pour évaluer l'inhibition de la croissance mycélienne par des particules antimicrobiennes innovantes [140]. En général, il a été souligné un degré différent d'inhibition par chaque composé Cu(II), en fonction de leur dosage appliqué. Comme prévu, CuSPHy était très efficace pour inhiber la croissance mycélienne *de B. cinerea* à toutes les concentrations appliquées et grâce à la haute solubilité de ce sel de cuivre(II), qui dissout en solution la plus forte concentration de Cu(II). L'activité antifongique inférieure rapportée par les autres sels de cuivre(II), tels que CuTBS, CuOxCl et CuHyOx, est le résultat de leur plus faible solubilité, car ces composés tendent à rester en suspension, libérant lentement et dans certaines conditions le Cu(II) les ions [20].

2.2.1 Essai préliminaire *in vitro* pour tester l'effet antifongique

L'activité antimicrobienne non significative décrit dans la littérature pour les particules à base de HA [98, 133] pourrait expliquer le rôle contradictoire de la formulation HA sur chaque composé de cuivre(II) (Fig. 2). Pour CuSPHy, le sel de cuivre(II) le plus efficace, la concentration la plus élevée de HA (6%) réduit significativement l'activité inhibitrice de CuSPHy lorsqu'il est appliqué à la plus faible concentration de Cu(II) (0,05%). Ceci était supposé être lié à l'excès de HA, sans activité antimicrobienne, comparé aux ions Cu(II) en solution. Des observations différentes ont été rapportées pour CuHyOx. En général, cet effet a été montré sur tous les composés de Cu(II) appliqués à Cu(II) 0,2%, dans lesquels le HA 6% a augmenté significativement l'inhibition de *B. cinerea* au cours du temps.

Ces observations ont suggéré d'étudier *in planta* le rôle de libération potentiellement joué par l'HA par rapport au sel fonctionnel de cuivre(II) et au rapport de concentration entre les ions HA et Cu(II) appliqués dans la formulation.

2.2.2 Essais d'efficacité *in planta*

Dans des conditions de serre, des résultats prometteurs sur l'activité biologique de formulations à base de Cu(II) et de particules nanostructurées de HA ont été obtenus par les présentes expériences. Les formulations précédemment appliquées *in vitro*, ont été pulvérisées dans l'essai EA1 sur le feuillage des plantes à la même concentration en ions Cu(II) (0,025%) du fongicide cuprique de référence (Table 4).

Dans cette condition, CuSPHy formulé avec 3% de HA réduisait significativement l'incidence et la sévérité de la maladie tandis qu'avec une teneur plus élevée en HA (6%), la formulation HA-CuSPHy perdait une activité protectrice contre *P. viticola*, confirmant la tendance détectée sur l'inhibition de la croissance de *B. cinerea* par les mêmes formulations. L'activité biologique non

significative montrée par la même concentration de HA confirme que le rendement élevé de la formulation de HA-CuSPHy n'est sûrement pas une conséquence de l'activité fongicide des particules à base de HA. D'autre part, un très bon contrôle de la maladie a également été réalisé par le traitement basé sur le CuSPHy pur, mais cela a entraîné une phytotoxicité élevée sur les feuilles traitées. Comme décrit par Gessler *et al.* (2011) [20], CuSPHy est le constituant principal de la bouille bordelaise, qui est un fongicide cuprique développé en neutralisant et fixant les ions Cu(II) hautement solubles et phytotoxiques de ce sel de cuivre (Fig. 3).

La microscopie électronique a été utile pour étudier la distribution des particules et l'agrégation dans les plantes, car l'application de l'HA est due à sa propriété de générer des revêtements biologiquement actifs nanostructurés [94]. De telles particules agrégées ont été détectées sur les échantillons de feuilles traitées avec HA. Une configuration similaire a été observée sur les feuilles traitées avec HA-CuSPHy. Alors que les traitements basés sur les composés insolubles Cu(II) et HA apparaissent distribués dans les plus petites agrégations de particules, montrant différents agrégats par rapport aux amas d'HA (Fig. 6).

La conformation et la distribution de particules diverses reflètent les différents résultats montrés par l'essai à effet de serre EA1. Cet aspect souligne l'importance de la propagation et de la distribution des ions Cu(II) sur la surface des feuilles en ce qui concerne le contrôle de l'infection fongique précoce et de leurs agents, les zoospores de *P. viticola* [121, 122]. En outre, la réponse variable des traitements dans EA1 peut s'expliquer par l'effet de l'agrégation irrégulière des particules et donc de leur taille irrégulière, qui ont un impact direct sur la taille des gouttelettes générées lors de l'application de la substance protectrice, en modifiant l'efficacité du traitement [112].

La formulation HA-CuSPHy a été étudiée ultérieurement en tant que fongicide cuprique potentiel par rapport à la formulation HA-CuTBS, basée sur le sel Cu(II) insoluble (Fig. 4). Dans les deux formulations, l'efficacité de diverses concentrations de HA a été étudiée par rapport aux concentrations de Cu(II). En général, les résultats (EA2) ont confirmé que la formulation de CuSPHy la plus efficace (contenant 5,2% de Cu(II)) est basée sur l'HA variable entre 2,4% et 3,6%, comme on le voit dans l'EA1 en serre. Ce résultat encourageant a été démontré par le surdosage (Cu (II) 0.05%) et de manière inattendue, lors d'un traitement sous dosé (Cu(II) 0,01%). Cette dernière donnée ouvre des perspectives intéressantes par rapport aux premières études visant à étudier l'efficacité de la réduction du dosage du cuivre contre *P. viticola* [24, 28, 142, 143] et des restrictions futures probables de l'utilisation du cuivre en agriculture. Les données recueillies sur les formulations à base de CuTBS (EA3) indiquent une activité dissemblable du facteur "HA%" sur le sel de cuivre insoluble. En effet, malgré une efficacité

généralement plus faible des formulations de CuTBS que celles basées sur CuSPHy, une moindre gravité de la maladie a été montré au plus haut % de HA et à la sur- et sous-dose d'ions Cu(II). Cette observation suggère une étude plus approfondie sur les méthodes et les matériaux applicables pour la co-formulation optimale entre la suspension des particules nanostructurées d'HA et les suspensions du composé Cu(II) insoluble, tel que CuTBS.

Dans les essais en serre réalisées sous l'effet de mouillage, il a été possible d'évaluer la stabilité et la persistance sur les feuilles de vigne des formulations à base de HA-CuSPHy et de HA-CuTBS. Contrairement à l'étude de Dagostin *et al.* (2010) [120], les traitements cupriques ont été appliqués à plus forte dose d'ions Cu (II) (0,075%) et une plus grande quantité de mouillage simulée (50 mm) reçue par les plantes pour mettre en évidence dans cette condition les performances potentielles les moins efficaces (Fig. 5). Cependant, malgré la forte solubilité dans l'eau de CuSPHy, les formulations à base de ce sel ont fortement réduit la gravité et l'incidence de la maladie dans les deux conditions (0 et 50 mm). En excluant le contrôle positif montré par le traitement CuSPHy pur en raison de la phytotoxicité sévère et diffuse sur les feuilles traitées, il a été confirmé le contrôle remarquable obtenu par le HA-CuSPHy sans aucun effet secondaire sur les plantes, indiquant ainsi une interaction significative entre le Cu(II), les ions et les particules HA nanostructurées. L'activité de contrôle moins importante sous l'effet de lavage de mouillage par le traitement HA-CuTBS a été davantage corrélée à une méthode de co-formulation non optimale considérant la tendance similaire rapportée également par le traitement CuTBS pur, en comparaison à la sévérité de la maladie montrée par le fongicide CuTBS. A cet égard, la pertinence du processus de formulation sur l'efficacité des fongicides cupriques a déjà été commentée dans la littérature, notamment dans des conditions environnementales telles que les conditions climatiques et la croissance des plantes, capables d'influencer fortement l'efficacité du produit [24].

Cette étude indique la possibilité d'augmenter l'activité biologique *in vitro* et *in planta* des ions Cu(II) en modifiant leur distribution, leur persistance et leur distribution à la surface des feuilles, à travers une formulation avec des particules nanostructurées d'hydroxyapatite. Cela forme un matériau biocompatible capable de générer des revêtements biologiquement actifs sur la surface traitée. Dans ce contexte, une étude plus approfondie des modèles fonctionnels pourrait encourager la recherche d'autres applications intéressantes sur des outils agricoles basés également sur des composés organiques et inorganiques, applicables à des fins de protection durable des plantes.

2.3. Chapitre 3 – Distribution et efficacité de l'hydroxyapatite fonctionnalisée par deux composés de cuivre(II) dans les tissus ligneux de la vigne colonisés par un agent pathogène fongique vasculaire et caractérisation des réponses de défense activées par la plante

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Battiston E., Compant S., Antonielli L., Simoni A., Di Marco S., Mugnai L. and Fontaine F. (2018). *In planta* activity of copper(II) based formulations to control the colonisation of the esca-associated fungus *Phaeoacremonium minimum*.

Les maladies du bois de la vigne (MDB) deviennent une menace sérieuse et croissante dans les vignobles du monde entier. La nécessité de stratégies innovantes de gestion des maladies persiste puisque les pesticides appliqués pour contrôler certains des MDB ont été interdits et jusqu'à présent, aucun traitement aussi efficace n'a été développé. Dans ce contexte, l'application de méthodes d'imagerie, déjà appliquées à l'étude des interactions plante-microbe [146], pourrait représenter un outil crucial pour comprendre l'effet des traitements expérimentaux sur les pathogènes liés aux MDB et sur les tissus colonisés.

Dans la présente étude, des essais ont été menés pour évaluer l'efficacité de traitements à base de deux composés de cuivre(II) libérés par un système de transporteurs (« drug delivery ») basé sur l'hydroxyapatite inorganique biomimétique et appliqués pour protéger les plants en pépinière de *Phaeoacremonium minimum*, un pathogène fongique associé aux MDB.

Des traitements ont été appliqués sur ce matériel de propagation lors du processus de réhydratation des porte-greffes (*Vitis Berlandieri* x *Vitis riparia* cv K5BB) et des greffons (*Vitis vinifera* L, cv Chardonnay). Après la soudure, les plants greffées ont été cultivés en serre. Les vignes ont été inoculées avec un implant d'agar contenant le pathogène (gfp7 transformé ou non transformé) au niveau du porte-greffe.

Quinze semaines après l'inoculation, les plantes ont été récoltées et les tissus ligneux ont été observés par microscopie confocale à balayage laser (CSLM). Après ces observations, le cuivre a été quantifié dans le même matériel végétal par l'instrument ICP-OES. Enfin, les réponses de défense de la vigne ont été étudiées dans les feuilles traitées des mêmes plantes, en analysant l'expression de gènes dont l'expression est modifiée dans le cas des MDB, d'un traitement au cuivre ou de molécules naturelles.

2.3.1 Essai *in vitro* d'inhibition de la croissance du pathogène.

Le GI% (Growth Inhibition) de mycélium de *P. minimum::gfp7* a été calculé 28 jours après l'inoculation. Les produits ont été appliqués dans le milieu solide de sorte qu'il a été ajouté avec les concentrations suivantes de Cu(II) : 0,05 ; 0,1 et 0,2% p/p. En observant la courbe de GI, les

deux composés de Cu(II) étaient efficaces. En revanche, l'HA n'a montré aucun effet fongicide mais à l'inverse un effet stimulant initial sur la croissance mycélienne a été observée. De plus, cet effet n'a pas réduit l'efficacité de CuSPHy, confirmant son interaction positive avec HA, qui a été moins noté avec le CuSTB (Fig. 2).

2.3.2. Observations microscopiques pour étudier la colonisation du pathogène.

Les images CLSM (Fig. 3 and 4) sont présentées selon (i) le type de *P. minimum*, souche de type sauvage et *P. minimum::gfp7*, (ii) la partie du site d'inoculation, la cavité, la bordure du parenchyme medullaire et le xylème avec le parenchyme, (iii) et le traitement, le contrôle, HA, CuSPHy, CuTBS, CuSPHy+HA et CuTBS+HA. Aucun signal n'a été détecté dans les plantes témoins inoculées avec *P. minimum* souche sauvage. L'inoculation de *P. minimum::gfp7* a réussi dans les plantes témoins et a conduit à une colonisation plus significative des hyphes de ce champignon dans le site de la cavité par rapport aux autres tissus. Des hyphes de *P. minimum::gfp7* ont été détectés dans les plants traités avec HA, en particulier dans le xylème et dans le parenchyme. Le signal GFP (Fig. 5) a également été détecté dans le matériel végétal de chaque traitement, montrant clairement une intensité et une distribution différentes des hyphes entre les traitements et dans la partie du site d'inoculation. Concernant les traitements à base de cuivre, un signal vert très faible a été détecté au point d'inoculation dans tous les traitements. Dans le parenchyme medullaire, seul le traitement avec HA+CuSPHy n'a pas montré la présence du pathogène, alors que dans les vaisseaux du xylème, il n'a jamais été détecté indépendamment des traitements. Dans cette étude, les méthodes d'imagerie appliquées semblent révéler distinctement la présence fongique et la colonisation à l'intérieur et autour du site d'inoculation dans les porte-greffes, traitées avec des substances expérimentales et non traitées.

2.3.3 Quantification du cuivre dans les tissus.

La quantification du cuivre (Fig. 6 and 7) est présentée en fonction des tissus végétaux (parenchyme médullaire, tissus vasculaires, écorce), et du temps de détection (post-traitement, post-récolte). L'étude de l'écorce avait pour objectif de caractériser la dynamique du traitement à la surface de la plante, mise en évidence par une différence significative de fonction du composé mais aussi une persistance inattendue. Dans les tissus vasculaires et le parenchyme médullaire, l'échelle change considérablement : dans le parenchyme médullaire, il y a une très faible accumulation de cuivre, significative seulement avec CuSPHy, expliquant ainsi les résultats rapportés par les images CLSM. Dans les tissus vasculaires, il n'a pas été

possible de conclure, en raison d'une teneur élevée en cuivre observée dans le contrôle peut être due à une contamination.

2.3.4. Étude des réponses de la vigne par biologie moléculaire en réponse aux traitements et à l'infection artificielle par le pathogène.

En analysant les valeurs d'expression relatives des gènes associés aux défenses de la vigne 8 h après le traitement (Fig. 8), une réponse différente a été observée en fonction du composé de cuivre, en particulier pour les formulations à base de HA. Malgré les résultats significatifs obtenus avec le CuSPHy pur, il est responsable de l'effet phytotoxique constant déjà connu dans la littérature et lié à la solubilité élevée du composé et au pH très bas. En effet, un tel composé doit être neutralisé avec de l'hydroxyde de calcium pour être appliqué ensuite pour la protection des cultures sous le nom commun de bouille bordelais. En ce qui concerne les valeurs d'expression relatives de ces gènes 24 h après le traitement (Fig. 9), une induction plus élevée a été signalée pour les deux composés de cuivre(II). La CuSPHy pure n'est pas satisfaisante en raison de la forte phytotoxicité détectée ; les valeurs élevées notées avec le CuTBS indiquent un coût énergétique élevé pour le métabolisme de la plante. Ainsi, les résultats obtenus par les traitements à base de composés de cuivre(II) et de HA, en particulier à 8 h post-traitement, suggèrent une étude plus poussée prenant en compte différentes pathologies, agents pathogènes et méthodes d'application. Donc l'analyse transcriptomique a révélé le rôle potentiel des formulations innovantes dans la stimulation des réponses de défense de la plante. En conclusion, la recherche a mis en évidence la pertinence de l'étude de l'interaction plante-pathogène par des méthodes d'imagerie. Dans cette perspective, en particulier pour la gestion des MDB, les stratégies expérimentales de contrôle devraient également être liées à la distribution et à la persistance de la substance appliquée et à son rôle biologique potentiel, en tant que fongicide et biostimulant du végétal.

3. Conclusions et perspectives

La protection des végétaux est de plus en plus orientée pour encourager le développement des approches innovatrices à faible impact sur l'environnement. Ceci est encore plus difficile dans la lutte contre des maladies encore mal comprises et qui peuvent affecter gravement l'agriculture. C'est le cas des maladies du bois de la vigne (MDB) qui deviennent un problème sérieux en viticulture, tant en Europe que dans le monde (pour revue Mondello *et al.*, 2018) [53]. Dans ce contexte, la présente recherche a été développée en tant qu'étude pluridisciplinaire visant à répondre à des objectifs cruciaux : la réduction considérable de la quantité de substances actives habituellement utilisées pour la protection de la vigne et l'optimisation de l'activité biologique des mêmes substances contre des pathogènes vasculaires liés à des maladies complexes.

La recherche a conduit à plusieurs résultats prometteurs, qui soutiennent l'application potentielle d'une hydroxyapatite (HA) spécifique pour la protection des plantes en améliorant l'efficacité des composés de cuivre(II). Dans la perspective d'approches innovantes et durables, cette recherche a été testée pour le contrôle de maladies fongiques chez *Vitis vinifera* L.

L'HA fonctionnalisée avec des composés de cuivre(II) a été entièrement caractérisée et la stabilité biologique a été établie en effectuant des applications sur les tissus foliaires de *V. vinifera* à des fins de protection des plantes (Battiston *et al.*, 2018, accepté).

La morphologie, la structure et la composition de l'HA fonctionnalisée avec différents composés de cuivre(II) et pulvérisée sur des feuilles de vigne ont été détectées et enfin, un modèle de transporteurs (« drug delivery ») différent et spécifique a été développé pour chaque sel de cuivre(II). Sur la base des résultats obtenus, l'agrégation micrométrique et la forme de l'HA ont été décrites en relation avec sa stabilité lorsqu'elle est formulée avec des ions Cu(II). Une affinité particulière a été mise en évidence pour les formulations à base de sulfate de cuivre pentahydrate et de sulfate de cuivre tribasique. La microscopie électronique sur les substances et dans la plante a permis de révéler un modèle de livraison différent pour le sulfate de cuivre pentahydrate et le sulfate de cuivre tribasique, et la non homologation de leurs agrégats aux nanostructures. Cela a été également confirmé par le test en serre sur *Plasmopara viticola*, dans lequel l'HA pure n'a montré aucun effet toxique sur la plante et le pathogène, tandis que les formulations à base de sulfate de cuivre pentahydrate et de sulfate de cuivre tribasique ont montré un contrôle plus élevé du pathogène que les composés de cuivre pur correspondants.

Sur la base de cette première approche, l'activité biologique de l'HA fonctionnalisée avec des composés de cuivre(II) a été étudiée plus en détail *in vitro et in vivo*, sur le contrôle des pathogènes fongiques de la vigne.

Les résultats recueillis évaluant la sévérité et l'incidence de la maladie sur les vignes en pot traitées, inoculées avec *P. viticola*, ont montré l'effet de l'HA avec une tendance significativement différente pour chaque composé de cuivre(II) (Battiston *et al.* Phytopathology, soumis). Compte tenu de ces résultats prometteurs, d'autres essais ont été effectués pour évaluer la stabilité des traitements à l'effet de lavage des précipitations, obtenant des résultats positifs sur l'efficacité et la persistance des traitements expérimentaux par rapport aux fongicides commerciaux.

La distribution et l'efficacité de l'HA fonctionnalisée avec des composés de cuivre(II), dans les tissus ligneux de la vigne colonisés par des pathogènes vasculaires ont été étudiées. Pour cela, une étude de l'interaction plante-pathogène par des méthodes d'imagerie à l'aide de matériel végétal de propagation en pépinière a été menée. L'étude a révélé comment ces paramètres peuvent être très variables, avec des conséquences sur la colonisation des agents pathogènes. Les données concertants la quantification des éléments ont révélé une concentration, une distribution et une persistance différentes du cuivre sur le matériel végétal traité et analysé en deux temps : après le traitement et après le cycle végétatif. Les différences sont également liées au composé de cuivre appliqué, confirmant les modèles de livraison précédemment proposés.

Une étude parallèle basée sur l'analyse transcriptomique a permis d'évaluer l'effet de l'HA et des composés de cuivre(II) pour stimuler les réponses de défense de la plante. De tels traitements pourraient être appliqués en tant qu'outil de biostimulation pendant le processus de soudure des greffons et/ou pendant la croissance des jeunes plants. L'activité fongicide du cuivre pourrait contribuer au contrôle des agents pathogènes associés aux MDB.

Encore une fois, il montre son rôle potentiel, notamment pour la gestion de maladies en agriculture biologique. Toutefois, la question de sa durabilité à long terme reste posée avec d'un côté, la réduction conséquente de la quantité de base de cuivre appliquée, et de l'autre, la biostimulation potentielle qui représenterait un atout pour la plante.

Concernant les perspectives ouvertes par l'application de l'HA pour protéger les végétaux, des considérations globales seront formulés sur la base des résultats des essais parallèles dans le vignoble et en pépinière, toujours en cours en raison de la nécessité d'observer plusieurs cycles végétatifs.

En conclusion, en combinant les résultats des approches physiques, chimiques, microscopiques, biologiques et moléculaires, nous avons étudié une alternative intéressante pour protéger la vigne contre deux maladies fongiques pertinentes, le mildiou et l'esca.

En particulier, sur la base des objectifs communs, les conclusions relatives aux MDB ont été présentées et discutées dans le cadre de l'action COST FA1303 portant sur le « contrôle durable des maladies du bois de la vigne ». Ainsi, afin d'améliorer la compréhension des MDB, ces travaux ont été présentés car ils ont apporté des connaissances sur : l'apparition de pathogènes, l'interaction entre la vigne et les pathogènes, l'écologie des micro-organismes vivant dans le bois et l'élaboration de nouveaux protocoles de gestion et d'approches de lutte biologique.

Riassunto sostanziale in Italiano

1. Introduzione generale

La vite (*Vitis vinifera* L.) è una delle colture più importanti a livello mondiale, in relazione alla produzione di vino e uva da tavola [1]. Sfortunatamente, la maggior parte delle cultivar utilizzate per la vinificazione sono altamente suscettibili a diversi agenti patogeni [2]. Nel corso dell'ultimo secolo, la lotta chimica delle malattie è stata la misura più efficace utilizzata in tutto il mondo per controllare i patogeni, specialmente nella viticoltura moderna. Considerando che le infezioni fungine sono principalmente responsabili dei danni alla qualità dell'uva e alle perdite di resa, i pesticidi più utilizzati in viticoltura sono i fungicidi [9].

A partire dalla loro diffusione dal continente americano nel XIX secolo, *Plasmopara viticola* (l'agente causale della peronospora) e *Erysiphe necator* (l'agente causale dell'oidio) sono le malattie fungine più importanti della vite, e insieme alla *Botrytis cinerea* (l'agente causale di muffa grigia) causano il maggior numero di trattamenti nei vigneti [3].

La protezione della vite è ancora più difficile nel controllo delle malattie ancora poco conosciute, responsabili delle perdite di produzione, della morte delle viti e di un generale deperimento della vite, che sta compromettendo gravemente la produttività dei vigneti in tutto il mondo. È il caso delle malattie del legno della vite (GTDs) che stanno diventando un serio problema in viticoltura. Le malattie del legno sono state conosciute e ben descritte in letteratura per le colture arboree, ma la loro attuale diffusione e l'aumento della gravità in tutto il mondo [37, 38] sono probabilmente la conseguenza dei cambiamenti climatici e indubbiamente legate alle pratiche moderne della viticoltura intensiva [39]. Le GTDs colpiscono il tronco principale e i cordoni della vite e si presentano come un complesso di varie malattie causate da patogeni associati a sintomi specifici, come i danni causati dalla secrezione di *Botryosphaeriaceae*, l'eutipiosi causata da patogeni fungini *Diatrypaceae* e il piede nero, malattia causata da specie *Dactylonectria* e *Ilyonectria*. Tra le GTDs, la forma più comune e dannosa in Europa è il complesso dell'esca associato a patogeni fungini vascolari, agenti cancerogeni e agenti di deperimento e la loro interazione. In questo quadro complesso, un aspetto cruciale per lo sviluppo delle GTDs è l'interazione tra quei diversi patogeni (compresa l'interazione dei loro fattori di patogenicità) e lo stato fisiologico della pianta [40].

Al giorno d'oggi, la protezione della vite è sempre più orientata a incoraggiare lo sviluppo di approcci sostenibili e innovativi a basso impatto per controllare in particolare i patogeni fungini. I problemi legati all'impatto ambientale e alla tossicologia dei vecchi fungicidi chimici sintetici e la crescente domanda di prodotti privi di residui, hanno incoraggiato la ricerca di nuovi strumenti per la protezione delle colture. Le aziende agrochimiche stanno sviluppando nuove

formulazioni basate su sostanze bioattive con un minore impatto sulla salute umana e sull'ambiente e un nuovo meccanismo di azione per ridurre l'allarmante aumento di ceppi fungini resistenti ai fungicidi. Inoltre, la ricerca di alternative ai fungicidi organici sintetici è sempre più orientata allo studio e all'applicazione di principi attivi basati su microrganismi e «botanicals» [3].

Le conoscenze sull'attività biologica di numerosi «botanicals» sono notevolmente aumentate negli ultimi decenni e le ricerche sull'applicazione di tali sostanze a fini fitosanitari sono in aumento. Miscele di sostanze naturali provenienti da varie parti di piante, principalmente semi, ma anche frutta e diversi tessuti, sono comunemente riconosciute come «botanicals», tra le quali gli oli essenziali vegetali sono profondamente studiati. Diversi aspetti critici stanno ancora limitando l'applicazione di «botanicals» in strategie di protezione delle piante su larga scala, quindi sono necessari ulteriori studi e formulazioni innovative per migliorare l'efficienza nel controllare i patogeni e ridurre l'alto costo dei prodotti commerciali.

In questo contesto, il rame è l'elemento più antico e più comune usato per controllare la peronospora in viticoltura. Oltre al suo effetto diretto sul patogeno, il maggiore livello di indicatori di difesa delle piante come perossidasi, fenoli, resveratrolo e antociani sono stati dimostrati su piante di vite trattate con composti a base di rame(II). Oggigiorno, il rame rimane il fungicida più efficiente nella viticoltura biologica essendo un composto non sintetico con un ampio spettro di attività. D'altra parte, l'uso a lungo termine è responsabile dell'ampio accumulo di rame metallo negli ecosistemi agricoli che sta causando gravi problemi ambientali. Di conseguenza, la normativa europea limita l'uso del rame a un massimo di 6 kg di Cu ha^{-1} all'anno o un massimo di 30 kg di Cu ha^{-1} per 5 anni [24].

Per quanto riguarda il controllo sostenibile delle GTDs, nel corso degli anni una vasta gamma di attività di ricerca è stata promossa a livello internazionale, producendo una comprensione più profonda delle malattie, della loro eziologia, biologia, epidemiologia, ma rimangono ancora molte questioni aperte sulla gestione della malattia [43]. Questo è legato a diversi aspetti critici, come una comprensione ancora non del tutto chiara degli agenti causali, oltre alla conoscenza ancora incompleta dei meccanismi di espressione dei sintomi, specialmente nella più grave malattia delle foglie tigrate (GLDS).

Un primo aspetto critico è legato alla mancanza di prodotti con bassa tossicità ma una buona efficacia per il controllo di queste malattie. L'arsenito di sodio era considerato l'unico agente chimico efficace su GLSD ("esca"), agendo per il suo accumulo nei tessuti trattati [44]. Ad ogni modo, a causa della sua elevata tossicità, l'arsenito di sodio non è più consentito in viticoltura [45]. Una promettente - sebbene bassa - riduzione dell'incidenza della malattia è stata ottenuta

recentemente in prove sul campo con formulati di rame che potrebbero parzialmente penetrare nel legno [46], probabilmente compromettendo la crescita e il metabolismo dei patogeni fungini coinvolti. Questa scoperta ha aperto un nuovo campo di ricerca sull'applicazione dei prodotti penetranti del legno, piuttosto che su quelli sistemici, poiché finora l'unico prodotto sistemico che ha dato una certa efficacia costante e prolungata nella riduzione dell'incidenza di GLSD è il fosetil alluminio, un fungicida usato per controllare *P. viticola*, la cui attività appariva legata alla sua capacità di attivare risposte di difesa [47].

Un secondo aspetto critico è legato alla protezione inefficace delle ferite da potatura, un importante punto di penetrazione per tutti gli agenti fungini del legno. I prodotti disponibili in commercio si sono rivelati efficaci *in vitro* [48], ma non in grado di garantire una protezione persistente rispetto alla prolungata suscettibilità delle ferite da potatura [49]. A tale scopo, è stata studiata l'applicazione di agenti di biocontrollo basati su specie di *Trichoderma* spp. I risultati ottenuti in diversi anni di esperimenti hanno rivelato alcuni effetti positivi sui sintomi di GLSD poiché l'agente di biocontrollo colonizza la ferita, consentendo un'interazione prolungata con la pianta [50].

Un terzo aspetto critico, rispetto alle difficoltà nel controllo di queste malattie del legno, e in particolare di GLSD, è che i patogeni sono localizzati nel tessuto legnoso, ma le cause dell'espressione dei sintomi nelle foglie sono legate alla reazione di difesa attivata a livello fogliare dalla produzione delle fitotossine nel legno, poi traslocate attraverso il tessuto vascolare, sotto l'influenza di altri cofattori ambientali non ben determinati. A questo proposito, alcuni risultati positivi sono stati ottenuti con applicazioni di nutrienti fogliari, la cui efficacia potrebbe essere fortemente ottimizzata da una migliore distribuzione e penetrazione del prodotto [51]. In questa direzione, un gruppo di ricerca dell'Università di Poitiers (Francia) ha recentemente studiato due tipi di composti con modalità d'azione differenti e complementari. Un primo studio è stato condotto utilizzando molecole esistenti modificate per ottenere prodotti in grado di muoversi, dopo un'applicazione fogliare, nel tronco della vite in cui sono localizzati gli agenti patogeni. Un secondo studio ha associato queste sostanze mobili a molecole in grado di stimolare le difese delle piante [52].

La presente ricerca nasce dalle prospettive aperte da precedenti applicazioni di cristalli inorganici basati costituiti da una fase specifica del fosfato di calcio biomimetico, l'idrossiapatite (HA), che è stata ampiamente studiata e applicata in molti campi [71, 72, 73].

Sulla base delle applicazioni di successo dell'HA eseguite in campo biomedico, tale materiale innovativo può potenzialmente agire come un sistema di rilascio di sostanze attive, inclusi ioni metallici, come rame e composti organici. Una delle principali indagini è stata svolta in

medicina, per sostituire la chemioterapia nel trattamento di un tumore osseo, grazie alla funzionalizzazione di HA con una molecola antitumorale [74, 75], mentre una seconda applicazione efficace è stata ottenuta attraverso lo sviluppo di un dentifricio basato sullo stesso materiale, funzionalizzato con ioni di zinco, al fine di sostituire fluoro, SLS, biossido di titanio e parabeni [76].

In questo quadro, la ricerca ha lo scopo di valutare, attraverso un approccio multidisciplinare, lo sviluppo e l'applicazione dell'HA per migliorare l'attività biologica delle sostanze attive per il controllo delle malattie fungine in *V. vinifera*.

Questo obiettivo risponde a obiettivi cruciali: (i) la riduzione in modo significativo la quantità di fungicidi chimici normalmente utilizzati per la protezione della vite, (ii) l'ottimizzazione della distribuzione del principio attivo, la penetrazione e la persistenza all'interno dei tessuti vegetali, (iii) la potenziale applicazione nel controllo dei patogeni anche nelle malattie vascolari e complesse.

Le attività sperimentali ed i relativi risultati sono presentati e discussi in base alle seguenti azioni: (i) caratterizzazione e stabilità dell'HA funzionalizzata con composti di rame(II), applicati ai tessuti fogliari di *V. vinifera* a scopo fitosanitario; (ii) valutazione *in vitro* e *in vivo* dell'attività biologica dell'HA funzionalizzata con composti di rame(II) sul controllo dei patogeni fungini della vite; (iii) valutazione della distribuzione e dell'efficienza dell'HA funzionalizzata con due composti di rame(II) in tessuti legnosi di vite colonizzati da un patogeno fungino vascolare e caratterizzazione delle risposte di difesa attivate dalla pianta.

2. Riassunti dei capitoli

2.1. Capitolo 1 – Caratterizzazione e stabilità dell'idrossiapatite funzionalizzata con composti del rame(II) e applicata a tessuti fogliari di *Vitis vinifera* L. per la protezione della pianta.

Articolo scientifico pubblicato su Pest Management Science

Battiston E., Salvatici M.C., Lavacchi A., Gatti A., Di Marco S. and Mugnai L. (2018). Functionalisation of a nano-structured hydroxyapatite with copper(II) compounds as pesticide: *in situ* TEM and ESEM observations of treated *Vitis vinifera* L. leaves.

Nel corso degli anni, la ricerca sulle nanotecnologie ha portato allo sviluppo di materiali intelligenti rivoluzionari in molti campi. In agricoltura, l'approccio nanotecnologico viene spesso applicato per migliorare la produttività delle piante, la qualità delle colture e la gestione delle malattie, attraverso materiali di dimensioni nano. In particolare, lo sviluppo di sistemi a rilascio lento per pesticidi apre nuove prospettive per ridurre la quantità di principio attivo applicato per la protezione delle piante e al controllo delle malattie. Considerando l'eventuale fitotossicità di tali materiali innovativi, che rimane ancora poco chiaro in letteratura, il presente studio considera un materiale intelligente biocompatibile per la protezione delle piante. In questo caso è stata considerata una idrossiapatite sintetica biomimetica (HA), studiata per la sua particolare bioattività. È stata esaminata l'aggregazione tra le nanoparticelle di HA e quattro composti di rame(II), applicati nelle foglie di *Vitis vinifera* L. come pesticida. Diverse formulazioni sono state caratterizzate per determinare le forme e le dimensioni degli aggregati mediante XRD, DLS e microscopia elettronica. Le stesse formulazioni sono state applicate *in planta* per verificare l'aggregazione delle particelle e l'efficienza nella protezione della pianta contro il patogeno fungino *Plasmopara viticola*. L'analisi a raggi X ha mostrato una diversa interazione tra HA e i composti di rame(II), possibilmente basato sulla solubilità e il pH delle diverse formulazioni. L'analisi DLS ha mostrato una distribuzione granulare che varia globalmente fuori dalla gamma dei nanometri. Ulteriori osservazioni mediante microscopia TEM ed ESEM hanno mostrato, in tutte le formulazioni, grossi aggregati parzialmente nanostrutturati, che sono stati riconosciuti come aggregati e non come cluster, e quindi stabili nella loro dimensione micrometrica. Le particelle rilevate, a base di calcio, fosforo e rame, non hanno mostrato alcun effetto fitotossico dopo la loro applicazione *in planta*. La formulazione HA e sale solubile di rame(II) ha mostrato risultati promettenti nel controllo del patogeno, confermando il ruolo potenziale dell'HA nel rilascio innovativo di ioni Cu(II). Il presente lavoro indica la possibilità di migliorare l'attività biologica di una sostanza bioattiva modificando la sua struttura attraverso una formulazione specifica e realizzabile con un materiale biocompatibile.

2.2. Capitolo 2 – Valutazione *In vitro* e *in vivo* dell'attività biologica dell'idrossiapatite funzionalizzata con composti del rame(II) per il controllo di patogeni fungini della vite.

Articolo scientifico sottomesso a Phytopathology

Battiston E., Antonielli L., Di Marco S., Fontaine F. and Mugnai L. (2018). Innovative delivery of copper(II) ions by a nano-structured hydroxyapatite: potential application *in planta* to enhance the sustainable control of *Plasmopara viticola*.

La vite (*Vitis vinifera* L.) è una delle colture da frutto che richiede il massimo impatto fungicida per il controllo delle malattie. *Plasmopara viticola* è probabilmente la più grave malattia della vite in grado di causare perdite di resa consistenti. Nel corso degli anni, sono stati sviluppati numerosi principi attivi per controllare il potenziale danno alla malattia. L'uso sistematico di fungicidi organici ha indotto la comparsa di ceppi resistenti di *P. viticola*. Solo un regime di trattamento a base di rame lo evita, grazie alla modalità d'azione non specifica dei fungicidi contenenti rame. Nella viticoltura biologica, la protezione contro la peronospora è essenzialmente assicurata dall'uso di fungicidi rameici, il cui uso in agricoltura dovrebbe essere ulteriormente limitato dai paesi europei, a causa del suo profilo ecotossicologico e fitotossico critico. La ricerca sulle forme innovative di rame e l'ottimizzazione della distribuzione e della persistenza dei pesticidi a base di rame è risultato l'approccio più promettente per il controllo della peronospora. La presente ricerca studia le proprietà di rilascio dell'idrossiapatite sintetica biomimetica (HA) per migliorare l'attività biologica degli ioni Cu(II). A questo scopo, quattro composti di rame(II) (CuSPHy, CuTBS, CuHyOx e CuOxCl) sono stati formulati con l'innovativo componente HA e applicati in un test preliminare antifungino *in vitro* contro *Botrytis cinerea*, patogeno della vite adatto per i test di attività *in vitro*, e infine nei saggi di efficacia *in planta* contro *P. viticola* in serra. I risultati *in vitro* hanno evidenziato un diverso grado di inibizione per ciascun composto di Cu(II) in base alla loro dose applicata e indicando anche il ruolo di rilascio potenzialmente giocato dall'HA, specialmente sui sali di rame insolubili. In condizioni di serra, sono stati ottenuti ulteriori risultati sull'attività biologica delle formulazioni applicate, in particolare sull'efficacia delle percentuali variabili di HA nelle formulazioni, sull'influenza della variazione di dosaggio della formulazione e sull'efficacia e la persistenza del trattamento sotto effetto del lavaggio da pioggia.

In conclusione, il presente studio ha rivelato risultati promettenti sulla formulazione basata sulle particelle di HA e sul sale di rame solubile (CuSPHy), che si sono rivelati estremamente efficaci nel ridurre sia la gravità della malattia che l'incidenza in tutte le condizioni sperimentali, suggerendo nel frattempo uno studio più profondo di modelli funzionali e del processo di co-formulazione sui composti di Cu(II) insolubili.

2.3. Capitolo 3 – Distribuzione ed efficacia dell'idrossiapatite funzionalizzata con due composti del rame(II), nei tessuti legnosi della vite colonizzati da un patogeno fungino vascolare e caratterizzazione delle risposte di difesa attivate dalla pianta.

Articolo scientifico in preparazione per Pesticide Biochemistry and Physiology

Battiston E., Compant S., Antonielli L., Simoni A., Di Marco S., Mugnai L. and Fontaine F. (2018). *In planta* activity of copper(II) based formulations to control the colonisation of the esca-associated fungus *Phaeoacremonium minimum*.

Le malattie del legno della vite (GTDs) stanno diventando una minaccia seria e crescente nei vigneti di tutto il mondo. La necessità di strategie di protezione innovative persiste poiché i pesticidi applicati per controllare alcune GTDs sono stati banditi e finora non sono stati sviluppati trattamenti efficaci. In questo contesto, l'applicazione di metodi di «imaging», già applicati per studiare le interazioni pianta-microbo, potrebbe rappresentare uno strumento cruciale per comprendere l'effetto dei trattamenti sperimentali sui patogeni correlati alle GTD nei tessuti colonizzati. Nella presente indagine sono stati considerati due composti di rame(II), rilasciati da aggregati di idrossiapatite inorganica biomimetica, e applicati per proteggere il materiale di propagazione dal patogeno *Phaeoacremonium minimum*, un agente patogeno associato alle GTDs. I trattamenti sono stati applicati durante il processo di reidratazione dei portinnesti (*Vitis Berlandieri* x *Vitis riparia* cv. K5BB) e marze (*Vitis vinifera* L, cv. Chardonnay). Dopo la forzatura, le viti innestate venivano coltivate in serra. Le viti sono state inoculate con un tappo di agar contenente il patogeno (gfp7 trasformato o non trasformato) praticando un foro nel portainnesto. Quindici settimane dopo l'inoculo, i tessuti legnosi delle piante sterrate sono stati osservati utilizzando una microscopia a scansione laser confocale (CSLM). Dopo le osservazioni, il rame è stato quantificato nello stesso materiale vegetale con lo strumento ICP-OES. Infine, le risposte di difesa della vite sono state studiate nelle foglie trattate delle stesse piante, analizzando l'espressione dei geni connessi alle GTDs. I risultati hanno mostrato la correlazione tra la colonizzazione del patogeno (immagini CLSM) e la distribuzione e la persistenza del rame(II) (dati ICP-OES) nel tempo e in diversi tessuti. Inoltre, questi parametri sono risultati altamente variabili, con conseguenze sulla colonizzazione del patogeno. Allo stesso tempo, l'analisi trascrittomica ha rivelato il ruolo potenziale delle formulazioni innovative nello stimolare le risposte di difesa della pianta. In conclusione, nel quadro sperimentale della prova, la ricerca ha evidenziato l'importanza della correlazione tra la distribuzione e persistenza della sostanza applicata e del suo potenziale duplice ruolo biologico, come fungicida e biostimolante della pianta.

3. Conclusioni e prospettive

La protezione delle piante è sempre più orientata a incoraggiare lo sviluppo sostenibile e approcci innovativi a basso impatto. Questo è ancora più difficile nel controllo di malattie ancora poco conosciute in grado di influenzare gravemente la resa del raccolto. È il caso delle malattie del legno della vite (GTDs) che stanno diventando un problema serio in viticoltura (Mondello *et al.*, 2018).

In questo contesto, la presente ricerca è stata sviluppata come studio multidisciplinare volto a rispondere a obiettivi cruciali: la notevole riduzione della quantità di sostanze attive normalmente impiegate sulla protezione della vite, l'ottimizzazione dell'attività biologica delle stesse sostanze, anche nel controllo patogeni vascolari correlati a malattie complesse. La ricerca ha portato a diversi risultati promettenti, che supportano la potenziale applicazione di una specifica idrossiapatite (HA) nella protezione delle piante per migliorare l'efficienza dei composti di rame(II), nella prospettiva di un approccio innovativo e sostenibile per il controllo delle malattie fungine in *Vitis vinifera* L.

L'idrossiapatite funzionalizzata con composti di rame(II) è stata completamente caratterizzata e la stabilità biologica è stata stabilita per l'esecuzione di applicazioni su tessuti fogliari di *V. vinifera* a scopo fitosanitario (Battiston *et al.*, 2018, accettato). Sono stati rilevati la morfologia, la struttura e la composizione dell'HA funzionalizzati con diversi composti di rame(II) e spruzzati su foglie di vite e, infine, è stato sviluppato un modello di rilascio diverso e specifico per ciascun sale di rame(II): i modelli sono basati sull'elevata differenza di pH tra i composti di rame(II). Sulla base dei risultati ottenuti, l'aggregazione micrometrica e la forma dell'HA sono stati descritti in relazione alla sua stabilità quando formulata con ioni Cu(II). Una particolare affinità è stata evidenziata per le formulazioni basate su solfato di rame pentaidrato e solfato di rame tribasico. La microscopia elettronica sulle sostanze e *in planta* ha permesso di rivelare un diverso modello di erogazione per il solfato di rame pentaidrato e il solfato di rame tribasico e la non omologazione dei loro aggregati alle nanostrutture. Ciò è stato confermato anche dal test della serra su *Plasmopara viticola*, in cui l'HA pura non ha mostrato alcun effetto tossico sulla pianta e sul patogeno, mentre le formulazioni basate su solfato di rame pentaidrato e solfato di rame tribasico hanno mostrato un controllo patogeno più elevato, se confrontato coi rispettivi composti puri del rame. Sulla base di questo primo approccio, l'attività biologica dell'HA funzionalizzata con composti di rame(II) è stata ulteriormente studiata *in vitro* e *in vivo*, sul controllo dei patogeni fungini della vite. I risultati raccolti valutando la gravità della malattia e l'incidenza sulle viti in vaso trattate, inoculate con *P. viticola*, hanno mostrato l'effetto HA con

una tendenza significativamente diversa per ciascun composto di rame(II). Considerando questi risultati promettenti, sono stati condotti studi complementari che hanno valutato la stabilità dei trattamenti all'effetto di lavaggio delle precipitazioni, ottenendo risultati positivi sull'efficacia e la persistenza dei trattamenti sperimentali rispetto ai fungicidi commerciali.

La distribuzione e l'efficienza dell'HA funzionalizzata con composti di rame(II), nei tessuti lignificati della vite colonizzati da patogeni vascolari è stata studiata nel quadro di uno studio dell'interazione pianta-patogeno attraverso metodi di imaging, in un esperimento sul materiale di propagazione. A questo proposito, lo studio ha rivelato come questi parametri possano essere altamente variabili, con conseguenze sulla colonizzazione degli agenti patogeni. I dati ICP hanno rivelato una diversa concentrazione, distribuzione e persistenza del rame sul materiale vegetale trattato analizzato dopo il trattamento e dopo la raccolta. Le differenze sono correlate anche al composto di rame(II) applicato, confermando i modelli di rilascio precedentemente ipotizzati. Un'indagine parallela basata sull'analisi trascrittomica ha permesso di apprezzare il ruolo potenziale di formulazioni innovative basate su composti HA e rame(II) per stimolare le risposte di difesa della pianta. Tali trattamenti potrebbero essere applicati come strumento biostimolante durante il processo di forzatura e/o di crescita nel vivaio. L'attività fungicida del rame potrebbe contribuire al controllo degli agenti patogeni associati alle GTDs e allo stesso tempo ad altri patogeni fungini. Ancora una volta il rame mostra il suo ruolo potenziale, soprattutto per la gestione della malattia nell'agricoltura biologica, ma la questione riguarda ancora la sostenibilità del rame a lungo termine: da una parte, la consistente riduzione della quantità minima applicata sarebbe una prima risposta positiva alla domanda ma dall'altra parte, la potenziale biostimolazione rappresenterebbe un ambiguo costo energetico per le piante.

Per quanto riguarda le prospettive aperte dall'applicazione dell'HA per proteggere le piante, verranno formulate considerazioni globali sulla base dei risultati di prove parallele in vigna e vivaio, ancora in corso a causa della necessità di osservare diversi cicli vegetativi.

In conclusione, combinando i risultati degli approcci fisici, chimici, microscopici, biologici e molecolari, la ricerca ha studiato un interessante modo alternativo per proteggere la vite da due malattie fungine rilevanti. In particolare, sulla base degli obiettivi comuni, i risultati relativi alle GTDs sono stati presentati e discussi nel quadro dell'azione COST FA1303 sul "Controllo sostenibile delle malattie del legno della vite", al fine di migliorare la comprensione delle GTDs acquisendo conoscenze su insorgenza di agenti patogeni, interazione vite-patogeno, ecologia dei microrganismi che abitano il legno e sviluppo di nuovi protocolli di gestione e approcci di biocontrollo.

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Annex I – Co-authored research paper

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RESEARCH PAPERS

Unmanned Aerial Vehicle (UAV)-based remote sensing to monitor grapevine leaf stripe disease within a vineyard affected by esca complex

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Summary. Foliar symptoms of grapevine leaf stripe disease (GLSD, a disease within the esca complex) are linked to drastic alteration of photosynthetic function and activation of defense responses in affected grapevines several days before the appearance of the first visible symptoms on leaves. The present study suggests a methodology to investigate the relationships between high-resolution multispectral images (0.05 m/pixel) acquired using an Unmanned Aerial Vehicle (UAV), and GLSD foliar symptoms monitored by ground surveys. This approach showed high correlation between Normalized Differential Vegetation Index (NDVI) acquired by the UAV and GLSD symptoms, and discrimination between symptomatic from asymptomatic plants. High-resolution multispectral images were acquired during June and July of 2012 and 2013, in an experimental vineyard heavily affected by GLSD, located in Tuscany (Italy), where vines had been surveyed and mapped since 2003. Each vine was located with a global positioning system, and classified for appearance of foliar symptoms and disease severity at weekly intervals from the beginning of each season. Remote sensing and ground observation data were analyzed to promptly identify the early stages of disease, even before visual detection. This work suggests an innovative methodology for quantitative and qualitative analysis of spatial distribution of symptomatic plants. The system may also be used for exploring the physiological bases of GLSD, and predicting the onset of this disease.

Key words: precision viticulture, disease detection, asymptomatic plant, trunk disease.

Introduction

Fungal trunk diseases (mainly Eutypa dieback, Botryosphaeria dieback, esca complex) are responsible for significant economic losses to the wine industry worldwide, and are the most difficult grapevine diseases to control (Di Marco *et al.*, 2011a). Among these diseases esca complex is the most widespread in Europe (Surico *et al*., 2008; Bertsch *et al*., 2013; Gubler *et al*., 2015), including wood decay (in Europe mainly caused by *Fomitiporia mediterranea*) and grapevine leaf stripe disease (GLSD) mainly associated with wood vascular infections by *Phaeomoniella chlamydospora* and *Phaeoacremonium minimum* (*"P. aleophilum", sensu* Gramaje *et al*., 2015), but also with a not yet well characterized vascular disfunction. In older vines, wood decay and GLSD frequently occur together on the same plant (Mugnai *et al*., 1999; Surico *et al*., 2008; Andolfi *et al*., 2011). Symptoms of GLSD include foliar interveinal necrosis, giving affected leaves the typical tiger-stripe appearance (Figure 1). Affected vines produce poor quality grapes

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Figure 1. Typical foliar symptoms of grapevine leaf stripe disease (GLSD) show interveinal chlorosis and/or necrosis, typically surrounded by a reddish, purple or yellow margin. While there is variability in the appearance of symptoms in different cultivars, the so called "tiger stripe" pattern is fairly typical.

(Calzarano *et al*., 2004b; Lorrain *et al*., 2012), have reduced production and a high incidence of yearly death, representing an increasing threat for grape growers around the world, but especially in Europe (Bertsch *et al*., 2013; Gubler *et al*., 2015).

The most typical characteristics of GLSD are the absence of correlation between the severity of wood deterioration and the appearance and severity of leaf symptoms (Calzarano *et al*., 2007; Fontaine *et al*., 2016), and the intermittent expression of leaf symptoms. These may not appear in every growing season on each affected same vine, even if it is infected and has shown symptoms in previous years. This discontinuity of leaf symptoms on individual vines makes it extremely difficult to determine the true incidence of the disease in a vineyard at any given time because many infected vines may not show symptoms every year (Surico *et al*., 2000; Marchi *et al*., 2006). As a consequence, annual monitoring of leaf symptoms becomes fundamental for evaluating disease expression over progressive years, and to acquire cumulative indices of the real incidence of the disease. A plant affected by GLSD or, in general terms, by esca disease, can give normal production if it remains symptomless in that year. Thus, it is only the manifestation of symptoms that is directly related to a loss of quality of the final product in each year (Calzarano *et al*., 2004b), besides leading to a progressive weakening and finally to death of the vine.

There is considerable debate about the factors leading to development of foliar symptoms (Fontaine *et al*., 2016). These include the possible involvement of phytotoxic substances (Andolfi *et al*., 2011) produced by the pathogens, a relevant role of environmental factors, specifically rain (Marchi *et al*., 2006), and general vascular dysfunction triggered by inappropriate cultural practices (Lecomte *et al*., 2012). Whatever the causes (most probably a combination of factors) many authors have demonstrated drastic alteration of photosynthetic functions as well as stimulation of defense responses in affected grapevines several days before the appearance of the first foliar symptoms (Bertamini *et al*., 2002; Christen *et al*., 2007; Letousey *et al*., 2010; Mattii *et al*., 2010; Magnin-Robert *et a1*., 2011; Calamai *et al*., 2014). Early detection of a disease is important in studies of symptom development, and for evaluation of the efficacy of the few control strategies available (Di Marco *et al*., 2011b; Calzarano *et al*., 2014; Smart, 2015). Early

detection can also support understanding of the factors that incite symptom development, leading to prevention of yield and quality losses. Furthermore, despite the general lack of effective control methods, early detection of symptomatic vines can enhance effectiveness of local trunk treatment or management (Lafon, 1921; Calzarano *et al*., 2004a; Darrieutort and Lecomte, 2007; Smart, 2015).

The strong relationship between foliar symptoms and alteration of photosynthetic activity has been the key to suggest new methodology to investigate the onset of symptoms, based on optical techniques aimed to monitor parameters linked to leaves, and therefore host physiological state. Recent developments in optical technology have provided rapid and non-destructive methods for disease detection based on reflectance data and spectral vegetation indices (Johnson *et al*., 1996; Zhang *et al*., 2002; Bravo *et al*., 2003; Steddom *et al*., 2003; Steddom *et al*., 2005; Graeff *et al*., 2006; Delalieux *et al*., 2007; Huang *et al*., 2007; Larsolle and Muhammed, 2007; Delalieux *et al*., 2009; Naidu *et al*., 2009; Wang *et al*., 2009; Sankarana *et al*., 2010; Reynolds *et al*., 2012; Bellow *et al*., 2013; Mirik *et al*., 2013; Berdugo *et al*., 2014; Elarab *et al*., 2015; Martinelli *et al*., 2015). In particular, plant spectral properties at visible and near-infrared wavelengths can assist development of specific signatures for specific stresses in different species (Hatfield and Pinter, 1993; Peñuelas and Filella, 1998; West *et al*., 2003). The green vegetation spectral reflectance in the red band is most sensitive to leaf chlorophyll content, while the near infrared band is most related to biomass (Thomas and Oerther, 1972; Toler *et al*., 1981; Blazquez and Edwards, 1983; Kurschner *et al*., 1984; Blakeman, 1990). In that respect, the Normalized Difference Vegetation Index (NDVI) is a good parameter to evaluate leaf chlorophyll content, which is directly correlated with the health status of plants. NDVI is calculated from the following equation:

$$NDVI = (\varrho NIR - \varrho R) / (\varrho NIR + \varrho R)$$

where ϱNIR and ϱR are, respectively, the reflectance in near infra-red and red bands (Rouse *et al*., 1973). An example is the work of Bauer *et al*. (2011), which described a laboratory method to provide early and reliable detection of sugar beet leaf diseases based on high resolution multispectral images realized with Tetracam ADC-Lite. Since the end of the 1960s remote sensing has been used in plant disease detection with increasing frequency. Remote sensing techniques are replacing traditional methods in field or laboratory analysis when repetitive large-scale measurements are required, representing the only feasible approach for obtaining these data (Steven and Clark, 1990; Fitzgerald *et al*., 2004). Even from early reports, many authors have suggested that remote sensing could be used for provisional plant disease detection some days before visual symptoms became apparent (Manzer and Cooper, 1967; Burns *et al*., 1969). Those observations could be applicable also for GLSD or esca complex of grapevine (Christen *et al*., 2007; Mattii *et al*., 2010; Magning-Robert *et al*., 2011).

The principle on which remote sensing techniques is based in plant disease detection is to investigate physiological disturbances by recording changes in foliar reflectance in the near infrared portion of the spectrum, which is not perceptible by eye. Thus, remote sensing provides a quick and low cost tool to analyze biotic and abiotic stress from differences in the spectral characteristics of the crop canopy. The performance of aircraft remote sensing in precision viticulture is well explored (Johnson *et al*., 1996; Johnson *et al*., 2003b; Hall *et al*., 2003). The approach is currently increasing in flexibility and spatial resolution of up to 0.4 m per pixel, as compared with satellite platforms, which at most reach resolution of 1.2 m per pixel in multispectral bands (Worldview-3). However, disease detection requires greater spatial resolution (Calderón *et al*., 2013) in order to discriminate row from inter-row, and even each vine within a row. Technological automation developments have provided a new solution for remote monitoring, the Unmanned Aerial Vehicles (UAVs). These fixed-wing or rotary platforms can be remotely controlled by a pilot or fly autonomously on user-planned routes by means a complex flight control sensors. The key strength of UAV application in remote sensing is the high spatial ground resolution (centimeters), and a reduced planning time, which allows for highly flexible and timely monitoring (Johnson *et al*., 2003a; Baluja *et al*., 2012; Colomina and Molina, 2014; Mathews 2014; Matese *et al*., 2015; Mathews, 2015; Zaman-Allah *et al*., 2015).

Since 2011, the Precision Viticulture group of the Institute of Biometeorology of the National Research Council, in collaboration with the Section of Plant pathology and Entomology (DISPAA, University of Florence), has carried out a series of experiments focused on GLSD in a vineyard in Chianti Classico

Domain (Tuscany, Italy). The purpose was to study the relationships between NDVI, derived from high resolution images acquired by UAV platforms, and symptomatic plants monitored by ground based observations. Despite the general lack of clear and consistent explanations of the factors that incite foliar symptoms, one aspect that is widely accepted is the role of rain in late spring-early summer in increasing the appearance of GLSD symptoms (Marchi *et al*., 2006; Guérin-Dubrana *et al*., 2012; Andreini *et al*., 2015). The years 2012 and 2013 differed markedly for summer rain parameters, so it is relevant to compare the data obtained by the UAV surveys in those two years. A second objective was to develop an innovative methodology aimed primarily at quantitative and qualitative analysis of spatial distribution of symptomatic plants, and then to develop a predictive model for the onset of the foliar symptoms of GLSD.

Materials and methods

Experimental site and climate data

The research was conducted on a 1.22 ha vineyard located in the Chianti Classico Domain (43°40′11.48″N-11°8′30.23″E) in Tuscany, Italy. The vineyard was planted in 1998 with Cabernet Sauvignon vines, with rows aligned NW-SE, 2.8 m between rows and the vines spaced 1.0 m apart within the rows. The vineyard was trained as upward vertical shoot positioning and pruned as spur cordons. Irrigation was not applied. The vineyard is located on a south exposed slope (8%) at 150 m above sea level. The experimental plot consisted of ten adjacent rows 50 m long, providing a sample of 500 vines to be monitored. This plot was chosen because of the high GLSD incidence (almost every year over 30% of vines affected) when monitored at the single plant level since 2003, without variability between vines in terms of vigour and of the land slope (less than 5%). Ground observations were collected between May and September, at weekly intervals in 2012, and at monthly intervals in 2013, while the UAV flight survey was made at full bloom (25 May 2012) as a preliminary flight, fruit-set (25 June 2012 and 2013) and beginning of veraison (25 July 2012 and 2013). Climate was characterized with agrometeorological data acquired from a MeteoSense weather station (Netsens srl) located 10 m from the East side of the vineyard.

Ground observation

All vines in the experimental plot were classified in the following categories: S = symptomatic vines with pronounced symptoms related to 20–100% disease severity; C = control vines that never showed foliar symptoms; or A = asymptomatic vines that had shown symptoms in the previous years. The vines that showed less than 20% disease severity were not included in the S category because the symptoms were generally located in basal leaves, which has low impact on yield quality and quantity and are barely detectable by the UAV flight survey. The ground disease monitoring was performed by two well-trained surveyors by observing the foliage of each vine, on both sides of each row, including branches, shoots and bunches. A percentage value for disease severity was ascribed using an arbitrary disease scale, where 0 = no symptoms; 1 = 0.1–10%; 2 = 11–20%; 3 = 21–40%; 4 = 41–60%; and 5 = 61–100%. The typical wood alterations associated with the esca complex were verified in a random sample of symptomatic vines (S category), by inspecting transverse stem sections as described in previously (Surico *et al*., 2008).

Ground-based ecophysiological measurements

During the 2013 growing season, plant ecophysiological measurements on leaves and pruned wood were introduced. Stomatal conductance (gs) and leaf temperature were recorded using an infrared gas analyser LI-COR 6400 (LI-COR, Lincoln, NE, USA). To avoid the environment variability, Photosynthetic Active Radiation (PAR) was set at 1000 μmol m^{-2} s^{-1}, CO_2 air concentration at 400 ppm, and cuvette temperature at 30°C. Measurements were made in four fully expanded and even-aged leaves from five different plants per treatment from 09:00 to 16:00 h (solar time). Measurements considered only control (always asymptomatic) and asymptomatic (that showed symptoms in previous years) plants, because the leaves of symptomatic plants show large necrotic areas that do not allow correct measurement with the gas analyser.

Epidermal polyphenols, which are representative of total leaf phenols (Kolb and Pfundel, 2005; Barthod *et al*., 2007), were non-destructively measured on the same sample leaves using a portable leaf-clip device (Dualex; Force-A, Orsay, France). This determines epidermal absorbance in the UV-A wavelength (315–400 nm), which is mainly due to flavonoids (Goulas

et al., 2004; Cartelat *et al*., 2005). Two adaxial and two abaxial measurements were recorded in sequence from the middle part of each leaf avoiding the main veins, and flavonoid contents was calculated following the method of Pollastrini *et al*., (2011).

As a further test to evaluate differences among the three vine classes (Symptomatic, Asymptomatic, Control), the distance between nodes and the weight of the canes were measured as indicators of the rate of shoot growth during the 2012 season (Pratt, 1974; Mullins *et al*., 1992). In January 2013 a cane was collected from of each vine from 30 vines from each of the three vine classes (S, A, C). The cane portion between the 2nd and 7th node from the base of each 1-year-old cane grown was selected in the 2012 growing season to measure length and fresh weight.

Unmanned aerial platform

Multispectral images were acquired with a UAV platform, based on a modified multi-rotor Mikrokopter OktoXL (HiSystems GmbH) equipped with a nadir-facing Tetracam ADC-lite camera (Tetracam, Inc.) (Figure 2a). The camera weighed 200 g and had remote power and display features for optimized placement on UAV platforms. The primary use of this camera is to record vegetation canopy reflectance for derivation of several vegetation indices (NDVI, Soil Adjusted Vegetation Index, canopy segmentation and Near Infrared/Green ratios). Images were recorded in the visible red (R) wavelength (520–600 nm) and green (G; 630–690) and near infrared (NIR; 760–900 nm) spectra. Camera features such as 3.2 megapixel CMOS sensor (2048 ´ 1536 pixels), 8.5 mm lens and 43° field of view, provided a 0.05 m/pixel ground resolution at a flight height of 150 m. All images were taken between 12:00 and 13:00 h (solar time) each day, in clear sky conditions. A white reference image was taken to compute reflectance by framing a Teflon calibration panel just before take-off. The flight altitude was fixed at 150 m (above ground level), and the UAV flight speed was of 4 m s^{-1}. These settings allowed a 72% image forward overlap, while a waypoints route planned *ad hoc* ensured a 40% image side overlap, great enough to guarantee optimal photogrammetric processing. The UAV platform, camera features and image processing were described previously (Matese *et al*., 2015). Sample plants were georeferenced at high resolution (0.02 m) with a differential GPS (Leica GS09 GNSS, Leica Geosystems AG) to precisely discriminate vines along the rows (Figure 2b).

Figure 2. Instrumentation used in the study. A, Multirotor UAV platform (Mikrokopter, HiSystems GmbH, Moomerland, Germany), inset shows the multispectral camera (Tetracam ADC-Lite, Tetracam), B, Differential GPS (Leica GS09 GNSS, Leica Geosystems AG).

Data processing

PixelWrench2 software (Tetracam Inc.) was used to manage and process multispectral images, providing a batch file conversion from RAW to TIFF. Two captured images were assembled into a mosaic by Autopano Pro 3.6 Software (Kolor SARL,). Twenty white PVC panels, each of 0.25 × 0.25 m, were used as Ground Control Points (GCPs) and located at the beginning and end of each vine row of the plot. The panels were georeferenced in the field during image acquisition using the high-resolution differential GPS.

The QGIS software (Quantum GIS Development Team 2014, Quantum GIS Geographic Information System, Open Source Geospatial Foundation Project, http://qgis.osgeo.org) was used to georeference the mosaics utilizing GCP white panel coordinates in the georeferencing plugin. A thin plate spline (TPS) function was applied using a nearest neighbor resampling method to render the geometrically corrected mosaic. NDVI values were then calculated from the multispectral mosaic. Data extraction for single plants was performed from the NDVI maps with an *ad hoc* developed algorithm on Matlab software platform (MATLAB version 7.11.0.584, (2010), The MathWorks Inc.), by means of average values contained in 0.80 × 0.30 m polygons along the row axes, centered on each georeferenced vine. The polygon size was chosen in order to better distinguish each plant from the adjacent ones, providing a buffer of 0.40 m between consecutive vines as a consequence of the vine spacing of 1.0 × 2.8 m. The homogeneity of the canopy and the availability of high spatial resolution images allowed correct extraction of the pure canopy pixels of sample vines, and verification of the exclusion of pixels of the underlying grass or bare soil.

Statistical analysis

Statistical analysis of the pruning wood (weight and length) and NDVI data, were analyzed by one-way ANOVA, with R Stat (R Project for Statistical Computing; www.R-project.org). Means were then separated using the Tukey's HSD test at $P \leq 0.05$. Predictive power of UAV was statistically evaluated by comparing, for each flight of 2012, the symptom classes. Leaf parameters (stomatal conductance, leaf temperature and flavonoid content) were analyzed by one way ANOVA at $P \leq 0.05$.

Results

Climate analysis

According to the Winkler index (Winkler et al., 1974; Hall and Jones 2010) and other bioclimatic indices (Huglin Index, Sum of daily temperature excursion, Gladstones Index, cumulative rainfall, sum of daily min, max and mean temperatures, number of days of temperatures over 35°C), the growing season of 2012 showed a higher thermal regime than 2013 (Table 1, Figure 3).

The 2012 growing season was dry, characterized by high temperatures (37 d above 35°C), minimum rainfall events concentrated in April and May and an extreme summer drought (no more than 50 mm of rain between June and August). The 2013 season, on the other hand, had high total rainfall well distributed throughout the year, with 200 Growing Degree Days, which was less than the previous year. 2013 was a wet season with moderate temperatures (only 12 d with temperatures above 35°C). In summary, analyzing the experimental period, conditions were similar during April and May in

Table 1. Bioclimatic indices during the period between the years 2012–2013.

Year	WI[a]	HI[a]	SET[a]	GI[a]	Rain[b] 01/03–31/08	ST[c] min	ST[c] max	ST[c] mean	Days Tmax>35°C[d] 01/03–31/08
2012	1915	2630	2364	1914	257	2668	5032	3739	37
2013	1715	2406	2246	1715	631	2553	4799	3541	12

[a] WI, Winkler Index; HI, Huglin Index; SET, Sum of daily temperature excursion; GI, Gladstones Index.
[b] Cumulative rainfall (01/03–31/08).
[c] ST, Sum of daily min, max and mean temperatures (STmin, STmax, STmean).
[d] Number of days of temperatures over 35°C (01/03–31/08).

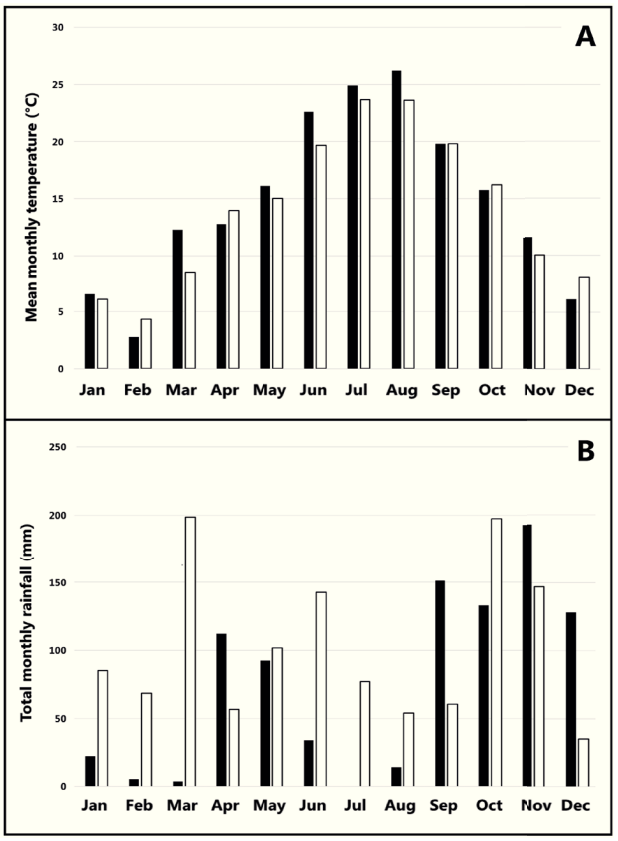

Figure 3. Climate analysis report. Climate data for 2012 (black) and 2013 (white): A, monthly mean temperature and B, total monthly rainfall.

both seasons, with moderate rainfall and moderate temperatures, while the summer months were hot and dry in 2012, while they were moderate and wet in 2013.

Image processing analyses

With the combined use of the UAV remote sensing platform and differential GPS, NDVI maps with high-resolution on the ground (0.05 m/pixels) were obtained. These allowed accurate analyses at single plant level. The algorithm developed in the Matlab platform allowed precise row identification and data extraction for each plant from the NDVI map (Figure 4). For each vine, base zonal statistics (mean, minimum, maximum and standard deviation) were calculated relative to each pixel contained inside the different polygons.

Comparison between field ground observation and UAV-based remote sensing NDVI

Stomatal conductance of leaves was significantly less in A vines compared to C vines, while leaf temperature in A vines was significantly greater than in C vines (Figure 5). Mean optical estimation of phenolic compounds showed significant differences between C and A vines ($F = 28.16$, $P \leq 0.0001$), with greater mean flavonoid values in the A vines (1.98 ± 0.10) compared with the C vine samples (1.85 ± 0.16).

The analysis of the pruning wood carried on the lengths and fresh weights of the shoots in the three vine classes showed that mean lengths of canes from C vines (49.7 cm ± 3.9) were significantly greater than those from A vines (46.1 cm ± 3.6; $F = 13.8$, $P<0.001$) and symptomatic (42.7 cm ± 3.6; $F = 52.19$, $P<0.001$) (Figure 6A). The mean weights of C vine canes (44.4 g ± 6.8) were significantly longer than those from A vines (32.1 g ± 6.6; $F = 49.95$, $P<0.001$) or S vines (25.9 g ± 5.0; $F = 142.4$, $P<0.001$) (Figure 6B). Mean weights and lengths of pruning wood showed a clear negative trend from C to S vines (Figure 6).

Figure 7 presents the NDVI values of the three vine classes (C, A and S) for 2012 and 2013, confirming this negative trend. Greater NDVI values were recorded for healthy vines (C) and lower NDVI values occurred for symptomatic vines (S). In both years, the asymptomatic plants (A) were characterized by lower NDVI values than healthy samples (C). NDVI values acquired in 2013 were greater than in the dryer 2012 season.

This trend could indicate a method to discriminate between possibly healthy vines and those that are known to be infected, since the asymptomatic vines had displayed GLSD foliar symptoms in the past. Anova analysis (Table 2) shows that the UAV approach correctly discriminated ($P \leq 0.05$) healthy vines from asymptomatic and symptomatic vines, and asymptomatic from symptomatic vines ($P \leq 0.05$) for each year, also in different meteorological conditions. The discrimination increased during each season: NDVI data analysis showed significant differences ($P \leq 0.05$) since the beginning of the monitoring period (May in 2012 and June in 2013), with maximum differences between each class in the last flight acquisition in July in both years ($P<0.001$).

This result suggests that further analysis should be carried out to determine if UAV remote sensing data can provide information about the onset of GLSD symptoms. Ground observations at weekly

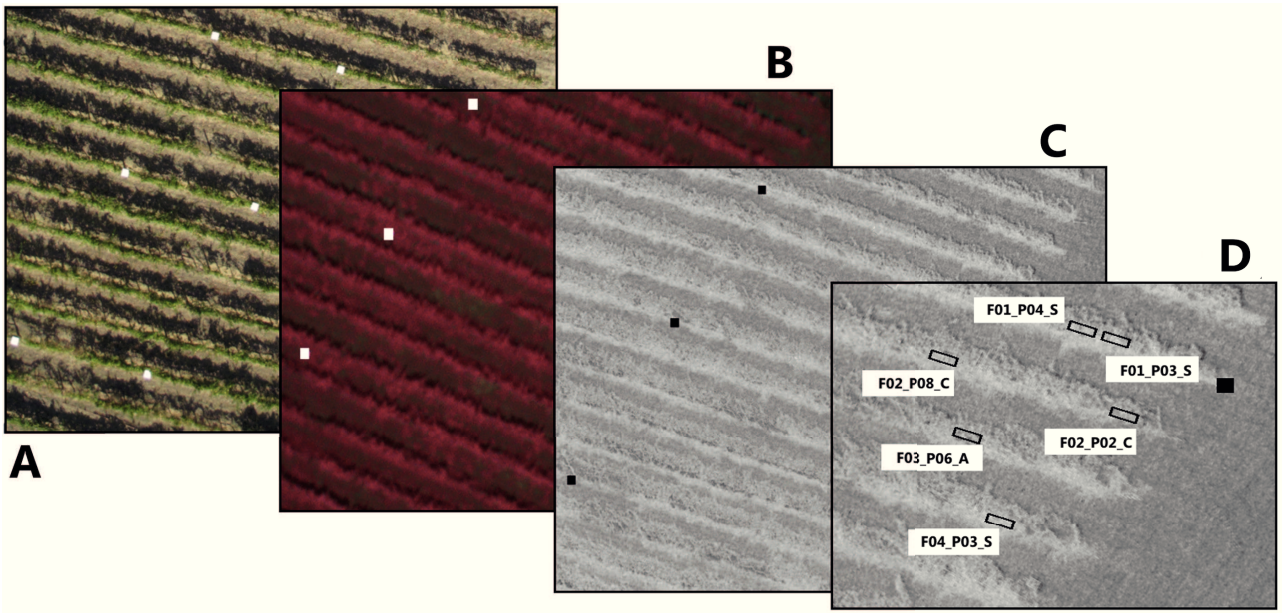

Figure 4. Image processing workflow. A, visible image, B, multispectral image, C, NDVI image, D, row detection and plant extraction data output.

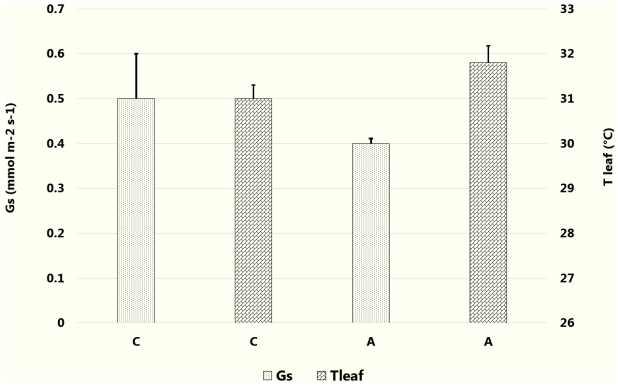

Figure 5. Ground-based ecophysiological measurements. Stomatal conductance (gs) and leaf temperature were recorded using an infrared gas analyser, on healthy (C) and asymptomatic (A) grapevines.

intervals were carried out in the 2012 season to produce accurate analysis of the predictive approach on the 2012 data. NDVI data of classes C (control), A (asymptomatic) and S (symptomatic) were compared to another class, that we define X, including only the vines that, despite being asymptomatic at the flight time, showed symptoms just 2–3 weeks after each UAV flight. Data analysis (Table 3) shows that the remote sensing methodology always identified apparently healthy vines that were going to show symptoms in the following few weeks. This approach had the best resolution ($P \leq 0.05$) both in June and July 2012, to discriminate C and X class, while no significant correlation could be detected in May. Furthermore the X class (vines going to be soon symptomatic) were quite close to the S class (symptomatic vines).

Discussion

Climate was identified as an important distinguishing element between the two growing seasons investigated in this study, and meteorological parameters are known to have a crucial influence on vegetative response of grapevines (Matese *et al.*, 2012). 2012 was a very low rainfall year, while 2013 had high rainfall which was well distributed throughout the season. This most likely stimulated the vegetative metabolism of the plants in 2013, which maintained a photosynthetic efficiency of the canopy until the end of September (Palliotti *et al.*, 2014). In 2012, on the other hand, the greatest NDVI values were reached in July. It is very likely that leaf

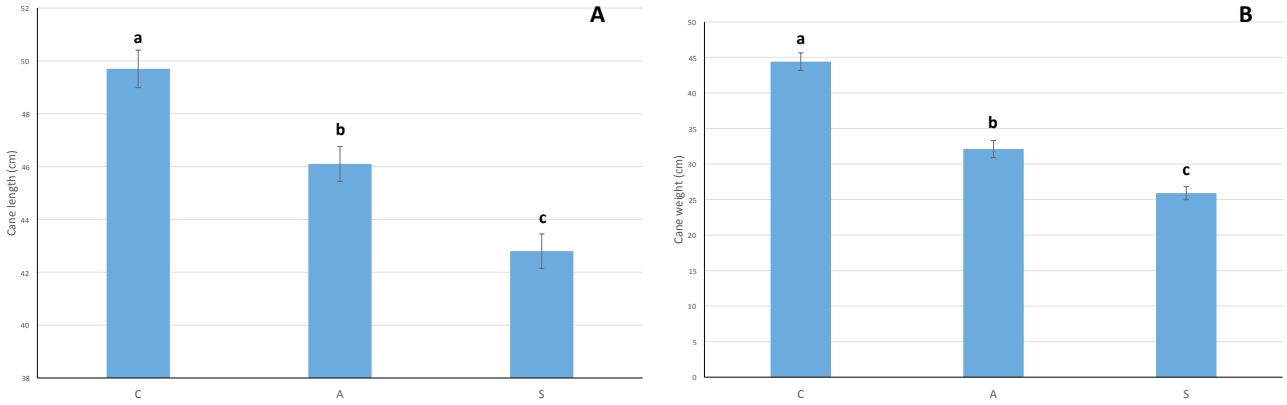

Figure 6. Mean lengths and fresh weights of the cane portion between the 2nd and 7th nodes from the base of canes grown in the 2012 season and collected at pruning in January 2013, from vines classified in the 2012 growing season in the three classes: control, always symptomatic or asymptomatic.

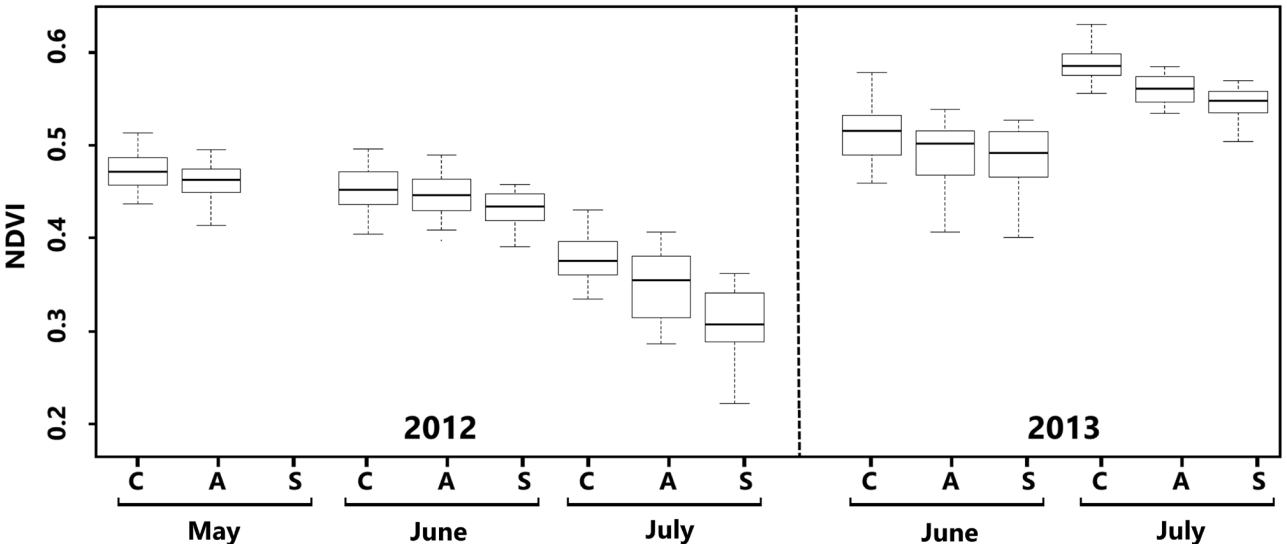

Figure 7. Relationship between NDVI data and ground observations. Boxplot graphical output of the correlation between NDVI values and GLSD symptoms for May, June and July of 2012 and 2013. The graph shows a negative trend in NDVI, with greater values in healthy vines (C) and lesser values in symptomatic vines (S), with intermediate values for the asymptomatic vines (A).

metabolism was affected by the disease history of the vines. Even when visually fully indistinguishable, leaves of asymptomatic vines had alterations (reduced stomatal conductance and increased leaf temperature; Petit *et al*. (2006)) that were related to the reduced NDVI values obtained by remote sensing. Differences between healthy and asymptomatic plants also emerged from the preliminary results on epidermal polyphenol contents. The role of phenolic compounds in the defense mechanism protecting grapevine against the fungi involved in esca disease, and against grapevine pathogens in general, has been well explored by several authors (Del Rio *et al*., 2004; Lattanzio *et al*., 2006; Amalfitano *et al*., 2011; Lambert *et al*., 2012). It could be hypothesized that the accumulation of flavonoids within the asympto-

Table 2. Detection power of UAV methodology was statistically evaluated (one-way ANOVA, Tukey's HSD test at $P \leq 0.05$) by comparing for each flight of 2012 and 2013, NDVI data of classes C (control), A (asymptomatic) and S (symptomatic).

Plant category	May 2012	June 2012	July 2012	June 2013	July 2013
Control	a	a	a	a	a
Asymptomatic	b	b	b	b	b
Symptomatic	n.d. [a]	c	c	c	c

[a] n.d., no symptomatic vines detected in May 2012.

Table 3. Predictive power of UAV was statistically evaluated (one-way ANOVA, Tukey's HSD test at $P \leq 0.05$) by comparing, for each flight of 2012, classes C (control), A (asymptomatic), X (asymptomatic vines at flight time that showed symptoms 2–3 weeks after the flight) and S (symptomatic).

Plant category	May 2012	June 2012	July 2012
C	a	a	a
A	a	b	ab
X	a	bc	b
S	n.d.[a]	c	b

[a] See Table 2.

matic plant leaves is the response of infected plants that have been able to prevent the manifestation of symptoms during the growing season.

The statistical analyses of the data collected in 2012 and 2013 shows that the very high resolution multispectral images acquired by UAV can discriminate different stress levels in grapevines, in relation to the visual symptoms of GLSD. In particular, Figure 7 shows good distinction between healthy plants (C) and symptomatic plants (S) in both years of this study, despite the different climatic conditions of the two growing seasons. The characterization of asymptomatic plants (A) is still relatively unexplored in the literature, but preliminary results confirm that the median of values of NDVI of asymptomatic vines is in an intermediate position with respect to the median of the other two disease classes, although with a partial overlap of boxplots. This intermediate position also clearly emerges from the data of cane lengths and weights obtained during a season when vines had been symptomatic, asymptomatic, disease-free ("control" vines). It is well known that a vine showing symptoms one growing season may not show symptoms in the next, and continues to give good quality wine. Nevertheless, the vigour of these vines is affected, as growth and wood production are reduced, with a clear trend in these parameters from control to symptomatic vines. Cane weights and lengths were less for the symptomatic vines, as expected, greater for the control vines that had never showed symptoms, and intermediate in the asymptomatic vines which had bee previously symptomatic vines. These data relate closely to the NDVI gradient recorded using UAV-assisted remote sensing.

The analysis of meteorological conditions confirmed substantial differences between the two seasons (Table 1), which were reflected in plant vigour. The 2012 season, was characterized by high temperatures and minimal precipitation, and produced significantly lower NDVI values than recorded in the 2013 season (Figure 5). Table 2 shows differences between healthy and diseased plants, but these differences were more evident when the foliar symptoms drastically lowered the photosynthetic efficiency, and were most pronounced in July, when symptoms progressively increased on the foliage. Table 3 explores an experimental approach where the sample of plants was reduced to consider only the plants that would manifest symptoms after 2–3 weeks. The sample is limited to about 15–20 plants per month, but this confirms the findings of other authors (Bertamini *et al*., 2002; Christen *et al*., 2007; Letousey *et al*., 2010; Mattii *et al*., 2010; Magnin-Robert *et a1*., 2011; Calamai *et al*., 2014). They showed that alteration of the photosynthetic activity of the leaves occurs a few weeks before the onset of GLSD symptoms, suggesting that further exploration of remote sensing methodology could be useful for the development of disease forecasting methods and further potential applications. In the present study, a homogeneous vineyard plot was used that was small, but had been monitored since 2003 at single plant level. This was to reduce other variables that could alter the data analyses. In this way it was possible to attribute changes of photosynthetic efficiency of the canopy to the onset of GLSD symptoms by excluding other potential causes of biotic or abiotic stresses. The

experimentation used here explored the relationship between remotely acquired NDVI values, and symptoms of GLSD obtained by visual observation of both the symptomatic plants and those that were asymptomatic in the year in which the field survey was carried out. These asymptomatic plants would have been incorrectly considered as healthy plants if the disease history of the vineyard was not known.

Conclusions

The methodology outlined in this paper has produced valuable results that indicate new possibilities for the use of remote sensing technologies for precision viticulture. UAV features, such as low cost, capability of timely provision of high resolution images, and flexibility of use in flight planning, provide an approach that can be easily applied for investigation and disease control strategy planning in vineyards. These methods are likely to be useful, both for research and for practical applications in wine production. The results of NDVI data analyses revealed in two growing seasons that asymptomatic vines in the year of the observation were distinguished from the plants that had never showed foliar symptoms from 2003 to 2013. This allowed the hypothesis that in the asymptomatic vines the spectral differences cannot be detected by in the human visible spectrum, but are detectable in the near-infrared wavelength (NIR) used by the detection system, and therefore can be identified and discriminated from the "healthy" vines (category "C"). However, additional experimentation will be required, across different growing seasons and climatic conditions, to validate and confirm this hypothesis, and to provide a useful tool for elucidating complex and not fully understood disease. Analysis of NDVI images could allow prompt identification of the early stages of the symptoms, even before they can be visually detected. However, the NDVI measurements have clear limits, because they provide indices capable of identifying alterations in photosynthetic efficiency, but do not discriminate the causes of biotic or abiotic stress afflicting plants. Our work has been possible because the experimentation could be carried out on a sample of plants monitored in detail, so that other causes of stress could be eliminated, confirming that alterations of NDVI were exclusively linked to symptoms of GLSD. It will also be possible to integrate our system with new optical technologies such as a hyperspectral camera. This powerful sensor allows qualitative measures on symptomatic leaf spectral responses with very high detail. A future perspective will be to identify the spectral signature specific for GLSD symptoms, to strictly discriminate GLSD from other vineyard stresses or diseases. Early detection could also support the planning and testing of economically rewarding field treatments, or the application of specific control methods on the single vines before they show the symptoms. The applications of such a predictive tool could open new perspectives in the knowledge and control of this important grapevine disease, within the increasing development of the precision agriculture approaches in viticulture.

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Grapevine Trunk Diseases: A Review of Fifteen Years of Trials for Their Control with Chemicals and Biocontrol Agents

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Grapevine trunk diseases (GTDs) are a group of diseases of grapevine caused by several fungal pathogens (Table 1) that live in and colonize the wood of the perennial organs causing wood necrosis, wood discoloration, vascular infections, and white decays (Bertsch et al. 2013; Mugnai et al. 1999). Affected vines show, externally, a general and progressive decline (delayed budburst, dead buds, dieback, stunted development, chlorosis, apoplexy, etc.) often associated with specific foliar symptoms according to the different diseases, that initially can cause loss of productivity and eventually death of the vine (Fig. 1).

One of the oldest of these diseases, known as "Esca," was the white rot caused by basidiomycetes, to which several symptoms in the crown had been linked. In the early 1990s, this was shown to be just one manifestation of a larger problem, a complex of different diseases known as the Esca complex. Many actors are involved: different pathogens, causing different wood symptoms and interacting with the vine, the environment, and each other in different and still not clear ways (Box 1). These different diseases overlapping in the same vine or developing at different stages of the vine life were soon recognized as forming one of the most important problems in grapevine growing areas. Due to the various names often used in the literature during these 15 years, here we use "Esca complex" to generally indicate the target pathogens of the tests, when not differently specified and addressing to the relative papers for further and more specific information.

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*The *e*-**X**tra logo stands for "electronic extra" and indicates that one supplementary file is published online.

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Since the 1970s, Eutypa dieback (formerly Eutypa dead arm) has been recognized as causing damage in some regions, especially in Australia, France, and California (Carter 1991; Carter and Price 1977; Munkvold et al. 1994; Péros and Berger 1994). When attention and research on GTDs and on their agents increased (Chiappara 2000), the importance of other canker agents, causing black dead arm (BDA, caused by *Diplodia mutila*) or Botryosphaeria dieback, became widely recognized (Phillips 2002; Urbez-Torres 2011). Nowadays, GTDs are seen as a serious threat to viticulture worldwide. A recent International Organisation of Vine and Wine (OIV) publication reported that in Italy, depending on the cultivar, on 15- to 18-year-old plants, incidence of GTDs ranged from 8 to 19% and around 10% in Spain. In France alone, approximately 13% of French vineyards are unproductive, with losses estimated at around €1 billion in 2014. In California, losses of at least US $260 million per year have been attributed to trunk diseases, while in Australia, mainly Eutypa dieback is responsible for damages of AU $8.3 billion to the wine industry (Fontaine et al. 2016a). Different causes could be considered responsible for the increasing incidence of GTDs (Lecomte et al. 2012; Surico et al. 2004) and among these, changes in cultural practices are surely a major inciting factor (Graniti et al. 2000): the lack of effective strategies and means to control these diseases surely contributed to their becoming widespread in all vine-growing areas.

In some countries in Europe, such as France, Portugal, and Spain, the sole product available to reduce the foliar symptoms within the Esca complex diseases (GLSD, Esca proper) was, for some decades, sodium arsenite. Despite this, incidence of the disease increased throughout Europe starting from the end of the 1980s. After the definitive ban of sodium arsenite in Europe in 2003, due to its high risks for humans and the environment (Larignon et al. 2008; Spinosi et al. 2009), no other control measure or any active ingredient (a.i.) was available to reduce the impact of the Esca complex. Meanwhile, the control of Eutypa and Botryosphaeria diebacks was based on pruning wound protection by treatments with carbendazim and benomyl (Magarey and Carter 1986; Ramsdell 1995). The banning of the two latter substances in most viticultural areas, especially in Europe, also put these two GTDs in the same situation as Esca complex. During the last decades, a large portion of research efforts was devoted to

selecting a.i.s and biological control agents (BCAs) able to limit or control GTDs by reducing wood infections.

This review does not cover the complexity of control strategies for GTDs, but aims to explain the main achievements of the scientific community worldwide in the search for products, i.e., a.i.s and BCAs, to control GTDs. After a short introduction on the problematic aspects of GTD control, the first part will be focused on a.i.s (organic and inorganic, natural or synthetic), and the second part on the trials with BCAs tested in the last 15 years. For each one, the methodology used and the results obtained against Botryosphaeria dieback, Esca complex, and Eutypa dieback in both vineyard and nursery are reported (Fig. 2). In the meantime, their efficacy and their sustainability for the winegrowers and the environment are discussed.

The Complexity of GTDs in Brief

The control of GTDs represents a big challenge for winegrowers, nurserymen, technicians, and scientists, mainly because of their complexity compared with other grapevine diseases such as powdery and downy mildew. In field trials, one of the intriguing and problematic aspects of GTDs is related to their undetermined latency period (asymptomatic status). All these diseases are linked to several wood pathogens able to infect the vines through wounds in the nursery or through pruning wounds in the field, causing wood discoloration and necrosis (Bertsch et al. 2013; Mugnai et al. 1999). Some of them also cause visible foliar symptoms. When the first external foliar symptoms occur, the wood may already be severely compromised (Calzarano and Di Marco 2007), giving few chances for viticulturists to reduce disease impact in the vineyard. This problematic aspect is confounded by the "erratic" behavior of the foliar symptoms displayed, especially for diseases in the Esca complex (GLSD, Esca proper). In the same vine, symptoms may appear in year n and not in year n+1, under the influence of environmental, climatic, and cultural factors (Marchi et al. 2006; Murolo and Romanazzi 2014; Sosnowski et al. 2011; Van Niekerk et al. 2011a) and that can lead to an underestimation of the real incidence in the vineyard in any given year. Latent infections are also dangerous in the propagation process since infected asymptomatic cuttings can transfer GTD pathogens by cross-contamination during the several steps of plant production (hydration, cold storage, grafting, callusing, etc.). If these infections are not controlled, an unsuspected spread of infected plants first in the nursery and then in the vineyard could occur (Aroca et al. 2010; Gramaje and Armengol 2011; Gramaje and Di Marco 2015).

The studies on GTDs conducted in several wine-growing areas worldwide have shown the large number of fungal genera and species associated with the diseases in the Esca complex, Eutypa and Botryosphaeria dieback, which are often different based on climate and geographical areas (Fischer 2006; Mostert et al. 2006; Van Niekerk et al. 2011a). These studies have also demonstrated how

Table 1. Fungal wood pathogens isolated from wood showing discoloration, necrosis, or decays of grapevines affected by major grapevine trunk diseases (Esca complex diseases, Botryosphaeria dieback, or Eutypa dieback). Species that proved pathogenic, at least as discoloration or necrosis agents, by artificial inoculations are indicated in bold (revised and adapted from Carlucci et al. 2015; Cloete et al. 2014; Úrbez-Torres 2014).

Family	Genus	Species
Botryosphaeriaceae	*Botryosphaeria*	***B. dothidea***
	Diplodia	***D. corticola*, *D. mutila*, *D. seriata***
	Dothiorella	***D. americana*, *D. iberica*, *D. sarmentorum***, *D. vidmadera*
	Lasidiplodia	***L. citricola*, *L. crassipora***, *L. exigua*, ***L. mediterranea*, *L. missouriana*, *L. theobromae*, *L. viticola***
	Neofusicoccum	***N. australe*, *N. luteum*, *N. macroclavatum*, *N. mediterraneum*, *N. parvum*, *N. ribis*, *N. viticlavatum*, *N. vitifusiforme***
	Phaeobotryosphaeria	***P. porosa***
	Spencermartinsia	***S. viticola***
Phaeomoniellaceae	*Phaeomoniella*	***P. chlamydospora***
Togniniaceae	*Phaeoacremonium*	***P. minimum*, *P. angustius***, *P. alvesii*, *P. argentiniense*, ***P. armeniacum***, *P. australiense*, *P. austroafricanum*, ***P. canadense*, *P. cinereum*, *P. croatiense*, *P. globosum*, *P. hispanicum*, *P. hungaricum*, *P. inflatipes*, *P. italicum*, *P. iranianum*, *P. krajdenii*, *P. mortoniae*, *P. occidentale***, *P. roseum*, *P. scolyti*, *P. sicilianum*, ***P. tuscanum*, *P. venezuelense*, *P. viticola***
Helotiales	*Cadophora*	***C. luteo-olivacea*, *C. melinii***
Hymenochaetaceae	*Fomitiporia*	*F. australiensis*, *F. capensis*, ***F. mediterranea***, *F. polymorpha*, ***F. punctata***
	Fomitiporiella	*F. vitis*
Stereaceae	*Inocutis*	*I. jamaicensis*
	Phellinus	*P. igniarius*
	Stereum	*S. hirsutum*
Diatrypaceae	*Eutypa*	***E. lata*, *E. laevata*, *E. leptoplaca***, *Eutypa sp.*, ***E. citricola***
	Eutypella	*E. cryptovalsoidea*, ***E. microtheca*, *E. vitis*, *Eutypella* spp.**
	Cryptosphaeria	***C. lignyota*, *C. pullmanensis***
	Cryptovalsa	***C. amplelina*, *C. rabenhorstii***
	Diatrype	*D. brunneospora*, ***D. oregonensis*, *D. stigma*, *D. whitmanensis*, *Diatrype* sp.**
	Diatrypella	*D. verrucaeformis*, ***D. vulgaris***
Pleurostomataceae	*Pleurostomophora*	***P. richardsiae***
Diaporthaceae	*Diaporthe*	***D. ampelina*, *D. eres***

their life cycles are strictly connected with some cultural practices typical of viticulture, especially dormant pruning. Pruning wounds are the main point of entry for GTD pathogens into the vines. Depending on the pathogen, wounds can remain susceptible to infection by GTD pathogens for up to 2 to 4 months (Eskalen et al. 2007a; Rolshausen et al. 2010; Serra et al. 2008; Van Niekerk et al. 2011b). As the wood of diseased vines can be simultaneously infected by various pathogens typically associated with different

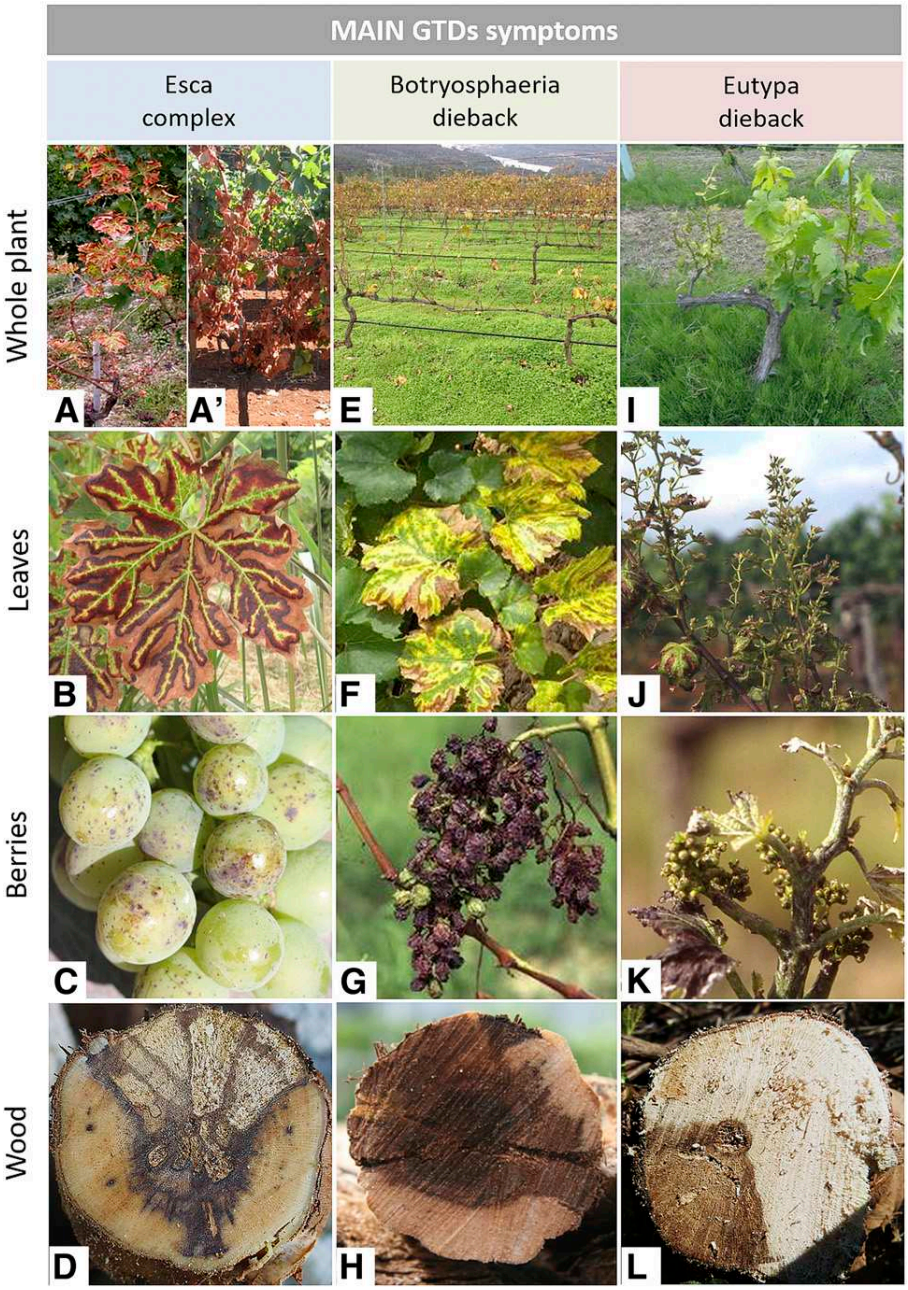

Fig. 1. Symptomatology of the main grapevine trunk diseases (GTDs) occurring in adult vineyards. The different diseases included in the Esca complex have different symptoms but some wood symptoms in common. In the vineyard, grapevine leaf stripe disease (GLSD) or Esca proper symptoms are visible in summer and the whole vine can show: **A,** leaf stripe symptoms; **A',** sudden wilting of the whole plant or part of the plant, i.e., apoplectic symptoms or apoplexy, a nonspecific but frequent symptom in older vineyards. **B,** A detail of the typical tiger-stripe pattern of symptomatic leaves. The external symptoms of GLSD can also include minute black spots called "black measles" on berries (**C**). The wood of vines affected by GLSD shows black spots, vascular streaking, discoloration, necrosis, and especially in vines more than 8 years old, can also show white decay (giving the complete Esca proper syndrome including all wood and foliage symptoms associated with Esca complex) (**D**). The first symptoms of Botryosphaeria dieback (often confused with black dead arm [BDA] by *Diplodia mutila*, and synonym of Botryosphaeria canker) are visible from budburst in spring and consist of dead spurs/buds and in a stunted or delayed growth (**E**). It can also appear in a severe form similar to Esca apoplexy. Foliar symptoms, when present, can be similar to chlorosis or to Esca (**F**). Botryosphaeriaceous fungi could determine also bunch rot on grapes (**G**). The wood of Botryosphaeria-infected plants shows the presence of brown necrotic sectors, typically arc-shaped and sectorial, wedge shaped necrosis, (**H**). The external symptoms of Eutypa dieback (formerly Eutypiose) appear early in the vegetative season, with stunted shoots with shortened internodes (forming witch's broom symptoms) (**I**). Leaves are small, chlorotic, cupped, and deformed (**J**); the grapes on the affected canes do not form at all or are small, poorly developed, and straggly (**K**). The internal wood of Eutypa-infected vines shows wedge-shaped grayish necrotic sectors, indistinguishable from Botryosphaeria dieback cankers (**L**). Simultaneous presence of pathogens of different GTDs in the same vine can result in overlapping symptoms. (Esca pictures: **A, B** courtesy of Institute of Agriculture and Tourism, Poreč, Croatia; **A'** courtesy of Maurizio Gily, Italy; **C** courtesy of DRL Rheinpfalz, Germany; **D** courtesy of Plant pathology sector SAF dept., University of Palermo, Italy. Botryosphaeria dieback pictures: **E**, courtesy of Feuga, Spain; **F** and **G**, courtesy of P. Larignon, France; **H** courtesy of Plant pathology sector SAF dept., University of Palermo, Italy. Eutypa dieback pictures: **I**, courtesy of ADVID, Portugal; **J**, **K** courtesy of P. Larignon, France; **L**, courtesy of Eger Food and Wine Research Center, Hungary.)

Box 1
The Esca complex of diseases.

The studies on Esca carried out from the 1980s have led to a deep revision of this trunk disease, on pathogens involved, and on symptom expression, in particular. Since then, researchers collected information and clues to explain the complexity not only in the internal symptoms but also in the association of the pathogens to different external symptoms, as only the wood symptoms could be reproduced by artificial infections, while the external symptoms could not be fully reproduced. The interpretation of the data collected led to an evolution in the description of the disease and a succession of different proposals. The more recent one revealed Esca as a "complex of diseases," characterized by different diseases (in particular wood decay and a vascular disease) and different syndromes according to the stage of the vine life, to the type of wood and foliar symptoms, and/or to the pathogens infecting and acting into the vine. The old denomination of "Esca" as a wood white decay causing "apoplexy" (sudden wilting of the vine) and typical foliar symptoms is replaced by five different diseases/syndromes (dark wood streaking, Petri disease, grapevine leaf stripe disease [GLSD], white rot, and Esca proper) grouped under the name of "Esca complex." These syndromes differ based on the age of the symptomatic vines and the pathogens involved (in white decay and Esca proper, basidiomycete decay agents are involved, while in all the others, vascular ascomycete species are the common factor, as *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., and more recently [in Petri disease], *Cadophora* spp.). The dark wood streaking is a wood symptom proving the presence of the pathogens in vines from the nursery, while the Petri disease is typical of very young vines, a decline developing in new plantations. The typical wood symptoms in both is the vascular discoloration, often coupled with the emission of dark tarry drops (see related pictures). The Petri disease could also determine foliar symptoms (chlorosis and decline, up to death of the vines). The GLSD syndrome is characterized by the typical tiger-stripe pattern on symptomatic leaves and is often associated with a partial or complete apoplexy in affected plants. It can be found in both young and adult vineyards, both in vines showing only vascular pathogens infections or those also showing wood decay. A longitudinal orange-brown stripe under the bark can usually be noticed removing the bark. The white decay is determined by basidiomycete fungi (in Europe, *F. mediterranea*), which determine only symptoms on the infected wood. Finally, the name Esca proper was retained to refer to the original name (Esca as decayed wood used as tinder), typical of adult vineyards, and characterized by the contemporary presence of the main vascular pathogens plus the basidiomycete species and the development of foliar symptoms (for more information, see Gubler et al. [2015] and Surico [2009]). Pictures: Dark wood streaking, V. Mondello; Petri disease, courtesy of Feuga (Spain); GLSD, courtesy of Plant pathology sector SAF dept., University of Palermo (Italy), DRL Rheinpfalz (Germany), and Maurizio Gily (Italy); white rot, upper picture courtesy of DRL Rheinpfalz (Germany); Esca proper, courtesy of Plant pathology sector SAF dept., University of Palermo (Italy), Feuga (Spain), and ADVID (Portugal).

GTDs, the different internal and external symptoms can overlap. Furthermore, some of the wood infections caused by GTD agents lead to foliar symptoms due to physiological factors (Lecomte et al. 2012), but also because of phytotoxins produced by the pathogens (Abou-Mansour et al. 2015; Andolfi et al. 2011; Sparapano et al. 2000; Tabacchi et al. 2000). In addition, grapevine cultivars differ in their sensitivity to the development of foliar symptoms in some GTDs even if no cultivar or species in the genus *Vitis* has been found to express complete resistance to GTDs (Bertsch et al. 2013; Guan et al. 2016).

Altogether, these factors are enough to complicate the linear relationship among symptom(s), disease diagnosis, and control strategy that exists in other plant diseases. For GTDs, external symptoms are not useful for rapid and early diagnosis. Diagnostic tools in terms of traditional, molecular, and serological methods may lead to identification of a group of associated pathogens with various virulence levels according to species or even to strains and that could eventually have different sensitivity to a specific a.i. or treatment. In this context, the possibility of an efficient control of GTDs has worsened, as reported before, by the lack of valid and simple control protocols.

Toward New Chemicals Through the Experience of Sodium Arsenite

While the mode of action of the two banned benzimidazoles, benomyl and carbendazim, against fungi is well known (they bind to spindle microtubules, interfere with spindle formation, inhibit mitosis, and thus inhibit germ tube and mycelial growth), to date, no definitive mechanism has been identified to explain how sodium arsenite acts in suppressing foliar symptoms within Esca complex (GLSD, Esca proper) and has some, even if lower, efficiency against Eutypa and Botryosphaeria dieback (Larignon and Dubos 2001; Larignon et al. 2008). It can penetrate into woody tissues and move inside the vine and has been found in sap, leaves, bunches, and roots. It can remain within wood, especially rotten wood, explaining the persistent effect even in years without treatment. However, its concentration in sap (in the order of µg/liter) is insufficient to inhibit fungal growth, which can be obtained in the laboratory only at mg/liter concentrations (Larignon et al. 2008; Santos et al. 2006). Some senescence effects (lower chlorophyll content and fluorescence levels) and lower growth rate in plantlets due to sodium arsenite have been reported (Santos et al. 2006), while no toxic concentrations were found inside bunches or roots. The suppression of GLSD foliar symptoms

by sodium arsenite could thus be related to a complex of interactions between the a.i., the plant microbiome, pathogen metabolism, and host metabolism. As a consequence of this hypothesis, no a.i. can be excluded a priori in the search for an alternative to sodium arsenite. Thus, the evaluation of either existing or novel a.i.s that can be used to reduce the upsurge of GTDs has been a major priority for industry and researchers during the last two decades. Although only one paper reports promising results with this type of winter application to the wood, applying a copper based product (Di Marco et al. 2011b), it does confirm that this type of applications warrants further exploration.

To date, the first barrier in this challenge is to identify the targets due to the lack of knowledge about the etiological agents of GTDs, except for diseases such as Eutypa dieback clearly related to *E. lata*, or cankers caused by Botryosphaeriaceae species. The second challenge is to manage GTDs at all stages from the production of new plants in nurseries to the growth of healthy vines in vineyards over the years and during a long period. Therefore, a.i.s have been tested by various researchers for preventive/sanitation protocols in the nursery, while they were tested in the field for their ability to reduce GTDs incidence by i) protecting pruning wounds, avoiding new infection; ii) suppressing foliar symptom expression and the related productivity and quality losses; and iii) improving or stimulating plant defenses against GTD pathogens.

Main Strategies Used in Testing Active Ingredients Against GTDs

In the search for GTD control agents, particular attention has been devoted to detect antifungal compounds to be employed against the main pathogens that have been revealed by research. This wide-range search has considered different organic and inorganic compounds, both synthetic and natural, and tested by various researchers (Tables 2 to 6).

Active ingredients have been assayed by different methods according to the kind of test (in vitro, in planta, and in the field), their usage (in nurseries, to reduce disease incidence, wound protection, symptom suppression, or improve plant defense mechanisms), and the specific GTD to manage. In vitro trials have been used to evaluate the efficiency of the a.i. in terms of its EC value (EC_{50} and, sometimes, EC_{90}) calculated either on percentage conidial germination or inhibition of mycelial growth of the pathogens on nutrient media amended with different concentrations of the a.i. (Bester et al. 2007; Cobos et al. 2015; Fleurat-Lessard et al. 2011; Gramaje et al. 2012; Halleen et al. 2010; Jaspers 2001; Martín and Martín 2013; Mazzullo et al. 2000; Nascimento et al. 2007; Pitt et al. 2012; Santos et al. 2006; Sosnowski et al. 2013). Some authors used both assays (Amponsah et al. 2012; Di Marco et al. 2011b; Rolshausen and Gubler 2005; Sosnowski et al. 2008, 2013) supplemented by studies on morphological modifications of the pathogen's mycelium (Fleurat-Lessard et al. 2011). In planta bioassays have also been useful to analyze other parameters on infected/treated plants such as wood colonization by GTD pathogens and plant mortality (Cobos et al. 2015; Nascimento et al. 2007), phytotoxic effects of the a.i. and its accumulation in host tissues (Di Marco et al. 2011b; Fleurat-Lessard et al. 2011), host plant growth (Cobos et al. 2015; Nascimento et al. 2007; Santos et al. 2006), and physiological

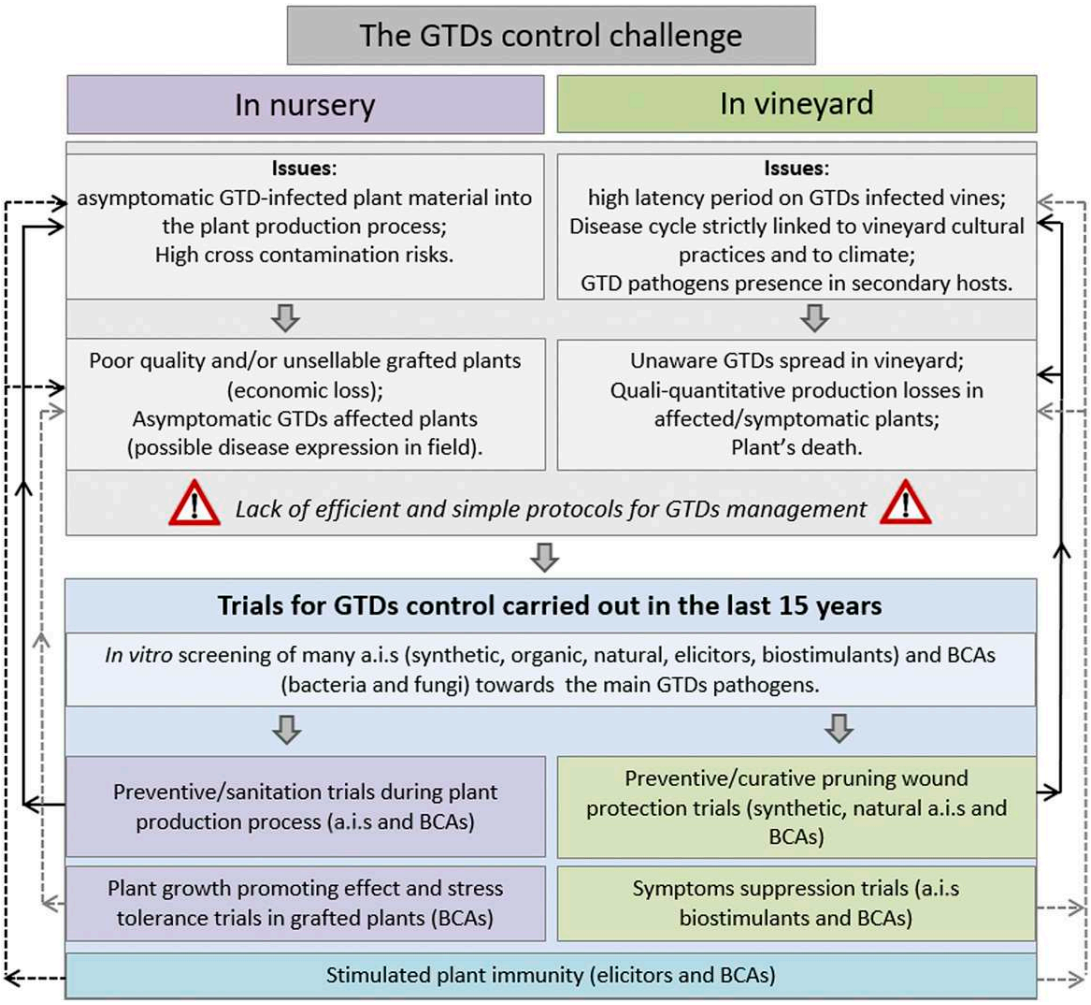

Fig. 2. The scheme followed by this review reflects the problems related to grapevine trunk disease (GTD) presence in both nursery and vineyard (in gray boxes), reporting the different strategies (colored boxes) set up by the research to solve the lack of suitable and efficient disease control protocols so far existing. a.i.: active ingredient; BCA: biocontrol agent.

changes (oxidative stress: Cobos et al. 2015; Santos et al. 2006; induction of defense mechanisms: Cobos et al. 2015; Di Marco et al. 2011a; Pierron et al. 2015; chlorophyll contents and fluorescence or leaf gas exchange: Di Marco et al. 2011a; Santos et al. 2006). The plant material used included plantlets, cuttings, and potted vines depending on the research aims, the trial timespan, and the a.i. selected for potential use against GTD pathogens. Plantlets were used for the evaluation of the a.i. impact on vine physiology by Santos et al. (2006) in laboratory and controlled conditions, avoiding the influence of environmental biotic and abiotic factors that are normally difficult to control in greenhouse and field experiments. Bioassays with cuttings allowed the evaluation of a.i. efficiency after a relatively short period, ranging from 5 to 90 days after inoculation and mainly by pathogen reisolation, thus revealing the disease control capacity of an a.i. to reduce internal symptoms in terms of lengths of vascular discoloration (Bester et al. 2007; Cobos et al. 2015; Díaz and Latorre 2013).

Table 2. List of the synthetic organic compounds tested, singly or mixed in lab or as found in commercial products, against the three main grapevine trunk diseases in field and nursery

		Tested on			Tested in		
Chemical group	**Active ingredient**	**Botryosphaeria dieback**	**Esca complex**	**Eutypa dieback**	**Lab**	**Vineyard**	**Nursery**
Anilinopyrimidine	Cyprodinil	x			x		
Anilopyrimidine	Pyrimethanil	x	x	x	x	x	
Arylamilopyrimidine	Fluazinam	x		x	x	x	
Benzimidazoles	Benomyl	x	x	x	x	x	
	Carbendazim	x	x	x	x	x	x
	Thiophanate methyl	x	x	x	x	x	x
Benzonitrile	Chlorothalonil	x		x	x		
Carboxamide	Boscalid	x			x		
Dicarboximide	Iprodione	x	x	x	x	x	x
	Procymidone	x			x	x	
Disinfectans	Didecyldimethylammonium chloride						x
	Alcohol-phenol-iodine solution						x
Dithiocarbammate	Mancozeb	x	x	x	x	x	
	Thiram		x		x		x
	Ziram		x	x	x		
Phenoxyquinoline	Quinoxyfen	x		x	x		
Hydroxianiline	Fenhexamid	x	x	x	x	x	
Imidazole	Prochloraz	x	x	x	x	x	x
	Imazalil			x	x	x	x
Morpholine	Dimethomorph			x	x		
Nitrile	Dithianon	x			x		
Phenylpyrrole	Fludioxonil	x			x		x
Phthalimide	Captan	x	x		x		x
Pyrimidine	Fenarimol	x	x	x	x	x	
Quinoline	Hydroxyquinoline sulfate	x	x		x		x
Spyroketalamine	Spiroxamine	x	x	x	x		x
Strobilurins	Azoxystrobin	x		x	x		x
	Kresoxym-methyl	x	x	x	x		
	Pyraclostrobin	x	x	x	x	x	
	Trifloxystrobin			x	x	x	
Sulphamid	Tolyfluamid	x	x		x		
Triazoles	Cyproconazole		x		x	x	x
	Difenoconazole		x			x	
	Flusilazole	x	x	x	x	x	x
	Myclobutanil	x		x	x	x	
	Penconazole	x	x	x	x	x	
	Propiconazole		x	x		x	
	Tebuconazole	x	x	x	x	x	x
	Tetraconazole	x	x	x	x	x	
	Thiabendazole		x		x	x	
	Triademenol	x	x	x	x	x	
Commercial mix	Boscalid + pyraclostrobin			x	x	x	
	Carbendazim + flusilazole		x		x		
	Carboxine + thiram		x				x
	Cyproconazole + iodocarb	x	x	x		x	
	Cyprodinil + fludioxionil	x	x	x	x	x	x
	Mancozeb + metalaxyl-m			x	x		
	Prothioconazole + tebuconazole			x			
	Pyraclostrobin + metiram	x	x				x
	Tebuconazole + boric acid + octhilinone			x		x	
Organic salts	Copper ammonium acetate	x		x	x		
	Copper bis(ethoxy-dihydroxy-diethylamino) sulfate						x
	Fosethil-al	x	x			x	
Triazoles lab mix	Propiconazole + thiabendazole		x			x	
	Difeconazole + tebuconazole		x			x	

Tests in the vineyard have focused mainly on wound protection from GTD pathogens or reduction of GTD incidence, permitting the evaluation of alternative a.i.s selected under standard conditions, since the a.i. action was tested under the influence of cultural practices and geographical and climatic variables during the test lifetime. Usage has been focused mainly on wound protection against GTD pathogens or reduction of GTD incidence and/or severity. The methodology developed is closer to the hypothetical practical use, utilizing the usual agricultural equipment for a.i. distribution (Halleen et al. 2010; Sosnowski et al. 2013), or trying different application tools such as systemic a.i. pole injection in soil (Di Marco et al. 2000) or directly into vines by trunk injection (Calzarano et al.

Table 3. Inorganic compounds tested against the three main grapevine trunk diseases in field and nursery

		Tested on			Tested in		
Chemical group	**Active ingredient**	**Botryosphaeria dieback**	**Esca complex**	**Eutypa dieback**	**Lab**	**Vineyard**	**Nursery**
Inorganic acid	Boric acid	x		x	x	x	
	Phosphorous acid		x	x	x		x
Inorganic salt	Calcium polysulfides		x				x
	Phosphonic acid salt		x	x	x		
	Iron sulfate	x	x	x	x		
	Sodium arsenite	x	x	x	x		
	Copper oxychloride	x	x		x	x	
Inorganic base	Copper hydroxide	x	x	x	x		
Inorganic element	Sulfur		x	x	x		

Table 4. Natural compounds tested against the three main grapevine trunk diseases in field and nursery

		Tested on			Tested in		
Group	**Natural compound**	**Botryosphaeria dieback**	**Esca complex**	**Eutypa dieback**	**Lab**	**Vineyard**	**Nursery**
Organic extract	*Allium sativum*	x	x	x	x		
	Evernia prunastri lichen	x	x	x	x		
	Green coffee	x	x	x	x		
	Lemon peel	x	x	x	x		
	Melaleuca alternifolia oil			x	x		
Organic	Chitosan	x	x	x	x		
	Honey			x	x		
	Lactoferrin			x	x		
	Propolis	x	x	x	x		
	Saponins	x			x		
	Vanillin	x	x	x	x		
Inorganic	Potassium bicarbonate			x	x		
	Hydrogen peroxide	x	x	x	x		x
	Ozonated water		x		x		
Lab mix	Allium + chitosan + vanillin	x	x	x	x	x	
	Seaweed extract + $CaCl_2$ + $Mg(NO_3)_2$		x			x	

Table 5. Plant-defense stimulating compounds tested against the three main grapevine trunk diseases in field and nursery

		Tested on			Tested in		
Group	**Active ingredient(s) (commercial name)**	**Botryosphaeria dieback**	**Esca complex**	**Eutypa dieback**	**Lab**	**Vineyard**	**Nursery**
Commercial products	Aluminum lignin sulfate, gluconic acid, microelements (Brotomax)		x	x	x	x	
	Amino acids, peptides, peptones (Fitostim)		x		x	x	
	Glutatione, oligosaccharine (Marvita)		x		x	x	
	Aschophyllum nodosum extract (Kendal)		x		x	x	
Plant-defense elicitor	2-hydroxybenzoic acid		x	x	x		
	Benzothiodiazole		x		x		
	Seaweed extracts + Ca + Mg		x			x	
Phytoalexins	Resveratrol		x	x	x		
	Pterostilbene		x	x	x		
	P-coumaric acid		x	x	x		
	Pterostilbene + resveratrol		x	x	x		
Lab mix	Copper oxychloride + gluconates		x		x	x	
	Resveratrol + phosphorous acid		x	x	x		

2004; Darrieutort and Lecomte 2007; Dula et al. 2007), as well as manual applications for wound protection (Díaz and Latorre 2013; Sosnowski et al. 2008).

For the protection of pruning wounds, the duration of the trials reported in literature is variable, since the trials could be repeated identically or with some changes in a.i. and/or pathogen application time (Amponsah et al. 2012; Díaz and Latorre 2013; Halleen et al. 2010; Pitt et al. 2012; Rolshausen et al. 2010; Sosnowski et al. 2008, 2013). In the trials for disease incidence reduction in GTD-affected vineyards, the duration was highly variable, ranging from 1 to 9 years, with the last 1 to 2 years sometimes devoted only to the observation of the treatment residual effects (Calzarano et al. 2004; Darrieutort and Lecomte 2007; Di Marco and Osti 2009; Di Marco et al. 2000, 2011a, b; Dula et al. 2007).

Due to the diversity of fungal species associated with GTDs, several different pathogens have been used in the in vitro assays, while in both in planta and field tests the most used species are *Phaeomoniella chlamydospora* and several *Phaeoacremonium* species for Esca complex, *D. seriata*, *D. mutila*, *L. theobromae*, and *N. parvum* for Botryosphaeria dieback, and *E. lata* for Eutypa dieback (Amponsah et al. 2012; Díaz and Latorre 2013; Halleen et al. 2010; Pitt et al. 2012; Rolshausen et al. 2010; Sosnowski et al. 2008, 2013).

The Most Efficient Active Ingredients Against GTDs

According to our literature review, more than 90 a.i.s have been tested from 2000 until now against the Esca complex diseases, Botryosphaeria dieback and Eutypa dieback. Results are reported in Tables 7 to 10 and grouped according to their main characteristics (synthetic organic, inorganic, natural, biostimulants, and plant defense elicitors). For each a.i., efficiency in relation to the tests (in vitro, in planta, in the field), the specific aim of the study (wound protection, symptom suppression, GTD control in nurseries), and the targeted GTD pathogens are reported, taking into account the geographical area of the test.

Synthetic organic active ingredients and compounds tested. Most of the assayed a.i.s (55 out of 93 in Table 7) are synthetic organic compounds, used both singly (44) or in mixtures (11). Triazoles (10 a.i.s), strobilurins (4 a.i.s), and benzimidazoles (3 a.i.s) represent the chemical groups most often tested. The now banned benzimidazoles benomyl and carbendazim were used as positive controls in several tests. Eighteen a.i.s were tested against all three GTDs considered in this review and 19 against only one GTD. Considering the kind of test, the number of a.i.s tested decreased moving from in lab tests (in vitro and in planta) to the in field tests (42 and 31 a.i.s, respectively). Only nine synthetic organic compounds were tested in both field and nursery.

Among the benzimidazoles, the most efficient a.i.s were benomyl, carbendazim, and thiophanate-methyl, which always showed high efficiency in both lab and field for pruning wound protection and in nurseries, irrespective of the geographical area or GTD targeted (Amponsah et al. 2012; Díaz and Latorre 2013; Gramaje et al. 2009; Groenewald et al. 2000; Halleen et al. 2010; Jaspers 2001; Pitt et al. 2012; Sosnowski et al. 2008, 2013). Their efficacy toward the main GTDs was certainly due to their broad-range fungicidal activity, their persistence, and their systemic activity. In the in vitro tests, they showed high capability in reducing both mycelial growth and conidial germination in all species except *E. lata*. For pruning wounds, benzimidazoles protected vines from new infection mainly as preventive treatments and, to a lesser extent, in curative ones. Thus, Sosnowski et al. (2008) reported the long-lasting preventive effect of benomyl in protecting wounds from *E. lata* infections (14 days) and the slight curative effect of benomyl and carbendazim, observed only if the a.i.s were applied 1 day after inoculation with *E. lata*. Benzimidazoles sprayed or painted on pruning wounds showed similar efficiency. To date, only thiophanate-methyl is approved for use in agriculture and it is currently applied to control *Botrytis cinerea* in grapevine. A disadvantage of benzimidazoles is that fungi can develop resistance to this class of compounds, as observed in *Phaeoacremonium minimum* toward carbendazim (Martín and Martín 2013). Consequently, benzimidazoles in use should be mixed with another a.i. or used alternately with other a.i.s. In nurseries, benzimidazoles successfully reduced the presence of vascular Esca complex pathogens. For example, benomyl and carbendazim decreased the amount of pathogen inoculum in grafted plants when applied during hydration or before grafting (Fourie and Halleen 2006; Gramaje et al. 2009), while soaking the scions in thiophanate-methyl before grafting (Serra et al. 2011) followed by hot water treatment (Eskalen et al. 2007b) successfully controlled Esca complex associated pathogens.

Table 6. Grapevine trunk disease (GTD) pathogens employed in the active ingredient (a.i.) and biocontrol agent (BCA) evaluation trials. The most used species are in bold.

GTD	Target pathogen used in a.i.s tests	Target pathogen used in BCAs tests
Botryosphaeria dieback	*Botryosphaeria dothidea*	*Diplodia mutila*
	Diplodia mutila	*Diplodia corticola*
	Diplodia seriata	***Diplodia seriata***
	Dothiorella viticola	***Lasiodiplodia theobromae***
	Lasiodiplodia theobromae	*Lasiodiplodia mediterranea*
	Neofusicoccum australe	*Neofusicoccum australe*
	Neofusicoccum luteum	*Neofusicoccum luteum*
	Neofusicoccum parvum	*Neofusicoccum mediterraneum*
		Neofusicoccum parvum
Esca complex	*Fomitiporia mediterranea*	*Cadophora luteo-olivacea*
	Phaeomoniella chlamydospora	*Fomitiporia mediterranea*
	Phaeoacremonium minimum	***Phaeomoniella chlamydospora***
	Phaeoacremonium angustium	*Phaeoacremonium minimum*
	Phaeoacremonium parasiticum	
	Pleurostomophora richardsiae	
	Togninia minima	
	Stereum hirsutum	
Eutypa dieback	*Cryptovalsa ampelina*	***Eutypa lata***
	Diatrypella vulgaris	
	Eutypa lata	
	Eutypa leptoplaca	
	Eutypella citricola	
	Eutypella microtheca	

Triazoles were the most numerous group within the synthetic compounds, with 10 a.i.s tested. They are currently used in vineyards to protect vines from several diseases (powdery mildew, botrytis bunch rot, etc.). For this reason and due to their systemic properties, triazoles have also been tested to manage GTDs. In vitro assays revealed a high efficiency in controlling the mycelial growth of GTD pathogens and sometimes inhibit conidial germination (Gramaje et al. 2009). Some of these positive in vitro effects were confirmed by in planta bio-assays. Differences in efficiency of triazoles were recorded in similar tests carried out by different laboratories, probably due to the differences in protocols, pathogen and strains tested, and grapevine cultivars used. When tested for pruning wound protection, triazoles showed different behaviors according to the GTD. Apart from tebuconazole, they were ineffective against Botryosphaeria dieback pathogens, while flusilazole, penconazole, tebuconazole and, to a lesser extent, triadimenol, showed good results when tested on *E. lata*. Only tebuconazole and triadimenol were tested against vascular Esca complex pathogens, but with unsatisfactory results. For suppression of foliar symptoms, cyproconazole, penconazole, and thiabendazole gave some positive results only in the younger vines, with initial GLSD symptoms and no or limited wood decay (Di Marco et al. 2000). Difenoconazole and propiconazole showed contrasting results in France (Darrieutort and Lecomte 2007) and Hungary (Dula et al. 2007), and tetraconazole was inefficient. Propiconazole was also tested to suppress Eutypa dieback foliar symptoms, unsuccessfully. In the nursery, two triazoles were studied to control Esca complex vascular pathogens during the plant production process, with promising results. Flusilazole combined with carbendazim (see in commercial mix, Table 12) controlled the development of *Pa. chlamydospora* and *Pm. minimum*, and cyproconazole limited the incidence of *Pa. chlamydospora* but only when coupled with a hot water treatment (Serra et al. 2011).

Strobilurins are currently used to control downy and powdery mildew in vineyards. They were especially tested for pruning wound protection. The most efficient against GTD pathogens was pyraclostrobin, in both in vitro and wound protection trials. Other strobilurins were studied only in vitro and showed different efficiencies according to the considered GTD pathogen as reported by Sosnowski et al. (2008), Gramaje et al. (2009), Halleen et al. (2010), and Amponsah et al. (2012) (see Table 7).

In the arylamilopyrimidine family of compounds, fluazinam, which is especially used in vineyards to control botrytis bunch rot and downy mildew, was tested on both Botryosphaeria and Eutypa dieback pathogens. Being a broad-spectrum, contact fungicide with high activity in blocking conidial germination, it was tested in vitro directly on pathogens and for wound protection, showing a high level of efficiency when applied after pruning (Gramaje et al. 2012; Pitt et al. 2012; Sosnowski et al. 2013).

Among the imidazoles, prochloraz, currently not registered for the use in vineyards, blocked conidial germination of all the tested GTD pathogens in vitro, but this trend was not always confirmed in planta (Table 7). Bester et al. (2007) reported its efficiency in protecting wounds from infection of *D. seriata*, *L. theobromae*, *N. australe*, and *N. parvum.* On the contrary, Pitt et al. (2012) obtained high reisolation percentages of the pathogen 6 months after artificial inoculation of *N. luteum* onto pruning wounds treated with prochloraz.

Seven commercial products were tested mainly for wound protection and in nursery applications. A cyproconazole+iodocarb mix, registered as wound protectant in some countries against Eutypa dieback, also successfully controlled Botryosphaeria dieback and pathogens associated to GLSD and Esca proper in the Esca complex (Rolshausen et al. 2010). The other commercial mixtures showed better results in nursery trials. The mix of carbendazim+flusilazole reduced the inoculum of *Pm. minimum* and *Pa. chlamydospora* when applied in the cutting soaking water before cold storage (Gramaje et al. 2009). Similarly, cyprodinil+fludioxinil and pyraclostrobin+metiram mixes controlled some Botryosphaeriaceae in the nursery when diluted in the soaking water before grafting (Rego et al. 2009).

Two experimental lab mixtures of triazoles, propiconazole+thiabendazole and difeconazole+tebuconazole, gave a significant reduction in Esca foliar symptoms expression, more evident for the difenaconazole+tebuconazole mix (Dula et al. 2007).

The dithiocarbamates ziram and thiram, tested in soaking water before grafting, showed good efficiency in controlling the presence of Petri disease pathogens in the nursery (Eskalen et al. 2007b), even if Santos et al. (2006) reported ziram as inefficient in their in vitro test toward *Pm. angustius* and *Pa. chlamydospora*. Mancozeb gave satisfactory results for wound protection toward *N. luteum* (Amponsah et al. 2012).

For the other a.i.s tested, fosetyl-Al (organic salt) was able to reduce Esca complex foliar symptoms (Esca proper), and also to reduce infections by *D. seriata* and Esca complex vascular pathogens in wounds (Díaz and Latorre 2013; Di Marco et al. 2011a).

The phtalimide captan, thanks to its broad-spectrum activity, is currently used in nurseries in some countries to limit fungal infection in wood tissues. Its efficiency was validated on Esca complex vascular pathogens (Fourie and Halleen 2006; Gramaje et al. 2009). Some organic disinfectants (didecyldimethylammonium chloride, usually used in nurseries as biocides) showed positive effects in vitro toward Botryosphaeria and Esca vascular pathogens that were confirmed in the nursery (Fourie and Halleen 2006; Gramaje and Armengol 2011; Gramaje et al. 2009). Moreover, these products coupled a high efficiency with a low impact on grafted plant viability (Fourie and Halleen 2006).

Inorganic active ingredients tested such as boron, copper, sulfur, and phosphorous. Among inorganic compounds (Table 8), acids, bases, and salts of well-known elements in plant disease control, such as boron, copper, sulfur, and phosphorus, have been tested. Sodium arsenite, even if banned, was tested in France and Portugal to understand more about its mode of action in order to find other a.i.s that could mimic the same or similar mechanisms (Larignon et al. 2008; Santos et al. 2006). Boron, known for its ability to affect some wood-rotting fungi, is used in timber and wood industries. On pruning wounds, boron showed good protection toward all the tested GTD pathogens (Rolshausen et al. 2010), although no significant improvement toward *E. lata* infections was reported by Sosnowski et al. (2008), when boron was added in the sealing paste. A possible phytotoxic effect of boron-based treatments resulting in bud failure was hypothesized (Rolshausen and Gubler 2005).

Trying a new sustainable road: tests with natural active ingredients. The concerns related to the massive use of chemicals and the attention toward environmentally friendly production models have given a boost to the study of natural a.i.s in agriculture, especially in viticulture. Consequently, several natural a.i.s were evaluated to manage GTDs. To date, 14 natural organic or inorganic substances, ranging from simple molecules such as hydrogen peroxide (Fourie and Halleen 2006; Sosnowski et al. 2013) to complex mixtures like propolis (Cobos et al. 2015) have been studied (Table 9).

Chitosan is a nontoxic, biocompatible, and biodegradable polymer, known for its fungistatic and fungicide properties and for its capability to stimulate plant defense systems through the induced systemic resistance (ISR) mechanism. Chitosan is able to penetrate fungal conidia and hyphae causing membrane disorganization and the loss of the cellular content (Palma-Guerrero et al. 2008; Park et al. 2002). When tested against GTD pathogens in vitro, chitosan inhibited mycelial growth of Botryosphaeriaceae, Esca complex fungi, and to a lesser extent, Eutypa dieback pathogens. This potential was confirmed for wound protection. Moreover, Cobos et al. (2015) reported that low-molecular weight chitosan was more effective than medium- and high-molecular weight ones.

Extracts of *Allium sativum* were assayed toward GTD pathogens, giving good results both in vitro and in wound protection (Cobos et al. 2015; Sosnowski et al. 2013). When combined with chitosan and vanillin, it was more effective than the individual a.i.s in reducing the infections of *D. seriata* and *Pa. chlamydospora* artificially inoculated onto treated pruning wounds (Cobos et al. 2015). Furthermore, and according to the authors, the lower mortality rate of vines and low percentages of reisolation of the pathogens recorded in the field trials could be related to a putative synergism among the

Table 7. Synthetic organic compounds tested toward the three main grapevine trunk diseases (GTDs). Reported for each active ingredient are: the efficiency[a] recorded in the relative test (in vitro, in planta, and in field); the results when tested for wound protection (WP), symptom suppression (SS), or for nursery use (NU); the pathogens used in tests; and in superscript brackets, the country in which the studies were carried out.[b] The number in superscript brackets on an efficiency result indicates the country in which the test was carried out (see supplementary file for references regarding a specific a.i.).

		Botryosphaeria dieback						
Chemical group	**Active ingredient**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
Anilinopyrimidine	Cyprodinil	- -	nt	nt	nt	nt	nt	*B. dothidea* (1)
		nt	nt	nt	nt	nt	+++	*Botryosphaeria* spp. (11)
		- -	nt	nt	nt	nt	nt	*D. seriata* (1)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (1)
		- -	nt	nt	nt	nt	nt	*N. parvum* (1)
Anilopyrimidine	Pyrimethanil	- -	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- -	nt	nt	nt	nt	nt	*D. mutila* (1)(10)
		- -	- + (3)	nt	nt	nt	nt	*D. seriata* (3)(10)(12)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (1)(12)
		- -	nt	nt	nt	nt	nt	*N. australe* (10)(12)
		- -	nt	nt	nt	nt	nt	*N. luteum* (10)
		- -	nt	nt	nt	nt	nt	*N. parvum* (1)(12)
Arylamilopyrimidine	Fluazinam	+++	nt	nt	+++	nt	nt	*B. dothidea* (1)
		+++	nt	nt	nt	nt	nt	*D. mutila* (1)
		+++	nt	nt	+++	nt	nt	*D. seriata* (1)
		+++	nt	nt	+++	nt	nt	*L. theobromae* (1)
		+++	nt	nt	+++	nt	nt	*N. parvum* (1)
Benzimidazole	Benomyl	+++ (12)	+++ (3)	nt	+++ (12)	nt	nt	*D. seriata* (3)(12)
		+++	nt	nt	+++	nt	nt	*L. theobromae* (12)
		+++	nt	nt	+++	nt	nt	*N. australe* (12)
		+++	nt	nt	+++	nt	nt	*N. parvum* (12)
	Carbendazim	+++	nt	nt	nt	nt	nt	*B. dothidea* (1)
		+++	nt	nt	+++ (1)	nt	nt	*D. mutila* (1)(10)
		+++	nt	nt	+++	nt	nt	*D. seriata* (1)
		+++	nt	nt	nt	nt	nt	*L. theobromae* (1)
		+++	nt	nt	nt	nt	nt	*N. australe* (10)
		+++	+++	nt	+++	nt	nt	*N. luteum* (10)
		+++	nt	nt	nt	nt	nt	*N. parvum* (1)
	Thiophanate methyl	nt	nt	nt	+++	nt	nt	*B. dothidea* (15)
		+++	+	nt	nt	nt	nt	*D. mutila* (10)
		nt	nt	nt	+++	nt	nt	*D. seriata* (3)(13)(15)
		nt	nt	++	+++	nt	nt	*D. viticola* (15)
		nt	nt	++	+++ (15)	nt	nt	*L. theobromae* (4)(15)
		+	nt	nt	nt	nt	nt	*N. australe* (10)
		+	+	nt	++	nt	nt	*N. luteum* (10)
Benzonitrile	Chlorothalonil	- +	- +	nt	nt	nt	nt	*D. mutila* (10)
		+	- + (10)	nt	nt	nt	nt	*D. seriata* (1)(10)
		++	nt	nt	nt	nt	nt	*L. theobromae* (1)
		- +	- +	nt	nt	nt	nt	*N. australe* (10)
		- +	- +	nt	nt	nt	nt	*N. luteum* (10)
		++	nt	nt	nt	nt	nt	*N. parvum* (1)
Carboxamide	Boscalid	- -	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- -	nt	nt	nt	nt	nt	*D. mutila* (1)
		- -	nt	nt	nt	nt	nt	*D. seriata* (1)(12)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (1)(12)
		- -	nt	nt	nt	nt	nt	*N. australe* (12)
		- -	nt	nt	nt	nt	nt	*N. parvum* (1)(12)
Dicarboximide	Iprodione	- +	nt	nt	nt	nt	nt	*Botryosphaeria* sp. (3)
		- +	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- + (1)/+++ (10)	nt	nt	nt	nt	nt	*D. mutila* (1)(10)
		- + (1)(12) +++ (10)	nt	nt	nt	nt	nt	*D. seriata* (1)(10)(12)
		- +	nt	nt	+ (15)	nt	nt	*L. theobromae* (1)(12)(15)
		- +	nt	nt	nt	nt	nt	*N. australe* (10)(12)
		+++	+++ (10)	nt	+++ (10)	nt	nt	*N. luteum* (10)
		- +	nt	nt	nt	nt	nt	*N. parvum* (1)(12)
Dicarboximide	Procymidone	- +	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- + (1) +++ (10)	+ (10)	+ (10)	nt	nt	nt	*D. mutila* (1)(10)
		- +	nt	nt	nt	nt	nt	*D. seriata* (1)
		- +	nt	nt	nt	nt	nt	*L. theobromae* (1)
		+++	+	+	nt	nt	nt	*N. australe* (10)
		+++	+	+	nt	nt	nt	*N. luteum* (10)
		- +	nt	nt	nt	nt	nt	*N. parvum* (1)
Disinfectants	Didecyldimethylammonium chloride	nt	nt	nt	nt	nt	++	*Botryosphaeria* sp. (12)
		nt	nt	nt	nt	nt		
	Alcohol-phenol-iodine solution	nt	nt	nt	nt	nt	- -	*Botryosphaeria* sp. (12)

(*Continued on next page*)

[a] Efficiency: - - ineffective; - + less effective; + moderately efficient; ++ efficient; +++ highly efficient; nt, not tested.

[b] Countries: (1) Australia; (2) Austria; (3) Chile; (4) Egypt; (5) France; (6) Germany; (7) Greece; (8) Hungary; (9) Italy; (10) New Zealand; (11) Portugal; (12) South Africa; (13) Spain; (14) Switzerland; (15) U.S.A.

[c] Abbreviations used for pathogen species: *B.* = *Botryosphaeria*; *D.* = *Diplodia*; *L.* = *Lasiodiplodia*; *N.* = *Neofusicoccum*; *Pa.* = *Phaeomoniella*; *Pm.* = *Phaeoacremonium*; *F.* = *Fomitiporia*; *S.* = *Stereum*; *P.* = *Pleurostomophora*; *T.* = *Togninia*; *C.* = *Cryptovalsa*; *Di.* = *Diatrypella*; *E.* = *Eutypa*; *Eu* = *Eutypella*; *Li.* = *Libertella.*

Table 7. (*Continued from previous page*)

Esca complex							Eutypa dieback						
In vitro	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
							- -	nt	nt	nt	nt	nt	*C. ampelina* (13)
							- -	nt	nt	nt	nt	nt	*Di. vulgaris* (13)
							- -	nt	nt	+++ (1)	nt	nt	*E. lata* (1)(12)(13)
nt	- +	nt	nt	nt	nt	*Pa. chlamydospora* (3)	- -	nt	nt	nt	nt	nt	*E. leptoplaca* (13)
							- -	nt	nt	nt	nt	nt	*Eu. citricola* (13)
							- -	nt	nt	nt	nt	nt	*Eu. microtheca* (13)
							+++	nt	nt	nt	nt	nt	*C. ampelina* (13)
							+++	nt	nt	nt	nt	nt	*Di. vulgaris* (13)
							+++	nt	nt	++ (1)	nt	nt	*E. lata* (1)(13)
							+++	nt	nt	nt	nt	nt	*E. leptoplaca* (13)
							+++	nt	nt	nt	nt	nt	*Eu. citricola* (13)
							+++	nt	nt	nt	nt	nt	*Eu. microtheca* (13)
+++ (10)(12)	+++ (1)(3)	nt	nt	nt	+ (10)	*Pa. chlamydospora* (1)(3)(10)(12)	+++	nt	nt	+++	nt		*E. lata* (1)(12)
nt	nt	nt	nt	nt	+ (10)	*Pm. minimum* (10)							
+++	+++	nt	nt	nt	+++	*Pm. minimum* (13)	+++	nt	nt	nt	nt	nt	*C. ampelina* (13)
+++	nt	nt	nt	nt	+++ (13)	*Pa. chlamydospora* (10)(13)	+++	nt	nt	nt	nt	nt	*Di. vulgaris* (13)
nt	nt	nt	nt	- - (9)	nt	for foliar symptom suppression (9)	+++	nt	nt	+++ (1)	nt	nt	*E. lata* (1)(13)
							+++	nt	nt	nt	nt	nt	*E. leptoplaca* (13)
							+++	nt	nt	nt	nt	nt	*Eu. citricola* (13)
							+++	nt	nt	nt	nt	nt	*Eu. microtheca* (13)
nt	nt	nt	+++	nt	nt	*Pm. parasiticum* (15)	nt	nt	nt	+++	nt	nt	*E. lata* (15)
+++	nt	nt	+++ (15)	nt	++ (8)(13)	*Pm. minimum* (8)(10)(13)(15)							
+++ (10)	nt	nt	+++ (3)(13)(15)	nt	++ (4)(8)/ + (9)	*Pa. chlamydospora* (3)(4)(8)(9)(10)(13)(15)							
nt	nt	nt	+++	nt	nt	*P. richardsiae* (15)							
nt	nt	nt	+++	nt	nt	*T. minima* (15)							
+++	nt	nt	nt	nt	nt	*Pa. chlamydospora* (12)	+	nt	nt	nt	nt	nt	*E. lata* (1)
- -	nt	nt	nt	nt	nt	*Pa. chlamydospora* (3)(10)(13)	- -	nt	nt	nt	nt	nt	*E. lata* (1)
- -	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
- -	nt	nt	nt	nt	nt	*Phaeoacremonium* sp. (3)							
++ (13)	nt	nt	nt	nt	++	*Pa. chlamydospora* (12)(13)							
++ (13)	nt	nt	nt	nt	++	*Pm. minimum* (12)(13)							
nt	nt	nt	nt	nt	++	*Pa. chlamydospora* (12)							
nt	nt	nt	nt	nt	++	*Pm. minimum* (12)							

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Table 7. (*Continued from previous page*)

		Botryosphaeria dieback						
Chemical group	**Active ingredient**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
Dithiocarbammate	Mancozeb	++	nt	nt	nt	nt	nt	*D. mutila* (10)
		++	nt	nt	nt	nt	nt	*N. australe* (10)
		++	+++	nt	+++	nt	nt	*N. luteum* (10)
	Ziram							
	Thiram							
Hydroxianiline	Fenhexamid	- -	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- -	nt	nt	nt	nt	nt	*D. seriata* (1)(3)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (1)
		- -	nt	nt	nt	nt	nt	*N. parvum* (1)
Imidazole	Prochloraz	+++	+	nt	nt	nt	nt	*Botryosphaeria* sp. (3)
		++	nt	nt	+++	nt	nt	*D. seriata* (12)
		++	nt	nt	+++	nt	nt	*D. seriata* (12)
		+++	nt	nt	+++	nt	nt	*L. theobromae* (12)
		+++	nt	nt	+++ (12)	nt	nt	*N. australe* (10)(12)
		+++	- -	nt	- -	nt	nt	*N. luteum* (10)
		+++	nt	nt	+++	nt	nt	*N. parvum* (12)
	Imazalil							
Morpholine	Dimethomorph							
Nitrile	Dithianon	- -	nt	nt	nt	nt	nt	*D. mutila* (10)
		- -	nt	nt	nt	nt	nt	*N. australe* (10)
		- -	+	nt	+	nt	nt	*N. luteum* (10)
Phenylpyrrole	Fludioxonil	+++	nt	nt	nt	nt	nt	*B. dothidea* (1)
		nt	nt	nt	nt	nt	+	*Botryosphaeria* spp. (11)
		+++	nt	nt	nt	nt	nt	*D. mutila* (1)
		+++	nt	nt	nt	nt	nt	*D. seriata* (1)
		+++	nt	nt	nt	nt	nt	*L. theobromae* (1)
		+++	nt	nt	nt	nt	nt	*N. parvum* (1)
Phenoxyquinoline	Quinoxyfen	- -	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- -	nt	nt	nt	nt	nt	*D. mutila* (1)
		- -	nt	nt	nt	nt	nt	*D. seriata* (1)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (1)
		- -	nt	nt	nt	nt	nt	*N. parvum* (1)
Phthalimide	Captan	- -	nt	nt	nt	nt	nt	*Botryosphaeria* spp. (12)
Quinoline	Hydroxyquinoline sulfate	nt	nt	nt	nt	nt	- -	*Botryosphaeria* sp. (12)
Pyrimidine	Fenarimol	+	nt	nt	nt	nt	nt	*B. dothidea* (1)
		+	nt	nt	nt	nt	nt	*D. mutila* (1)(10)
		+ (1)/+++ (12)	nt	nt	+ (12)	nt	nt	*D. seriata* (1)(12)
		+ (1)/+++ (12)	nt	nt	+ (12)	nt	nt	*L. theobromae* (1)(12)
		+ (10)/+++ (12)	nt	nt	+ (12)	nt	nt	*N. australe* (10)(12)
		+	+	nt	nt	nt	nt	*N. luteum* (10)
		+ (1)/+++ (12)	nt	nt	+ (12)	nt	nt	*N. parvum* (1)(12)
Spyroketalamine	Spiroxamine	- +	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- +	nt	nt	nt	nt	nt	*D. mutila* (1)
		- +	nt	nt	nt	nt	nt	*D. seriata* (1)
		- +	nt	nt	nt	nt	nt	*L. theobromae* (1)
Strobilurin	Azoxystrobin	nt	nt	nt	++	nt	nt	*Botryosphaeria* spp. (11)
		- -	nt	nt	nt	nt	nt	*D. mutila* (10)
		- -	nt	nt	nt	nt	nt	*N. australe* (10)
		- -	nt	nt	nt	nt	nt	*N. luteum* (10)
	Kresoxym-methyl	- -	nt	nt	nt	nt	nt	*D. seriata* (12)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (12)
		- -	nt	nt	nt	nt	nt	*N. australe* (12)
		- -	nt	nt	nt	nt	nt	*N. parvum* (12)
	Pyraclostrobin	+++ (1)	nt	nt	+++ (15)	nt	nt	*B. dothidea* (1)(15)
		+++ (1)	nt	nt	+++ (15)	nt	nt	*D. mutila* (1)(15)
		nt	+ (3)	nt	+ (3)/+++ (15)	nt	nt	*D. seriata* (3)(15)
		nt	nt	nt	+++	nt	nt	*D. viticola* (15)
		nt	nt	nt	+++	nt	nt	*L. theobromae* (15)
	Trifloxystrobin							

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Table 7. (*Continued from previous page*)

Esca complex							Eutypa dieback						
In vitro	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
- -	nt	nt	nt	nt	++	*Pa. chlamydospora* (12)	- - (1) + (12)	nt	nt	nt	nt	nt	*E. lata* (1)(12)
nt	nt	nt	nt	nt	++	*Pm. minimum* (15)	- -	nt	nt	nt	nt	nt	*E. lata* (1)
- -	nt	nt	nt	nt	nt	*Pm. angustium* (11)							
- - (11)	nt	nt	nt	nt	++ (15)	*Pa. chlamydospora* (11)(15)							
++ (13)	nt	nt	nt	nt	++ (15)	*Pa. chlamydospora* (13)(15)							
++ (13)	nt	nt	nt	nt	++ (15)	*Pm. minimum* (13)(15)							
- -	nt	nt	nt	nt		*Pm. chlamydospora* (3)	- -	nt	nt	- -	nt	nt	*E. lata* (1)(12)
+++	+ (3)	nt	nt	nt	nt	*Pa. chlamydospora* (1)(3)(13)	+++	nt	nt	nt	nt	nt	*E. lata* (1)
+++	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
+++	+ (3)	nt	nt	nt	nt	*Phaeoacremonium* sp. (3)							
+++	nt	nt	nt	nt	nt	*Pa. chlamydospora* (13)	++	nt	nt	+	nt	nt	*E. lata* (1)
++	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
							+	nt	nt	nt	nt	nt	*E. lata* (1)
nt	nt	nt	nt	nt	- -	*Pa. chlamydospora* (11)							
							- -	nt	nt	nt	nt	nt	*E. lata* (1)
- + (13)	nt	nt	nt	nt	++ (12)	*Pa. chlamydospora* (12)(13)							
- + (13)	nt	nt	nt	nt	++ (12)	*Pm. minimum* (12)(13)							
+++ (2)/- + (13)	nt	nt	nt	nt	-+ (2)/- - (12)	*Pa. chlamydospora* (2)(12)(13)							
+++ (2)/- + (13)	nt	nt	nt	nt	- - (2)(12)	*Pm. minimum* (2)(12)(13)							
nt	+++	nt	nt	nt	nt	*Pa. chlamydospora* (1)	+++	nt	nt	- -	nt	nt	*E. lata* (1)(12)
+++	nt	nt	nt	nt	- +	*Pa. chlamydospora* (2)	+++	nt	nt	nt	nt	nt	*E. lata* (1)(12)
+++	nt	nt	nt	nt	- -	*Pm. minimum* (2)							
+++ (13)	nt	nt	nt	nt	nt	*Pa. chlamydospora* (13)	- -	nt	nt	nt	nt	nt	*E. lata* (1)(12)
+++ (13)	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
- - (3)	+ (1)	nt	nt	nt	nt	*Pa. chlamydospora* (1)(3)	- -	nt	nt	nt	nt	nt	*E. lata* (12)
nt	nt	nt	+++ (15)	nt	nt	*Pm. parasiticum* (15)	+	nt	nt	nt	nt	nt	*C. ampelina* (13)
nt	+ (2)	nt	+ (2)/+++ (15)	nt	nt	*Pa. chlamydospora* (3)(15)	+	nt	nt	nt	nt	nt	*Di. vulgaris* (13)
nt	nt	nt	+++ (15)	nt	nt	*P. richardsiae* (15)	+ (1)(13)	nt	nt	+++ (15)	nt	nt	*E. lata* (1)(13)(15)
nt	nt	nt	+++ (15)	nt	nt	*T. minima* (15)	+	nt	nt	nt	nt	nt	*E. leptoplaca* (13)
							+	nt	nt	nt	nt	nt	*Eu. citricola* (13)
							+	nt	nt	nt	nt	nt	*Eu. microtheca* (13)
							- +	nt	nt	- - (1)	nt	nt	*E. lata* (1)(12)

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Table 7. (*Continued from previous page*)

Chemical group	Active ingredient	Botryosphaeria dieback						
		In vitro	In planta	In field	WP	SS	NU	Pathogen[c]
Sulphamid	Tolyfluanid	- -	nt	nt	nt	nt	nt	*Botryosphaeria* sp. (3)
Triazole	Cyproconazole							
	Difenoconazole	nt	nt	nt	++	nt	nt	*Botryosphaeria* spp (11)
	Flusilazole	+	nt	nt	nt	nt	nt	*B. dothidea* (1)
		+ (1)/+++ (10)	nt	nt	nt	nt	nt	*D. mutila* (1)(10)
		+ (1)/+++ (12)	nt	nt	- - (12)	nt	nt	*D. seriata* (1)(12)
		+ (1)/+++ (12)	nt	nt	- - (12)	nt	nt	*L. theobromae* (1)(12)
		+++	nt	nt	- - (12)	nt	nt	*N. australe* (10)(12)
		+++	+++	nt	+++	nt	nt	*N. luteum* (10)
		+ (1)	nt	nt	- - (12)	nt	nt	*N. parvum* (1)(12)
		+++ (12)						
	Myclobutanil	+++	nt	nt	nt	nt	nt	*B. dothidea* (1)
		+++	nt	nt	- -	nt	nt	*Diplodia mutila* (1)
		+++	nt	nt	- -	nt	nt	*D. seriata* (1)
		+++	nt	nt	nt	nt	nt	*L. theobromae* (1)
		+++	nt	nt	nt	nt	nt	*N. parvum* (1)
	Penconazole	+++	nt	nt	nt	nt	nt	*B. dothidea* (1)
		+++	nt	nt	- -	nt	nt	*D. mutila* (1)
		+++	nt	nt	- -	nt	nt	*D. seriata* (1)
		+++	nt	nt	nt	nt	nt	*L. theobromae* (1)
		+++	nt	nt	nt	nt	nt	*N. parvum* (1)
	Propiconazole							
	Tebuconazole	+++ (3)	+ (3)	nt	++ (11)	nt	nt	*Botryosphaeria* sp. (3)(11)
		+++	nt	nt	nt	nt	nt	*B. dothidea* (1)
		+++	+++	nt	++ (1)	nt	nt	*Diplodia mutila* (1)(10)
		+++ (1)(12)	+++ (3)	nt	+++ (1)(12)	nt	nt	*D. seriata* (1)(3)(12)
		+++	nt	nt	nt	nt	nt	*L. theobromae* (1)(12)
		+++	nt	nt	nt	nt	nt	*N. australe* (10)(12)
		+++	+++	nt	nt	nt	nt	*N. luteum* (10)
		+++	nt	nt	nt	nt	nt	*N. parvum* (1)(12)
	Thiabendazole							
	Tetraconazole	nt	nt	nt	- -	nt	nt	*D. mutila* (1)
		nt	nt	nt	- -	nt	nt	*D. seriata* (1)
	Triademenol	- -	nt	nt	nt	nt	nt	*B. dothidea* (1)
		- -	nt	nt	nt	nt	nt	*D. seriata* (1)
		- -	nt	nt	nt	nt	nt	*L. theobromae* (1)
		- -	nt	nt	nt	nt	nt	*N. parvum* (1)
Carboxamide + strobilurin	Boscalid + pyraclostrobin *BASF516*							
Anilide + dithiocarbammate	Carboxine + thiram *Vitavax/T*			++			++	*L. theobromae* (4)
Benzimidazole + triazole	Carbendazim + flusilazole *Escudo*							
Triazole + carbammate	Cyproconazole + iodocarb *Garrison*	nt	nt	nt	+++	nt	nt	*B. dothidea* (15)
		nt	nt	nt	+++	nt	nt	*D. mutila* (1)
		nt	nt	nt	+++ (1)/+ (15)	nt	nt	*D. seriata* (1)(15)
		nt	nt	nt	++	nt	nt	*D. viticola* (15)
		nt	nt	nt	++	nt	nt	*L. theobromae* (15)
Anilinopyrimidine + phenylpyrrole	Cyprodinil + fludioxionil *Switch*	nt	nt	nt	nt	nt	++	*Botryosphaeria* spp. (11)
		nt	nt	nt	- +	nt	nt	*D. mutila* (1)
		nt	nt	nt	- +	nt	++ (5)	*D. seriata* (1)(5)
		nt	nt	nt	nt	nt	++	*N. parvum* (5)
Dithiocarbammate + depsipeptides	Mancozeb + metalaxyl-m *Ridomil Gold MZ*							
Triazoles	Prothioconazole + tebuconazole *Prosaro*							
Strobilurin + dithiocarbammate	Pyraclostrobin + metiram *Cabrio top*	nt	nt	nt	nt	nt	++	*Botryosphaeria* spp. (11)
		nt	nt	nt	nt	nt	++	*D. seriata* (5)
Triazole + inorganic acid + thiazole	Tebuconazole + boric acid + octhilinone *Gelseal ultra*							
Organic salts	Copper ammonium acetate	- -	- -	nt	nt	nt	nt	*D. seriata* (12)
		- -	- -	nt	nt	nt	nt	*L. theobromae* (12)
		- -	- -	nt	nt	nt	nt	*N. australe* (12)
		- -	- -	nt	nt	nt	nt	*N. parvum* (12)
	Fosethyl-al	nt	nt	nt	- +	nt	nt	*D. seriata* (3)
	Copper bis (ethoxy-dihydroxy-diethylamino) sulfate							
Triazole	Propiconazole + thiabendazole							
Triazole	Difeconazole + tebuconazole							

(*Continued on next page*)

Table 7. (*Continued from previous page*)

Esca complex							Eutypa dieback						
In vitro	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
- - [3]	nt	nt	nt	nt	nt	*Pa. chlamydospora* [3]							
- - [3]	nt	nt	nt	nt	nt	*Phaeoacremonium* sp. [3]							
++ [10]	nt	+ [9]	nt	++ [9]	++ [9]	*Pa. chlamydospora* [9][10]							
nt	nt	nt	nt	- - [5] +++ [8]	nt		nt	nt	nt	- -	nt	nt	*E. lata* [5]
+++	nt	nt	nt	nt	nt	*Pa. chlamydospora* [13]	+++	nt	nt	- - [1] +++ [12]	nt	nt	*E. lata* [1][12]
++ [9]	- - [13]	nt	nt	- - [9]	nt	*Pm. minimum* [9][13]							
							+ [1]/+++ [12]	nt	nt	- - [1]	nt	nt	*E. lata* [1][12]
nt	nt	nt	nt	+ [9]	nt	for foliar symptom suppression [9]	+++	nt	nt	+++	nt	nt	*E. lata* [1]
+++	nt	nt	nt	nt	+	*Pa. chlamydospora* [2]							
+++	nt	nt	nt	nt	+	*Pm. minimum* [2]							
nt	nt	nt	nt	- - [5] +++ [8]			nt	nt	nt	nt	- - [5]	nt	
++ [10][13]	- + [1]/+++ [3]	nt	- + [3]	nt	nt	*Pa. chlamydospora* [1][3][10][13]	+++	nt	nt	nt	nt	nt	*C. ampelina* [13]
+++	nt	nt	nt	nt	nt	*Pm. minimum* [13]	+++	nt	nt	nt	nt	nt	*Di. vulgaris* [13]
+++	+	nt	nt	nt	nt	*Phaeoacremonium* sp. [3]	+++	nt	nt	+++ [1][10][12]	nt	nt	*E. lata* [1][10][12][13]
							+++	nt	nt	nt	nt	nt	*E. leptoplaca* [13]
							+++	nt	nt	nt	nt	nt	*Eu. citricola* [13]
							+++	nt	nt	nt	nt	nt	*Eu. microtheca* [13]
+++	for phytotoxicity	nt	nt	nt	nt	*Pm. angustium* [11]							
+++	for phytotoxicity	nt	nt	nt	nt	*Pa. chlamydospora* [11]							
nt	nt	nt	nt	+++ [8]									
nt	nt	nt	nt	- - [9]	nt		+++	nt	nt	- -	nt	nt	*E. lata* [1]
+	nt	nt	- -	nt	nt	*Pa. chlamydospora* [1]	+++	nt	nt	- +	nt	nt	*E. lata* [1]
							++	nt	nt	- -	nt	nt	*E. lata* [1]
nt	nt	nt	nt	nt	++ [13]	*Pm. minimum* [13]							
++	nt	nt	nt	nt	++ [13]	*Pa. chlamydospora* [13]							
nt	nt	nt	- -	nt	nt	*Pm. parasiticum* [15]	nt	nt	nt	+++	nt	nt	*E. lata* [1][15]
nt	nt	nt	++	nt	nt	*Pa. chlamydospora* [15]							
nt	nt	nt	+++	nt	nt	*P. richardsiae* [15]							
nt	nt	nt	+	nt	nt	*T. minima* [15]							
+++ [10]	++ [11]	nt	nt	nt	++ [5]	*Pa. chlamydospora* [5][10][11]	+++	nt	nt	+	nt	nt	*E. lata* [1]
							- -	nt	nt	nt	nt	nt	*E. lata* [1]
							+++	nt	nt	nt	nt	nt	*C. ampelina* [13]
							+++	nt	nt	nt	nt	nt	*Di. vulgaris* [13]
							+++	nt	nt	nt	nt	nt	*E. lata* [1][12][13]
							+++	nt	nt	nt	nt	nt	*E. leptoplaca* [13]
							- +	nt	nt	nt	nt	nt	*Eu. citricola* [13]
							+++	nt	nt	nt	nt	nt	*Eu. microtheca* [13]
nt	++	nt	nt	nt	nt	*F. mediterranea* [6]							
nt	++ [6]	nt	nt	nt	++ [5]	*Pa. chlamydospora* [5][6]							
nt	++	nt	nt	nt	nt	*Pm. minimum* [6]							
							nt	nt	nt	+++	nt	nt	*E. lata* [1][10]
							- -	nt	nt	nt	nt	nt	*E. lata* [1]
nt	nt	nt	+	+	nt	*Pm. minimum* [9]							
nt	nt	nt	- + [3]	nt	nt	*Pa. chlamydospora* [3][9]							
- +	nt	nt	nt	nt	nt	*Pa. chlamydospora* [13]							
- +	nt	nt	nt	nt	nt	*Pm. minimum* [13]							
nt	nt	nt	nt	+ [8]	nt								
nt	nt	nt	nt	++ [8]	nt								

Table 8. Inorganics tested toward the three main grapevine trunk diseases (GTDs). Reported for each active ingredient are: the efficiency[a] recorded in the relative test (in vitro, in planta, and in field); the results when tested for wound protection (WP), symptom suppression (SS), or for nursery use (NU); the pathogens used in tests; and in superscript brackets, the country in which the studies were carried out.[b] The number in superscript brackets on an efficiency result indicates the country in which the test was carried out (see supplementary file for references regarding a specific a.i.).

		Botryosphaeria dieback						
Chemical group	**Active ingredient**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
Inorganic acid	Boric acid	- - [(1)]	nt	nt	++ [(15)]	nt	nt	*B. dothidea* [(1)(15)]
		- - [(1)]	nt	nt	++ [(15)]	nt	nt	*D. mutila* [(1)]
		- - [(1)]	nt	nt	++ [(15)]	nt	nt	*D. seriata* [(1)(15)]
		nt	nt	nt	++ [(15)]	nt	nt	*D. viticola* [(15)]
		- - [(1)]	nt	nt	++ [(15)]	nt	nt	*L. theobromae* [(1)(15)]
		- - [(1)]	nt	nt	++ [(15)]	nt	nt	*N. parvum* [(1)]
	Phosphorous acid							
	Phosphonic acid salt							
Inorganic salt	Calcium polysulfides							
	Iron sulfate	- +	for phytotoxicity	nt	nt	nt	nt	*D. seriata* [(5)]
		- +	for phytotoxicity	nt	nt	nt	nt	*N. parvum* [(5)]
	Sodium arsenite							
	Copper oxychloride							
Inorganic base	Copper hydroxide	- -	nt	nt	nt	nt	nt	*D. mutila* [(10)]
		- -	nt	nt	nt	nt	nt	*N. australe* [(10)]
		- -	+	nt	nt	nt	nt	*N. luteum* [(10)]
Inorganic element	Sulfur							

(*Continued on next page*)

[a] Efficiency: - - ineffective; - + less effective; + moderately efficient; ++ efficient; +++ highly efficient; nt, not tested.
[b] Countries: (1) Australia; (2) Austria; (3) Chile; (4) Egypt; (5) France; (6) Germany; (7) Greece; (8) Hungary; (9) Italy; (10) New Zealand; (11) Portugal; (12) South Africa; (13) Spain; (14) Switzerland; (15) U.S.A.
[c] Abbreviations used for pathogen species: *B.* = *Botryosphaeria*; *D.* = *Diplodia*; *L.* = *Lasiodiplodia*; *N.* = *Neofusicoccum*; *Pa.* = *Phaeomoniella*; *Pm.* = *Phaeoacremonium*; *F.* = *Fomitiporia*; *S.* = *Stereum*; *P.* = *Pleurostomophora*; *T.* = *Togninia*; *C.* = *Cryptovalsa*; *Di.* = *Diatrypella*; *E.* = *Eutypa*; *Eu* = *Eutypella*; *Li.* = *Libertella*.

Table 9. Natural compounds tested toward the three main grapevine trunk diseases (GTDs). Reported for each active ingredient are: the efficiency[a] recorded in the relative test (in vitro, in planta, and in field); the results when tested for wound protection (WP), symptom suppression (SS), or for nursery use (NU); the pathogens used in tests; and in superscript brackets, the country in which the studies were carried out.[b] The number in superscript brackets on an efficiency result indicates the country in which the test was carried out (see supplementary file for references regarding a specific a.i.).

		Botryosphaeria dieback						
Chemical group	**Active ingredient**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
Natural compounds	Ozonated water							
	Chitosan	++	nt	nt	nt	nt	nt	*Botryosphaeria* sp. [(11)]
		+++	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		+++	+++	nt	+++	nt	nt	*D. seriata* [(13)]
	Allium sativum extract	++	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		+++	+++	nt	+++	nt	nt	*D. seriata* [(13)]
	Honey							
	Hydrogen peroxide	nt	nt	nt	nt	nt	- -	*Botryosphaeria* sp. [(12)]
	Lactoferrin							
	Melaleuca alternifolia oil							
	Potassium bicarbonate							
	Saponins	- +	nt	nt	nt	nt	nt	*D. seriata* [(5)]
		- +	nt	nt	nt	nt	nt	*N. parvum* [(5)]
	Sard laundry powder							
	Evernia prunastri lichen extract	+++	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		++	- +	nt	nt	nt	nt	*D. seriata* [(13)]
	Green coffee extract	- -	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		- -	nt	nt	nt	nt	nt	*D. seriata* [(13)]
	Lemon peel extract	++	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		++	- +	nt	nt	nt	nt	*Diplodia seriata* [(13)]
	Propolis	++	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		++	+	nt	nt	nt	nt	*D. seriata* [(13)]
	Seaweed extract + $CaCl_2$ + $Mg(NO_3)_2$							
	Vanillin	+	nt	nt	nt	nt	nt	*B. dothidea* [(13)]
		++	+	nt	nt	nt	nt	*D. seriata* [(13)]
	Allium + chitosan + vanillin	nt	++	nt	+++	nt	nt	*D. seriata* [(13)]

(*Continued on next page*)

[a] Efficiency: - - ineffective; - + less effective; + moderately efficient; ++ efficient; +++ highly efficient; nt, not tested.
[b] Countries: (1) Australia; (2) Austria; (3) Chile; (4) Egypt; (5) France; (6) Germany; (7) Greece; (8) Hungary; (9) Italy; (10) New Zealand; (11) Portugal; (12) South Africa; (13) Spain; (14) Switzerland; (15) U.S.A.
[c] Abbreviations used for pathogen species: *B.* = *Botryosphaeria*; *D.* = *Diplodia*; *L.* = *Lasiodiplodia*; *N.* = *Neofusicoccum*; *Pa.* = *Phaeomoniella*; *Pm.* = *Phaeoacremonium*; *F.* = *Fomitiporia*; *S.* = *Stereum*; *P.* = *Pleurostomophora*; *T.* = *Togninia*; *C.* = *Cryptovalsa*; *Di.* = *Diatrypella*; *E.* = *Eutypa*; *Eu* = *Eutypella*; *Li.* = *Libertella*.

Table 8. (*Continued from previous page*)

Esca complex							Eutypa dieback						
In vitro	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
nt	nt	nt	+++ (15)	nt	nt	*Pa chlamydospora* (15)	++ (15)	nt	nt	- - (1) +++ (15)	nt	nt	*E. lata* (1)(15)
nt	nt	nt	+++ (15)	nt	nt	*P. parasiticum* (15)							
nt	nt	nt	+++ (15)	nt	nt	*P. richardsiae* (15)							
nt	nt	nt	+++ (15)	nt	nt	*T. minima* (15)							
- - (9)	nt	nt	nt	nt	nt	*F. punctata* (9)	+++	nt	nt	nt	nt	nt	*L. blepharis* (9)
- - (9)	nt	nt	nt	nt	- + (12)	*Pm. minimum* (9)(12)							
- - (9)(10)	nt	nt	nt	nt	- + (12)	*Pa. chlamydospora* (9)(10)(12)							
- - (9)	nt	nt	nt	nt	nt	*S. hirsutum* (9)							
							- -	nt	nt	nt	nt	nt	*E. lata* (1)
nt	nt	nt	nt	nt	++	*Pa. chlamydospora* (15)							
nt	nt	nt	nt	nt	++	*Pm. minimum* (15)							
+	for phytotoxicity	nt	nt	nt	nt	*Pm. minimum* (5)	+++	for phytotoxicity	nt	nt	nt	nt	*E. lata* (5)
+	for phytotoxicity	nt	nt	nt	nt	*Pa. chlamydospora* (5)							
- -	- - (11)	nt	nt	nt	nt	*F. mediterranea* (5)	- -	nt	nt	nt	nt	nt	*E. lata* (5)
- -	- - (11)	nt	nt	nt	nt	*Pm. minimum* (5)							
- -	- - (11)	nt	nt	nt	nt	*P. angustium* (11)							
- -	- - (11)	nt	nt	nt	nt	*Pa. chlamydospora* (5)(11)							
- +	nt	nt	nt	nt	nt	*Pa. chlamydospora* (9)(13)							
- +	nt	nt	nt	nt	nt	*Pm. minimum* (9)(13)							
- -	nt	nt	nt	nt	nt	*Pa. chlamydospora* (10)	- -	nt	nt	nt	nt	nt	*E. lata* (1)
- +	nt	nt	nt	nt	nt	*Pa. chlamydospora* (2)	- -	nt	nt	- -	nt	nt	*E. lata* (1)
- -	nt	nt	nt	nt	nt	*Pm. minimum* (2)							

Table 9. (*Continued from previous page*)

Esca complex							Eutypa dieback						
In vitro	**In planta**	**In field**	**WP**	**SS**	**NU**	**on Pathogen[c]**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
+++	+	nt	nt	nt	nt	*Pm. minimum* (5)							
++	nt	nt	nt	nt	nt	*F. mediterranea* (11)							*E. lata* (11)(13)
+++	+++	nt	+++ (13)	nt	nt	*Pa. chlamydospora* (11)(13)	- +	nt	nt	nt	nt	nt	
+++	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
+++	nt	nt	nt	nt	nt	*Pa. chlamydospora* (13)	+++ (1) + (13)	nt	nt	- + (1)	nt	nt	*E. lata* (1)(13)
+++	+++	nt	+++	nt	nt	*Pm. minimum* (13)							
							- -	nt	nt	nt	nt	nt	*E. lata* (1)
nt	nt	nt	nt	nt	- -	*Phaeomoniella* spp. (12)	- -	nt	nt	nt	nt	nt	*E. lata* (1)
nt	nt	nt	nt	nt	- -	*Phaeoacremonium* spp. (12)							
							+++	nt	nt	- +	nt	nt	*E. lata* (1)
							+	nt	nt	nt	nt	nt	*E. lata* (1)
							- -	nt	nt	nt	nt	nt	*E. lata* (1)
							- -	nt	nt	nt	nt	nt	*E. lata* (1)
++	- -	nt	nt	nt	nt	*Pa. chlamydospora* (13)	- +	nt	nt	nt	nt	nt	*E. lata* (13)
- +	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
- -	nt	nt	nt	nt	nt	*Pa. chlamydospora* (13)	- -	nt	nt	nt	nt	nt	*E. lata* (13)
- -	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
++	- -	nt	nt	nt	nt	*Pa. chlamydospora* (13)	++	nt	nt	nt	nt	nt	*E. lata* (13)
- -	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
++	+	nt	nt	nt	nt	*Pa. chlamydospora* (13)	++	nt	nt	nt	nt	nt	*E. lata* (13)
++	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
nt	nt	+++	nt	+++	*nt*	*for foliar symptoms reduction* (9)							
++	++	nt	nt	nt	nt	*Pa. chlamydospora* (13)	++	nt	nt	nt	nt	nt	*E. lata* (13)
++	nt	nt	nt	nt	nt	*Pm. minimum* (13)							
nt	++	nt	+++	nt	nt	*Pa. chlamydospora* (13)							

Table 10. Biostimulants and plant elicitors tested toward the three main grapevine trunk diseases (GTDs). Reported for each active ingredient are: the efficiency[a] recorded in the relative test (in vitro, in planta, and in field); the results when tested for wound protection (WP), symptom suppression (SS), or for nursery use (NU); the pathogens used in tests; and in superscript brackets, the country in which the studies were carried out.[b] The number in superscript brackets on an efficiency result indicates the country in which the test was carried out (see supplementary file for references regarding a specific a.i.).

		Botryosphaeria dieback						
Biostimulant	**Active ingredient**	**In vitro**	**In planta**	**In field**	**WP**	**SS**	**NU**	**Pathogen[c]**
Commercial	Aluminum lignin sulfate, gluconic acid, microelements (Brotomax)							
	Amino acids, peptides, peptones (Fitostim)							
	Glutatione, oligosaccharine (Kendral)							
	Aschophyllum nodosum extract (Marvita)							
Elicitor	2-hydroxybenzoic acid							
	Benzothiodiazole							
Phytoalexins	Resveratrol							
	Pterostilbene							
	P-coumaric acid							
Lab mix	Resveratrol + phosphorous acid							
	Pterostilbenes + phosphorous acid							
	Copper oxychloride + gluconates							

(*Continued on next page*)

[a] Efficiency: - - ineffective; - + less effective; + moderately efficient; ++ efficient; +++ highly efficient; nt, not tested.
[b] Countries: (1) Australia; (2) Austria; (3) Chile; (4) Egypt; (5) France; (6) Germany; (7) Greece; (8) Hungary; (9) Italy; (10) New Zealand; (11) Portugal; (12) South Africa; (13) Spain; (14) Switzerland; (15) U.S.A.
[c] Abbreviations used for pathogen species: *B.* = *Botryosphaeria*; *D.* = *Diplodia*; *L.* = *Lasiodiplodia*; *N.* = *Neofusicoccum*; *Pa.* = *Phaeomoniella*; *Pm.* = *Phaeoacremonium*; *F.* = *Fomitiporia*; *S.* = *Stereum*; *P.* = *Pleurostomophora*; *T.* = *Togninia*; *C.* = *Cryptovalsa*; *Di.* = *Diatrypella*; *E.* = *Eutypa*; *Eu* = *Eutypella*; *Li.* = *Libertella*.

Table 11. List of the bacterial biocontrol agents tested, singly or mixed, against the three main grapevine trunk diseases both in field and nursery

		Tested on			Tested in		
Genus	**Species**	**Botryosphaeria dieback**	**Esca complex**	**Eutypa dieback**	**Lab**	**Vineyard**	**Nursery**
Acinetobacter	*A. radioresistens*	x	x		x		
Bacillus	*B. amyloliquefacens*	x	x		x		
	B. cereus			x	x		
	B. firmus	x	x		x		
	B. ginsengihumi	x	x		x		
	B. licheniformis	x	x		x		
	B. pumilus	x	x		x		
	B. subtilis	x	x	x	x	x	x
	B. thuringiensis			x	x		
	Bacillus sp.	x		x	x		
Brevibacillus	*B. reuszeri*	x	x		x		
Burkholderia	*B. phytofirmans*	x			x		
Curtobacterium	*Curtobacterium* sp.	x	x		x		
Enterobacter	*E. cowanii*	x	x		x		
	Enterobacter sp.	x			x		
Erwinia	*E. erbicola*	x			x		
Paenibacillus	*P. barengoltzii*	x			x		
	P. illinoisensis	x	x		x		
	P. polymyxa	x			x		
	P. turicensis	x			x		
	Paenibacillus sp.	x			x		
Pantoea	*P. agglomerans*	x			x		
Pseudomonas	*P. aeruginosa*			x	x		
	P. fluorescens			x	x		
	Pseudomonas sp.			x	x		
Serratia	*S. plymuthica*			x	x		
Stenotrophomonas	*S. maltophylia*			x	x		
Streptomyces	*Streptomyces* spp.			x	x		
Xanthomonas	*Xanthomonas* sp.	x					x
Bacterial mix	*Azospirillum* sp. + *Pseudomonas* sp. + *Bacillus* sp.		x				x

Table 10. (*Continued from previous page*)

Esca complex							Eutypa dieback						
In vitro	In planta	In field	WP	SS	NU	Pathogen[c]	In vitro	In planta	In field	WP	SS	NU	Pathogen[c]
+ [10][13]	- -	nt	nt	- -	nt	*Pa. chlamydospora* [9][10][13]	nt	nt	+	nt	nt	nt	*E. lata* [1]
+	nt	nt	nt	nt	nt	*Pm. minimum* [10][13]							
nt	- -	nt	nt	- -	nt	*Pa. chlamydospora* [9]							
nt	- -	nt	nt	- -	nt	*Pa. chlamydospora* [9]							
nt	- -	nt	nt	- -	nt	*Pa. chlamydospora* [9]							
nt	nt	nt	nt	- + [7]	nt		nt	nt	nt	nt	- + [7]	nt	
nt	- -	nt	nt	nt	nt	*Pa. chlamydospora* [1]							
++	nt	nt	nt	nt	nt	*F. punctata* [9]	- -	nt	nt	nt	nt	nt	*L. blepharis* [9]
- -	nt	nt	nt	nt	nt	*Pm. minimum* [9]							
- +	nt	nt	nt	nt	nt	*P. angustium* [11]							
- - [9] - + [11]	nt	nt	nt	nt	nt	*Pa. chlamydospora* [9][11]							
++	nt	nt	nt	nt	nt	*S. hirsutum* [9]							
++	nt	nt	nt	nt	nt	*F. punctata* [9]	++	nt	nt	nt	nt	nt	*L. blepharis* [9]
++	nt	nt	nt	nt	nt	*Pm. minimum* [9]							
++	nt	nt	nt	nt	nt	*Pa. chlamydospora* [9]							
++	nt	nt	nt	nt	nt	*S. hirsutum* [9]							
- +	nt	nt	nt	nt	nt	*Pm. minimum* [13]	- +	nt	nt	nt	nt	nt	*E. lata* [9]
- -	nt	nt	nt	nt	nt	*P. inflatipes* [13]							
- +	nt	nt	nt	nt	nt	*Pa. chlamydospora* [13]							
- +	nt	nt	nt	nt	nt	*S. hirsutum* [13]							
- +	nt	nt	nt	nt	nt	*F. punctata* [9]	+++	nt	nt	nt	nt	nt	*L. blepharis* [9]
+	nt	nt	nt	nt	nt	*Pm. minimum* [9]							
+	nt	nt	nt	nt	nt	*Pa. chlamydospora* [9]							
- +	nt	nt	nt	nt	nt	*S. hirsutum* [9]							
- -	nt	nt	nt	nt	nt	*F. punctata* [9]	+++	nt	nt	nt	nt	nt	*L. blepharis* [9]
+	nt	nt	nt	nt	nt	*Pm. minimum* [9]							
+	nt	nt	nt	nt	nt	*Pa. chlamydospora* [9]							
+	nt	nt	nt	nt	nt	*S. hirsutum* [9]							
- +	nt	nt	nt	+	nt	*F. mediterranea* [9]							
- +	nt	nt	nt	+	nt	*Pm. minimum* [9]							
+	nt	nt	nt	+	nt	*Pa. chlamydospora* [9]							

different mechanisms of action of the three compounds. Similarly, foliar applications of a brown seaweed extract containing $CaCl_2$ + $Mg(NO_3)_2$ to Esca-affected vines (GLSD) reduced the incidence and severity of foliar symptoms (Calzarano et al. 2014, 2017, 2018). In this case, it was suggested that the treatments acted by interfering with the mechanisms involved in the development of leaf symptoms, without any phytotoxicity or plant growth inhibition.

Some products belonging to the biostimulants and elicitors group were also tested (Table 10). This complex category, which is now under revision at EU regulation level, includes some commercial products with very different composition (Di Marco and Osti 2009) and others recognized in the scientific literature that are able to either stimulate plant defense system like phytoalexins (Mazzullo et al. 2000; Santos et al. 2006) or induce systemic acquired resistance as elicitors (Darrieutort and Lecomte 2007; Santos et al. 2006). None of the assayed commercial products effectively reduced Esca complex foliar symptoms expression. Within elicitors, the application of 2-hydroxybenzoic acid, an aromatic compound able to stimulate systemic acquired resistance (SAR), seems to reduce foliar symptoms expression for both Esca complex diseases and Eutypa dieback (Darrieutort and Lecomte 2007). The phytoalexins resveratrol, p-coumaric acid, and pterostilbene were tested in vitro only with the vascular pathogens involved in the Esca complex, giving different results according to the phytoalexin and the pathogen. For example, pterostilbene inhibited all of the tested Esca complex pathogens (Mazzullo et al. 2000), while resveratrol showed different results according to pathogen (Mazzullo et al. 2000; Santos et al. 2006).

A Brief Introduction to Biological Control Agents (BCAs) Used for GTDs

Different reasons have led researchers to test BCAs to control GTDs beside chemical a.i.s. The most efficient a.i.s (benzimidazoles, for instance) are not able to protect pruning wounds throughout the entire period they are susceptible to GTD infections, which varies from 2 to 4 months (Kotze et al. 2011). The use of potential BCAs in colonizing woody tissues and maintaining a broad spectrum activity against GTD pathogens for extended periods could be a strategy for wound protection in vineyards. Furthermore, the positive effects of some microorganisms in host-plant physiology, such as the resistance to biotic or abiotic stresses or systemic induced resistance (SIR), are well known (Berg 2009; Handelsman and Stabb 1996; Pal and McSpadden Gardener 2006). Consequently, the main aims of BCA trials were i) to prevent GTD pathogen contamination in nurseries, in which several steps of the plant production process are critical for the spread of GTD pathogens (Aroca et al. 2010; Gramaje and Armengol 2011; Gramaje and Di Marco 2015; Waite et al. 2013; Waite and Morton 2007), and ii) to evaluate BCAs for durable pruning wound protection in vineyards. In particular, nursery trials also allowed the evaluation of the putative BCA effects on plant growth, induced or improved disease resistance, and globally on the development of healthier and sound vines (Di Marco and Osti 2007; Fourie et al. 2001).

Strategies and Main Aims of Potential BCA Tests Against GTDs

Similar to a.i.s, different BCAs have been tested in vitro, in planta, in the field, and during the nursery plant production process (Tables 11–12). Dual cultures in vitro are the primary means to detect antagonistic activity of a potential BCA (Di Marco et al. 2002; Haidar et al. 2016; Hunt et al. 2001; John et al. 2004; Kotze et al. 2011; McMahan et al. 2001; Mutawila et al. 2015; Schmidt et al. 2001). In vitro tests also allow us to decipher some aspects of the biocontrol mechanisms such as the detoxification of phytotoxins (e.g., eutypine, 4-hydroxybenzaldehyde, and 3-phenyllactic acid) considered to be involved in the expression of foliar symptoms in some GTDs (Christen et al. 2005; Tey-Rulh et al. 1991). BCAs have also been tested as wound protectants to limit annual contaminations in the field (Di Marco et al. 2002, 2004; Halleen et al. 2010; John et al. 2005; Kotze et al. 2011; Mutawila et al. 2011, 2015; Pitt

Table 12. List of the fungal biocontrol agents tested, singly or mixed, against the three main grapevine trunk diseases both in field and nursery

Genus	Species	Tested on			Tested in		
		Botryosphaeria dieback	Esca complex	Eutypa dieback	Lab	Vineyard	Nursery
Aureobasidium	*Aureobasidium* spp.		x		x		
Epicoccum	*Epicoccum* spp.		x		x		
	E. purpurascens	x			x		
Fusarium	*F. lateritium*		x	x	x	x	
	F. lateritium mutant Benzimidazole resistant			x	x		
	F. proliferatum	x			x		
Pythium	*P. oligandrum*		x		x		
Trichoderma	*T. atroviride*	x	x	x	x	x	x
	T. atroviride mutant Benzimidazole resistant	x	x	x	x	x	
	T. gamsii (ex *T. viride*)		x		x		
	T. hamatum		x		x		
	T. harzianum	x	x	x	x	x	x
	T. harzianum mutant Benzimidazole resistant	x	x	x	x		
	T. koningii			x		x	
	T. longibrachiatum	x	x		x	x	x
	T. polysporum		x	x	x		
	Trichoderma spp.	x					
	T. asperellum + *T. gamsii*		x	x		x	
Mix	*Trichoderma* + *Gliocladium*			x			x

Table 13. Bacterial control agents (BCAs) tested toward the three main grapevine trunk diseases (GTDs). Reported for each active ingredient are: the efficiency[a] recorded in the relative test (in vitro, in planta, and in field); the results when tested for wound protection (WP) or nursery use (NU); the pathogens used in tests; and in superscript brackets, the country in which the studies were carried out.[b] The number in superscript brackets on an efficiency result indicates the country in which the test was carried out (see supplementary file for references regarding a specific BCA).

Genera	Species	Botryosphaeria dieback					
		In vitro	In planta	In field	WP	NU	Pathogen[c]
Acinetobacter	*A. radioresistens*	nt	- -	nt	nt	nt	*N. parvum* (5)
Bacillus	*B. amyloliquefaciens*	+	nt	nt	nt	nt	*L. mediterranea* (9)
	B. cereus						
	B. firmus	nt	- -	nt	nt	nt	*N. parvum* (5)
	B. ginsengihumi	nt	- -	nt	nt	nt	*N. parvum* (5)
	B. licheniformis	nt	- -	nt	nt	nt	*N. parvum* (5)
	B. pumilus	nt	- -	nt	nt	nt	*N. parvum* (5)
	B. subtilis	+++ (12)	nt	nt	++ (12)	nt	*D. seriata* (12)
		+++ (12)	nt	nt	++ (12)	nt	*L. theobromae* (12)
		+++ (12)	nt	nt	+++ (12)	nt	*N. australe* (12)
		+++ (12)	nt	nt	+ (12)	nt	*N. parvum* (12)
	B. thuringiensis						
	Bacillus sp.	nt	+	nt	nt	nt	*N. parvum* (5)
Brevibacillus	*B. reuszeri*	nt	- -	nt	nt	nt	*N. parvum* (5)
Burkholderia	*B. phytofirmans*		- -	nt	nt	nt	*D. seriata* (5)
			+ -	nt	nt	nt	*N. parvum* (5)
Curtobacterium	*Curtobacterium* sp.	nt	- -	nt	nt	nt	*N. parvum* (5)
Enterobacter	*E. cowanii*	nt	- -	nt	nt	nt	*N. parvum* (5)
	Enterobacter sp.	nt	+++	nt	nt	nt	*N. parvum* (5)
Erwinia	*E. herbicola*						
Paenibacillus	*P. barengoltzii*	nt	- -	nt	nt	nt	*N. parvum* (5)
	P. illinoisensis	nt	- -	nt	nt	nt	*N. parvum* (5)
	P. polymyxa	nt	- -	nt	nt	nt	*N. parvum* (5)
	P. turicensis	nt	- -	nt	nt	nt	*N. parvum* (5)
	Paenibacillus sp.	nt	- -	nt	nt	nt	*N. parvum* (5)
Pantoea	*P. agglomerans*	nt	+++	nt	nt	nt	*N. parvum* (5)
Pseudomonas	*P. aeruginosa*						
	P. fluorescens						
	Pseudomonas sp.						
Serratia	*S. plymuthica*						
Stenotrophomonas	*S. maltophylia*						
Streptomyces	*Streptomyces* spp.						
Xanthomonas	*Xanthomonas* sp.	nt	- -	nt	nt	nt	*N. parvum* (5)
Bacterial mix	*Azospirillum* sp. + *Pseudomonas* sp. + *Bacillus* sp.						

(*Continued on next page*)

[a] Efficiency: - - ineffective; - + less effective; + moderately efficient; ++ efficient; +++ highly efficient; nt, not tested.

[b] Countries: (1) Australia; (2) Austria; (3) Chile; (4) Egypt; (5) France; (6) Germany; (7) Greece; (8) Hungary; (9) Italy; (10) New Zealand; (11) Portugal; (12) South Africa; (13) Spain; (14) Switzerland; (15) U.S.A.

[c] Abbreviations used for pathogen species: *B. = Botryosphaeria*; *D. = Diplodia*; *L. = Lasiodiplodia*; *N. = Neofusicoccum*; *Pa. = Phaeomoniella*; *Pm. = Phaeoacremonium*; *F. = Fomitiporia*; *S. = Stereum*; *P. = Pleurostomophora*; *T. = Togninia*; *C. = Cryptovalsa*; *Di. = Diatrypella*; *E. = Eutypa*; *Eu = Eutypella*; *Li. = Libertella*.

et al. 2012). Among these, *Trichoderma* species and strains present in registered products actually bring a clear reduction in the GLSD foliar symptoms, being at present the only tool for in field Esca complex reduction (GLSD) (Mounier et al. 2016). For BCA persistence within the trunk, the viability and colonizing capability of BCAs in grapevine woody tissues have been studied (Di Marco et al. 2002, 2004; Halleen et al. 2010; Hunt et al. 2001). Several BCA-based commercial products and different BCA strains have been tested, taking into consideration different grapevine cultivars and BCA distribution methods (Bourbos and Barboupoulos 2005; Di Marco et al. 2002, 2004; Halleen et al. 2010; John et al. 2005; Kotze et al. 2011; Mutawila et al. 2011, 2015, 2016).

In planta tests under controlled conditions have been carried out (Di Marco et al. 2002, 2004; John et al. 2005; Haidar et al. 2016; Yacoub et al. 2016) to study grapevine-BCA-pathogen interactions, especially the role of the BCA in systemic induction of resistance in grapevines.

Another useful application of BCA is in the nursery. Studies of BCA treatments often focus on their effects on the physiology of the plant such as root development (Di Marco et al. 2004; Fourie et al. 2001; Pertot et al. 2016), plant growth rate (Fourie and Halleen 2006; Fourie et al. 2001), and overall plant quality (Di Marco and Osti 2007; Fourie and Halleen 2004). The treatments are usually compared with a.i.s or other preventive/sanitation treatments, such as the hot water treatment (HWT) that is often used during the grapevine plant production process (Fourie and Halleen 2004, 2006; Pertot et al. 2016).

Similarly to the a.i. tests, the GTD pathogens most used in BCA tests were *D. seriata*, *L. theobromae*, and *N. parvum* for Botryosphaeria dieback, while *Pa. chlamydospora* and *E. lata* were used in tests on control of Esca complex and Eutypa dieback (see Table 6).

The Most Efficient Biocontrol Agents Against GTDs: Antagonistic Bacteria and Fungi

Since 2000, more than 40 BCAs have been tested against Esca complex, Botryosphaeria dieback, and Eutypa dieback pathogens (Tables 13 and 14). For each BCA, the efficiency in relation to the kind of tests (in vitro, in planta, in the field), the application strategy (wound protection, control in nursery process), and the targeted GTD pathogens are reported in Tables 13 and 14, taking into account the geographical area where the tests were carried out.

Among bacterial BCAs, *Bacillus subtilis* was the most tested toward GTDs. Its ascertained in vitro efficiency against GTD pathogens was confirmed as wound protectant, with different biocontrol degrees according to both the GTD and the selected pathogens (Halleen et al. 2010; Kotze et al. 2011; Schmidt et al. 2001). In nurseries, *B. subtilis* reduced the incidence of the vascular pathogen associated to Esca complex (Petri disease), but the severity of internal symptoms increased (Fourie and Halleen 2004). However, no effects of the *B. subtilis* treatment on callus formation or on the percentage of certifiable plants have been reported. Within the other assayed bacterial genera, some strains of *Enterobacter* spp. and *Pantoea*

Table 13. (*Continued from previous page*)

Esca complex						Eutypa dieback					
In lab	**In planta**	**In field**	**WP**	**NU**	**Pathogen[c]**	**In lab**	**In planta**	**In field**	**WP**	**NU**	**Pathogen[c]**
- -	nt	nt	nt	nt	*Pa. chlamydospora* (5)						
+	nt	nt	nt	nt	*F. mediterranea* (9)						
+	nt	nt	nt	nt	*Pm. minimum* (9)						
+	nt	nt	nt	nt	*Pa. chlamydospora* (9)						
						- +	nt	nt	nt	nt	*E. lata* (9)
+++	nt	nt	nt	nt	*Pa. chlamydospora* (5)						
++	nt	nt	nt	nt	*Pa. chlamydospora* (5)						
+	nt	nt	nt	nt	*Pa. chlamydospora* (5)						
- +	- -	nt	nt	nt	*Pa. chlamydospora* (5)						
nt	nt	nt	nt	- + (12)	*Phaeoacremonium* spp. (12)	- - (12)/+++ (6)(12)	++ (6)	nt	+ (1)/++ (12)	nt	*E. lata* (1)(6)(12)
+++ (12)	nt	nt	+ (12)	- + (12)	*Pa. chlamydospora* (12)						
						- +	nt	nt	nt	nt	*E. lata* (6)
						- +	nt	nt	nt	nt	*E. lata* (6)
+	++	nt	nt	nt	*Pa. chlamydospora* (5)						
- -	nt	nt	nt	nt	*Pa. chlamydospora* (5)						
- +	nt	nt	nt	nt	*Pa. chlamydospora* (5)						
++	+++	nt	nt	nt	*Pa. chlamydospora* (5)	+++	nt	nt	nt	nt	*E. lata* (6)
						+++	nt	nt	nt	nt	*E. lata* (6)
+	++	nt	nt	nt	*Pa. chlamydospora* (5)						
++	+++	nt	nt	nt	*Pa. chlamydospora* (5)						
- +	++	nt	nt	nt	*Pa. chlamydospora* (5)						
						- +	nt	nt	nt	nt	*E. lata* (6)
						- +	nt	nt	nt	nt	*E. lata* (6)
						- +	nt	nt	nt	nt	*E. lata* (6)
						+	nt	nt	nt	nt	*E. lata* (6)
						- -	nt	nt	nt	nt	*E. lata* (6)
						+	nt	nt	nt	nt	*E. lata* (6)
nt	nt	nt	nt	- -	*Phaeoacremonium* spp. (12)						
nt	nt	nt	nt	- -	*Pa. chlamydospora* (12)						

agglomerans reduced necrotic lesions when coinoculated in planta with *N. parvum* (Haidar et al. 2016).

Regarding fungi, the majority of the reviewed trials were carried out with *Trichoderma* spp. (Di Marco and Osti 2007; Di Marco et al. 2004; Fourie and Halleen 2004, 2006; Fourie et al. 2001; Pertot et al. 2016). Less-tested were some antagonistic and nonpathogenic *Fusarium* spp. and *Pythium oligandrum* within the Oomycetes. Within the eight *Trichoderma* species, tested singly or in mixtures, *T. atroviride* and *T. harzianum* were the most studied and are currently present in several commercial products. Besides competition for space and nutrients, *Trichoderma* spp. were able to produce both volatile (Hunt et al. 2001; John et al. 2004) and nonvolatile antibiotics, with various effects on fungal hyphae (lysis, degeneration, mycoparasitism, etc.) (Hunt et al. 2001; John et al. 2004; Kotze et al. 2011; Mutawila et al. 2015). *Trichoderma* spp. generally showed high efficiency in wound protection toward all GTD pathogens, were able to colonize the wood of pruned canes and remained viable for up to one year in the greenhouse (Di Marco et al. 2002) and up to 8 months under field conditions (Di Marco et al. 2002, 2004; Halleen et al. 2010; John et al. 2008). Intensity of colonization was more related to the grapevine cultivar than to the strain tested (Mutawila et al. 2011). Moreover, the influence of vine physiological status on *Trichoderma* colonization ability was reported and dormancy break is regarded as the best time for applications (John et al. 2005; Mutawila et al. 2016). Interestingly, a *T. asperellum* and *T. gamsii* mix (Remedier) as well as *T. atroviride* strain I-1237 (Mounier et al. 2016) reduced the incidence of and mortality in Esca complex affected vineyards (GLSD, Esca proper) starting from the

Table 14. Fungal control agents tested toward the three main grapevine trunk diseases (GTDs). Reported for each active ingredient are: the efficiency[a] recorded in the relative test (in vitro, in planta, and in field); the results when tested for wound protection (WP) or nursery use (NU); the pathogens used in tests; and in superscript brackets, the country in which the studies were carried out.[b] The number in superscript brackets on an efficiency result indicates the country in which the test was carried out (see supplementary file for references regarding a specific a.i.).

		Botryosphaeria dieback					
Genus	**Species**	**In vitro**	**In planta**	**In field**	**WP**	**NU**	**Pathogen[c]**
Aureobasidium	*Aureobasidium* spp.						
Chaetomium	*Chaetomium* spp.	++					*D. seriata* (5)
		++					*N. parvum* (5)
Epicoccum	*Epicoccum* spp.						
	E. purpurascens	++	nt	nt	nt	nt	*L. theobromae* (9)
Fusarium	*F. lateritium*						
	F. lateritium mutant Benzimidazole resistant						
Pythium	*P. oligandrum*						
Trichoderma	*T. atroviride*	++	nt	nt	nt	+	*B. dothidea* (10)
		++	nt	nt	nt	+	*B. stevensii* (10)
		++	++	nt	nt	nt	*D. corticola* (13)
		+++	nt	nt	+++	+ (10)	*D. seriata* (10)(12)
		+++ (12)	nt	nt	+++	nt	*L. theobromae* (12)
		+++ (12)/++ (13)	nt	nt	+++	nt	*N. australe* (12)(13)
		++	++	nt	nt	+ (10)	*N. luteum* (10)(13)
		++	++	nt	nt	nt	*N. mediterraneum* (13)
		+++ (12)/++ (13)	nt	nt	+++	+ (10)	*N. parvum* (10)(12)(13)
	T. atroviride mutant Benzimidazole resistant	+++ (12)	nt	nt	nt	nt	*D. seriata* (12)
		+++ (12)	nt	nt	nt	nt	*N. parvum* (12)
	T. gamsii (ex *T. viride*)						
	T. hamatum						
	T. harzianum	++	nt	nt	nt	nt	*B. stevensii* (10)
		+++ (12)	nt	nt	+ (1)/++ (12)	nt	*D. seriata* (1)(12)
		+++ (12)	nt	nt	++ (12)	nt	*L. theobromae* (12)
		+++ (12)	nt	nt	++ (12)	nt	*N. australe* (12)
		+++ (12)	nt	nt	++ (12)	nt	*N. parvum* (12)
	T. harzianum mutant Benzimidazole resistant	+++ (12)	nt	nt	nt	nt	*D. seriata* (12)
		+++ (12)	nt	nt	nt	nt	*N. parvum* (12)
	T. koningii						
	T. longibrachiatum	+++	nt	nt	nt	nt	*D. seriata* (8)
	T. polysporum						
	Trichoderma spp.	+++	nt	nt	nt	nt	*D. seriata* (8)
	T. asperellum + *T. gamsii*						
Genera mix	*Trichoderma* + *Gliocladium*						

(*Continued on next page*)

[a] Efficiency: - - ineffective; - + less effective; + moderately efficient; ++ efficient; +++ highly efficient; nt, not tested.

[b] Countries: (1) Australia; (2) Austria; (3) Chile; (4) Egypt; (5) France; (6) Germany; (7) Greece; (8) Hungary; (9) Italy; (10) New Zealand; (11) Portugal; (12) South Africa; (13) Spain; (14) Switzerland; (15) U.S.A.

[c] Abbreviations used for pathogen species: *B.* = *Botryosphaeria*; *D.* = *Diplodia*; *L.* = *Lasiodiplodia*; *N.* = *Neofusicoccum*; *Pa.* = *Phaeomoniella*; *Pm.* = *Phaeoacremonium*; *F.* = *Fomitiporia*; *S.* = *Stereum*; *P.* = *Pleurostomophora*; *T.* = *Togninia*; *C.* = *Cryptovalsa*; *Di.* = *Diatrypella*; *E.* = *Eutypa*; *Eu* = *Eutypella*; *Li.* = *Libertella*.

second or third year of a multiple-year treatment of a vineyard. This result was mainly attributed to the wound protective effect of *Trichoderma* against new infections by GTD pathogens.

In the nursery, *Trichoderma* species are applied exclusively for the prevention of infections by vascular pathogens associated with Esca complex. Grafted plants previously treated with a *Trichoderma*-based product showed a reduction in the incidence of vascular Esca pathogens comparable to that obtained with quintozene and procymidone applications (Fourie et al. 2001). Similarly, Di Marco and Osti (2007) and Di Marco et al. (2004) reported a significant reduction of *Pa. chlamydospora* wood necrosis in grafted plants treated with *Trichoderma* (Trichodex, Rootshield) at the rooting stage, linking this reduction more to a stronger defense reaction in the treated plants than to the direct effect of *Trichoderma* on the pathogen. Recently, Pertot et al. (2016) reported that use of the selected *T. atroviride* strain SC1 (Vintec) during the hydration steps before cold storage and grafting were the best treatments to prevent *Pm. minimum* and *Pa. chlamydospora* infections compared with the use of cryptonol (8-hydroxyquinoline sulfate) or iprodione during the same steps. On the contrary, Fourie and Halleen (2004, 2006) considered the exclusive use of *Trichoderma* (Trichoflow-T) not suitable for efficient GTD control in nurseries. As a matter of fact, in none of the nursery trials was the preventive *Trichoderma* application able to fully avoid infections, as expected, but it reduced them strongly. In addition to the antagonistic effect, a significant increase of root biomass was reported in grafted plants treated with *T. harzianum* or *T. longibrachiatum* (Di Marco and Osti 2007; Di Marco et al. 2004; Fourie et al. 2001). On the other hand, Pertot et al. (2016) observed no differences in the root systems between plants treated with a strain of *T. atroviride* and the untreated control. Overall plant quality may be also influenced by *Trichoderma* spp. (Di Marco and Osti 2007; Fourie and Halleen 2004). In previous trials with a different *Trichoderma* strain, the use of *Trichoderma* in all first steps of production (grafting, callusing, rooting) led to more plant growth failures, but also to a higher

Table 14. (*Continued from previous page*)

Esca complex						Eutypa dieback					
In vitro	**In planta**	**In field**	**WP**	**NU**	**Pathogen[c]**	**In vitro**	**In planta**	**In field**	**WP**	**NU**	**Pathogen[c]**
					Cadophora luteo-olivacea [13] *Pa. chlamydospora* [13] *Pm. minimum* [13]						
++					*Pa. chlamydospora* [5]						
++					*Pm. minimum* [5] *Cadophora luteo-olivacea* [13] *Pa. chlamydospora* [13] *Pm. minimum* [13]						
+++	nt	nt	nt	nt	for detoxification [14]	nt	nt	nt	+	nt	*E. lata* [1]
						+++	nt	nt	nt	nt	for detoxification [14]
						nt	+++	nt	nt	nt	*E. lata* [15]
nt	++	nt	nt	nt	*Pa. chlamydospora* [5]						
+++	nt	nt	nt	nt	for detoxification [14]	+++	nt	nt	nt	nt	for detoxification [14]
+++ [12]	nt	nt	++ [12]	+++ [9]	*Pm. minimum* [9][12]	+++ [12]	nt	nt	- - [5] /+++ [12]	nt	*E. lata* [5][12]
+++ [12]	nt	nt	++ [12]	+++ [9]	*Pa. chlamydospora* [9][12]						
nt	nt	++ [12]	nt	nt	for viability [12]						
+++ [12]	nt	nt	++ [12]	nt	*Pa. chlamydospora* [12]	+++ [12]	nt	nt	nt	nt	*E. lata* [12]
+++	nt	nt	nt	nt	*Pa. chlamydospora* [9]						
++	nt	nt	nt	nt	*Pa. chlamydospora* [9]						
+++ [12]	++ [9]	nt	++ [6]/+++ [9][12]	++ [9][12]	*Pa. chlamydospora* [6][9][12]	+++ [12]	++ [1]	nt	- - [5]/++ [1][7][12]	nt	*E. lata* [1][5][7][12]
+++ [12]	nt	nt	+ [6]/+++ [12]	nt	*Pm. minimum* [6][12]	nt	nt	++ [12]	nt	nt	for viability [12]
nt	+++ [9]	++ [9][12]	nt	nt	for viability [9][12]	+++	nt	nt	nt	nt	for detoxification [14]
+++	nt	nt	nt	nt	for detoxification [14]						
+++ [12]	nt	nt	nt	nt	*Pa. chlamydospora* [12]	+++ [12]	nt	nt	nt	nt	*E. lata* [12]
						nt	nt	nt	++	nt	*E. lata* [10]
nt	++ [9]	nt	+++ [9]	++ [9]	*Pa. chlamydospora* [9]						
nt	+++ [9]	++ [9]	nt	nt	for viability [9]						
+++	nt	nt	nt	nt	for detoxification [14]	+++	nt	nt	nt	nt	for detoxification [14]
nt	nt	++ [9]	++ [9]	nt	*Pa. chlamydospora* [9]						
nt	nt	nt	nt	++ [12]	*Phaeoacremonium* spp. [12]						
nt	nt	nt	nt	++ [12]	*Pa. chlamydospora* [12]						

percentage of certifiable plants at the end of the process, especially for the vines treated at the rooting stage (Di Marco and Osti 2007). Furthermore, *Trichoderma*-treated plants were more vigorous compared with the untreated ones, in terms of increased resistance to environmental stresses and to *Pa. chlamydospora* artificial inoculations.

Among the other fungal BCAs, *Fusarium lateritium* was able to degrade in vitro some phytotoxins involved in expression of GTD foliar symptoms (Christen et al. 2005). It was successfully tested as a wound protectant for the control of *E. lata* infections and the benomyl-resistant mutant strains were especially effective (John et al. 2005; McMahan et al. 2001). The nonpathogenic Oomycete *Pythium oligandrum* was able to stimulate host plant defenses when present in the rhizosphere of different plants (Benhamou et al. 2012) and reduced *Pa. chlamydospora* wood necrosis by a higher induction of the genes normally activated during pathogen infection (Yacoub et al. 2016).

Discussion

The complexity of GTD management is increasingly evident as research deepens our knowledge on the topic. Agronomical, environmental, ecological, host plant, and pathogen factors are all involved in the development and expression of GTDs, giving few possibilities in building a conceptual model of GTDs effective in all viticulture situations. In view of this complexity, the main goal of this review was to attempt to synthesize the results obtained in the last 15 years by scientific testing in the search for efficient tools, active ingredients, and biocontrol agents as a support for the control of the three main GTDs. The review of scientific literature gave us the chance to report the different approaches used to test and select products and methods for control of GTDs.

Assayed a.i.s ranged from systemic to protectant, from broad-spectrum to selective, and from synthetic to natural. Most of the reviewed a.i.s were tested in vitro while only 50% were further tested in bioassays. This proportion could be partially due to the lack of activity obtained in vitro but also to the complexity in setting up and following a long-term field study, which is unavoidable given the "erratic" appearance of some GTD foliar symptoms. The variation in efficiency often recorded for the same a.i. toward the same GTD pathogens in different studies reflects the well-known complexity of GTDs, but also attests to how other factors (a.i. formulation used, time of application, climatic conditions, etc.) can influence activity. Furthermore, the often very different applied protocols could lead to an under- or overestimation in a.i. efficiency. For example, Bester et al. (2007) linked the low Botryosphaeriaceae incidence recorded after a prochloraz treatment to both the short time of the test and the low stress condition of the vines growing in a glasshouse. Such conditions could have limited the colonizing ability of the inoculated pathogens. Again, a.i. efficiency is surely underestimated in the case of artificial inoculations by mycelial plug since wood pathogens usually infect by spores. In the same way, the spore concentration used in artificial GTD inoculations could also affect the treatment evaluation. If significantly higher than what is normally expected in natural conditions, this high advantage for the pathogen could lead to an underestimation of the efficiency of the chemical or biological treatment. The results of Ayres et al. (2017), observing different efficiency levels for the same a.i. in wound protection trials when different spore concentrations of *E. lata* were used, are in agreement with this remark. Regarding the field trials, some protocols seem too far from extensive and practical application. For wound protection, the only application method already in use by growers and economically feasible is the atomizer application of *Trichoderma*-based commercial products soon after pruning. Most of the other a.i.s were applied directly on pruning cuts by local spraying or hand painting. These modes of a.i. application are economically feasible only in high-value vineyards and are suitable in large vineyards only if special tools are used to speed up the application. Similarly, the pole and trunk injection methods used to distribute a.i.s in some experimental tests become both expensive and time-consuming if applied on a large scale in a vineyard, especially if they do not have a long lasting effect. Thus, field trials that set up applicable and feasible protocols, limiting the variability only to the unavoidable factors (pathogen, climate, cultivars, etc.), could have a better impact in setting up efficient tests.

Within the synthetic organic compounds, thiophanate-methyl was the most "flexible" a.i., showing good efficiency to control infections by GTD pathogens in vineyards and nurseries in Australia, Chile, New Zealand, and the U.S.A. Even if they do not reach the efficiency levels recorded for banned benomyl and carbendazim, thiophanate-methyl could be a valid substitute useful to manage GTDs in nurseries and to protect vines from new infections in the field. Different sensitivities toward some a.i.s, such as thiophanate-methyl, chlorotalonil, iprodione, procymidone, etc., recorded for Botryosphaeriaceae species and, in some cases, for the vascular Esca complex pathogens were highlighted. Such variability, which can limit the results of a treatment, could be overcome by the use of a.i. combinations with various different modes of action. As a consequence, tests with commercial products of two a.i.s have shown some promising results. Despite their large use in nurseries, some a.i.s were ineffective toward GTD pathogens. For example, 8-hydroxyquinoline sulfate showed inconsistent results when used toward a species of Botryosphaericeae, *Pa. chlamydospora* and *Pm. minimum* in soaking water (Gramaje et al. 2009).

Among natural compounds, the efficiency of a garlic+chitosan+vanillin mix for wound protection, and of an inorganic salt and seaweed extract mix in the reduction of GLSD symptoms indicate the possibility of finding tools, based on natural substances, to help and limit losses by GTDs in organic viticulture (Calzarano et al. 2014; Cobos et al. 2015). However, the results obtained up to now with biostimulants showed that they are largely inefficient in vineyards, sometimes leading to an increase in disease incidence, possibly because of faster and/or greater movement of fungal toxins to the leaves. Still, the biostimulants field is developing and it cannot be excluded that other products acting as biostimulants or defense inducers can bring different results and efficacy. The use of biostimulants in nurseries toward vascular pathogens appears to be promising, where they reduced the length of necrotic lesions in potted plants artificially infected with *Pa. chlamydospora* (Calzarano and Di Marco 2007; Di Marco and Osti 2007).

For BCAs, although several microorganisms were tested, currently only *Trichoderma* spp. have been shown to be the most suitable agent for biological control of GTDs, in both field and nursery. The reason for this supremacy probably stems from the synergistic action of different biocontrol mechanisms, in their ecological characteristics (saprotrophic, endophytic) and in the positive effects induced in the host plants (Handelsman and Stabb 1996; Harman 2006; Pal and McSpadden Gardener 2006). All these aspects allow *Trichoderma* spp. to be used in different environmental conditions with almost the same biocontrol efficiency observed in vitro, despite the recorded influence of biotic and abiotic factors. With regard to this, Mutawila et al. (2011, 2016) demonstrated how, following *Trichoderma* field application on different cultivars, the BCA incidence during the season could be highly variable and not related to its biocontrol efficiency toward GTD pathogens. Age of vineyard, cultivar, environmental conditions, the age of the vines of first application (as application at early stages is essential in a preventive control tool), and also phenological grapevine stages were considered to be the main factors responsible for either the variation in biocontrol efficiency or the inconsistent results obtained with *Trichoderma* and, more in general, for the low use of BCAs in vineyards for management of fungal diseases.

Trichoderma can be useful as a preventive and long lasting treatment for pruning wound protection. It is important to protect pruning wounds with *Trichoderma*-based treatments as soon as possible in young vineyards to avoid the increase of GTD infections. It also seems important to choose the most suitable *Trichoderma* strains according to the cultivar, the main GTD to control, and the climatic conditions. The contemporary use of different *Trichoderma* strains could reduce the variation in biocontrol levels due to environmental factors (John et al. 2008; Aloi et al. 2015). *Trichoderma* spp. also have several positive effects for nursery applications. Globally, grafted vines had a more developed root system able to support plants in stressful conditions such as transplantation in the vineyard.

Furthermore, *Trichoderma*-treated vines were more resistant to wood colonization by GTD pathogens and stimulate the general defense reaction of the vine.

Among other promising BCAs, the rhizospheric *P. oligandrum* showed high persistence in the root system and stimulated plant defenses against complex pathogens. As reported by Yacoub et al. (2016), *P. oligandrum* promotes a particular physiological condition called priming, in which the plant is able to mobilize its defense reactions more intensely in response to infection by the Esca complex pathogens.

Conclusions

All these studies clearly indicate that currently, there are many possible solutions even if none can be seen yet as a straightforward tool to add to the GTD management protocols. The control of Botryosphaeria dieback, the Esca complex diseases, and Eutypa dieback still represent a challenge for both end-users and scientists, even if the knowledge on GTDs so far acquired allowed the identification of some practices to limit the impact of GTDs in vineyards and nurseries (Fontaine et al. 2016a). These practices could widely benefit from the support of a.i.s and BCAs studied by scientists during recent years.

High infection risks in nurseries and vineyards and the lack of curative treatments have encouraged the idea of a transversal strategy for GTD control along the grapevine growing sector. According to Armengol (2014), any GTD control method could be useless if applied only in the nursery or in the vineyard. To date, the best strategy is to start the GTD control in the nursery to obtain healthier vines and to continue the control in the vineyard, limiting contamination by GTD pathogens. Recent studies support this strategy. For example, Larignon and Bruez (2016) demonstrated that after 15 years in a vineyard without any treatment for GTD control, nursery-GTD-treated plants were infected to a similar extent as those in the nursery-untreated group. Furthermore, the beneficial effect of early adoption of good practices to limit the economic impact of GTDs in vineyards was clearly reported by Baumgartner et al. (2014). According to that study, the use of highly efficient control methods could strongly limit the economic impact of GTDs up to its elimination, if the practices are adopted within 3 years of planting the vineyard. Another problem is that of knowledge transfer from scientists to end-users. To date, despite the broad knowledge obtained by research on GTDs, they are not well known in some countries and are often confused with one another by vine-growers, leading to underestimation or to the adoption of wrong practices and treatments. First results of the WINETWORK project (http://www.winetwork.eu/), funded by European Community and focused on the knowledge transfer on GTDs, are demonstrating that this transfer fault has boosted many empirical attempts to "solve" the problem, sometimes reporting a tool as "a cure" but without any scientific validation, or continuing to use inefficient a.i.s or practices. In this regard, a recent European survey on nursery practices attested how 11.6% of nurseries still use no fungicides or disinfectants during the entire production process and, when used, the most common one is based on an a.i. that was shown to be nonefficient for the control of GTDs when assayed in scientific trials (Gramaje and Di Marco 2015).

Trials on promising ways to develop tools to support GTD control are ongoing. For example, recent studies are focusing on the translocation of a.i.s inside plants. In particular, carboxylic acid function, methyl groups, amino-acids, or sugars can be added to a.i. molecules to increase their translocation in plant tissues or to transform nonsystemic molecules into a.i.s able to reach the pathogens in woody tissues (Chollet et al. 2004, 2005; Wu et al. 2015).

Regarding BCAs, they are at present the most efficient agents for sustainable disease management, both in the vineyard and nursery. They can be used not only in organic viticulture but also in integrated strategies, for example, by the use of benzimidazole-resistant *Trichoderma* mutants (Mutawila et al. 2015). The integrated a.i.-BCA approach could take advantage of the specific characteristics of both control methods toward GTDs, namely i) the immediate protective effect of the a.i. and ii) the broad-spectrum and lasting efficacy of the BCA, coupled with their positive effects on plants. For instance, BCAs could limit the effect of biotic and abiotic stress that often leads to higher symptom expression levels (Sosnowski et al. 2011; Spagnolo et al. 2014, 2017; Van Niekerk et al. 2011c). The BCA could have a positive effect through actions on host plant metabolism changes and disorders determined by plant defense responses and fungal toxins, respectively (Abou-Mansour et al. 2015; Burruano et al. 2016; Fontaine et al. 2016b). The study of both the grapevine microbiome and its interaction with grapevine could represent a technological development in the control of these diseases, permitting the identification of new BCAs useful for GTD pathogens and/or the host plant defense system (Pinto and Gomes 2016). Other promising opportunities to limit GTD spread and damage could be linked to the transfer of resistance factors from wild species into new GTD-resistant varieties, as was done for other important grapevine diseases such as downy or powdery mildew (Eibach et al. 2007). In this direction and according to the putative genetic basis of *Vitis vinifera* that confers a different sensibility to GTDs, ongoing studies are addressed to find GTD resistance genetic factors within the Vitaceae family for transfer into cultivated ones. Some promising results have been obtained for Botryosphaeria dieback (Guan et al. 2016). Nevertheless, the introduction of genetically modified grapevine cultivars could show different issues, linked either to the prohibition of their use in some countries or to the required adjustment in terms of cultural and enological practices (Pedneault and Provost 2016).

In conclusion, the perspectives for efficient GTD control could take advantage of both the GTD knowledge reservoir so far built and the ongoing new insights. A holistic view of the problem could be the key to define winning strategies for the control of GTDs, whose model could also be applied for the control of other complex plant diseases.

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Vincenzo Mondello

Vincenzo Mondello is a research scientist at the Induced Resistance and Plant Bio-Protection (RIBP) research unit of the University of Reims Champagne-Ardenne (URCA, France). Currently, he collaborates with the team of Prof. Florence Fontaine, studying grapevine physiological changes and gene defense induction determined by both grapevine trunk disease (GTD) pathogens and active ingredients and potential biocontrol agents used for their control. His research activity as a plant pathologist started at the University of Palermo (Italy) on fungal diseases of Mediterranean and subtropical crops (*Olea europaea*, *Citrus* spp., *Opuntia ficus indica*, *Mangifera indica*) with a focus on biocontrol agents and grapevine pathology. Besides epidemiological studies on the main recurrent diseases such as powdery and downy mildew, his activity since 2006 was mainly addressed to GTD epidemiology and etiology in Sicilian vineyards. He contributed in both national and international projects on GTDs, participating in the management of the European scientific working group on GTDs within the WINETWORK Project (2015–2017) and in the scientific committee of the 10th International Workshop on Grapevine Trunk Diseases (IWGTD).

Aurélie Songy

Aurélie Songy is a Ph.D. student at the Induced Resistance and Plant Bio-Protection (RIBP) research unit at the University of Reims Champagne-Ardenne (URCA, France) since 2016. She is an agronomy engineer who graduated from the Ecole Nationale Supérieure d'Agronomie et des Industries Alimentaires (ENSAIA) of Nancy (France) in 2015. She also received a master's degree in biology and ecology for forest, agronomy and environment (FAGE) from the University of Lorraine (Nancy, France). Her thesis aims to study the impact of heat stress, under different hydric regimes, on grapevine physiology linked with the aggressiveness of Botryosphaeriaceae. This work is part of the GTDfree Chair, first industrial research chair in agronomy INRA-Maison Hennessy-ANR. Previously, she participated to the CASDAR project V1301, concerning the aggressiveness of pathogenic fungi and mode of action of sodium arsenite, and to a part of the thesis project of Dr. Pinto dealing with the study of the biocontrol capacity of grapevine associated microorganisms against botryosphaeria dieback agents. She is also currently involved in a PHC Balaton project on the virulence of grapevine trunk diseases with the University of Eger in Hungary (2017–2018).

Enrico Battiston

Enrico Battiston is a former Ph.D. student in plant pathology at the University of Florence (Italy) and in plant physiology at the University of Reims Champagne-Ardenne (URCA, France), where he undertook a joint Ph.D. project on the development of an innovative delivery system to enhance the biological activity of active substances for the control of grapevine fungal diseases. He graduated with a B.Sc. in viticulture and enology at the University of Udine (Italy) and a M.Sc. in the same fields at the Hochschule Geisenheim University (Germany). He was introduced to grapevine trunk disease management during his first degree, performing a study on the composting process of grapevine pruning residues, monitoring the survival of *Diaphorte neoviticola* spores in the composted material. Dr. Battiston is actively involved in the development of sustainable practices for plant protection in vineyard and grapevine propagation in nursery with particular regard to the aspects related to grapevine young decline.

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Catia Pinto is a research scientist at the Induced Resistance and Plant Bio-Protection (RIBP) research unit at the University of Reims Champagne-Ardenne (URCA, France). Her main research interest is currently focused on the study of grapevine-microbiome interactions and the development of new sustainable solutions for grapevine protection against grapevine trunk diseases. She received a master's degree in molecular genetics in 2011 from the University of Tras-os-Montes and Alto Douro (UTAD) and completed a double Ph.D. degree in biology and molecular genetics in 2017 by a European cotutelle network with the University of Aveiro (UA, Portugal), University of Reims Champagne-Ardenne (URCA, France), and Biocant (Portugal). Her Ph.D. research was deep analysis of the dynamics of the natural microbial resources from vineyard until wine fermentation through NGS approaches, and to exploit potential beneficial microorganisms from grapevine to grapevine protection, namely to control Botryosphaeriaceae species associated with grapevine trunk diseases.

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Cindy Coppin is a research scientist at the Induced Resistance and Plant Bio-Protection (RIBP) research unit at the University of Reims Champagne-Ardenne (URCA, France). She graduated with a master's degree in enology and viticulture in 2015 from the Institute of Grapevine and Wine Sciences (ISVV), Bordeaux (France). She is working on a network biocontrol project named Advantage that aims to develop new technical management tools for a global management of grapevine trunk diseases at both nurseries and vineyards. While completing her master's thesis at INRA (National Institute for Agricultural Research, Bordeaux), she was strongly involved in the phenotypic characterization of Botryosphaeriaceae species and their interactions with plants.

Patricia Trotel-Aziz

Patricia Trotel-Aziz is associate professor in the research unit of Induced Resistance and Plant Bioprotection (RIBP) at the University of Reims Champagne-Ardenne (France). Her research activities within the general framework of plant responses to environmental constraints focus on plant-rhizobacterial interactions with pathogens and their virulence factors. She isolated the "PTA" beneficial bacteria and contributes to decipher their impacts on grapevine protection and tolerance against pathogens with different lifestyles through both direct- and indirect- effects (antagonisme, detoxication,ISR,...), and takes also advantage of *Arabidopsis* for functional approaches. She also explored the possibility to reduce the potential toxicity and diffused pollution due to organic pollutants introduced into soil and/or water in vineyards, by using plants associated with beneficial bacteria. She is currently mainly associated with the MALDIVE Chair 'Disease of grapevine wood' at RIBP, aiming to reduce the variability of plant protection in situ by using such beneficial microorganisms.

Christophe Clément

Christophe Clément a research scientist at the Induced Resistance and Plant Bio-Protection (RIBP) research unit at the University of Reims Champagne-Ardenne (URCA, France). The goal of his research is to decipher plant immunity in response to both biotic and abiotic stresses. This knowledge is further used to trigger plant immunity previously to stress appearance by eliciting/priming plant defense mechanisms, using either beneficial microorganisms or the elicitors they may synthesize. Plant defense response is more deeply investigated in the context of global warming, especially drought and high temperatures.

Laura Mugnai

Laura Mugnai is associate professor of plant pathology at the Department of Agrifood Production and Environmental Sciences at the University of Florence, where she runs the courses of grapevine plant pathology, diseases caused by fungal agents, and tropical plant pathology. She has conducted or collaborated in research on various aspects of plant pathology, first in forestry, where she focused on taxonomy and described a new intersterility group of *Heterobasidion annnosum*, and later in plant pathology of agricultural crops, including olive and grapes. In the last 25 years, she has concentrated on wood diseases of grapevine, in particular the esca complex. On this subject, she has been an invited speaker in Italy and many other grape-growing countries worldwide. She has been responsible for and collaborated in several national and international projects, including EU funded projects. She was co-founder and, for 12 years, chairperson of the subject matter committee on grapevine trunk diseases within the International Society of Plant Pathology (ISPP), for which she organized or co-organized many workshops and

meetings. She was in the management committee as the Italian representative of the COST action on this subject at the European level (COST ManaGTD – FA1303 Sustainable control of grapevine trunk diseases). She is actively engaged in running the activities of the Mediterranean Phytopathological Union and, since 2009, she has been co-chief editor of the international journal Phytopathologia Mediterranea.

Florence Fontaine

Florence Fontaine is a research scientist at the Induced Resistance and Plant Bio-Protection (RIBP) research unit at the University of Reims Champagne-Ardenne (URCA, France). She studied plant physiology and biology at URCA and earned her Ph.D. in biological sciences in 1999, investigating wood quality on oak trees. She has served as a teacher and researcher at URCA since 2002. Since 2002, she has been the head of the grapevine trunk disease (GTD) team, with main topics of identification of the molecules linked to the aggressiveness of Botryosphaeriaceae, pathogens associated to Botryosphaeria dieback, the impact of GTDs on vine physiology including the primary and secondary metabolisms; and the focus on friendly strategies to manage GTDs. During the last 5 years, she was the chair of the COST Action FA1303 entitled "Sustainable control of grapevine trunk diseases" (2013–2017), which includes 120 people from 24 European countries and the leader of the scientific working group on GTDs in the WINETWORK project (2015–2017). She is an active member of the International Council for Grapevine Trunk Diseases and member of the International Organization of Vine and Wine (OIV) expert group "Vine Protection." Starting in 2017, she is the leader of the Chaire MALDIVE, with a term through 2020, that suggests more research connected with private companies. She is currently teaching plant biology and physiology, plant pathology, and viticulture at the graduate level.

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Annex III – Co-authored research paper

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Di Marco S., Osti F., Bossio D., Nocentini M., Metruccio E., **Battiston E.** and Mugnai L. (2017). Activity of electrolyzed acid water towards trunk disease pathogens in grapevine nursery.

UTILIZZO DI ACQUA ACIDA ELETTROLIZZATA IN VIVAIO NEI CONFRONTI DI PATOGENI DEL LEGNO DELLA VITE

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RIASSUNTO

Phaeomoniella chlamydospora (*Pch*) e *Phaeoacremonium minimum* (*Pmi*) sono i principali funghi patogeni associati a malattie del legno della vite in vivaio. Questi patogeni possono causare nel materiale di propagazione infezioni latenti, spesso associate a forme di deperimento in impianti giovani. L'infezione può verificarsi durante diverse fasi del processo produttivo, in particolare durante l'idratazione. Per ridurre questo rischio si è inteso valutare l'attività di acqua acida elettrolizzata (EAW) utilizzata per idratare le talee prima dell'innesto. L'EAW è un igienizzante caratterizzato da pH 2,5, potenziale di ossidoriduzione (ORP)>1000 e cloro libero. Sono state effettuate prove su 3 portinnesti: 1103P, K5BB e SO4 (cultivar Trebbiano Romagnolo) in ambiente controllato e in vivaio, su materiale inoculato artificialmente con *Pch*. I principali risultati indicano una significativa riduzione dell'infezione di *Pch* rilevata alla fine del ciclo vegetativo, su piante idratate in EAW.

Parole chiave: *Phaeomoniella chlamydospora, Phaeoacremonium minimum,* idratazione portinnesto, strategie di difesa in vivaio viticolo

SUMMARY

ACTIVITY OF ELECTROLYZED ACID WATER TOWARDS TRUNK DISEASE PATHOGENS IN GRAPEVINE NURSERY

Phaeomoniella chlamydospora (*Pch*) and *Phaeoacremonium minimum* (*Pmi*) are the main grapevine trunk disease pathogens that cause infections in the nursery and are often associated to decline occurrence in young vineyards. The infection may occur during the grafting process, particularly during hydration. This study assessed the activity of electrolyzed acid water (EAW) to reduce the risk of infections during the hydration of cuttings before grafting. EAW is a sanitizing agent characterized by low values of pH (2.5), high oxidation-reduction potential (ORP>1000) and a certain amount of free chlorine. Trials were performed on three rootstocks 1103P, K5BB and SO4 (Trebbiano Romagnolo as scion), under controlled conditions and in the nursery where plants were artificially inoculated with *Pch*. The most important results showed a significant reduction of the infection severity on cuttings inoculated with *Pch* and hydrated in EAW assessed in the nursery at the end of the vegetative growing season.

Keywords: *Phaeomoniella chlamydospora, Phaeoacremonium minimum,* cutting hydration, graftling control strategy

INTRODUZIONE

Phaeomoniella chlamydospora (*Pch*) e, in misura minore, *Phaeoacremonium* spp. - *P. minimum* (*Pmi*) in particolare - sono i principali patogeni associati al complesso Esca, la più importante e diffusa malattia del legno della vite (Surico *et al*, 2008). In vivaio, *Pch* e *Pmi*

possono infettare il materiale di propagazione durante tutto il processo produttivo, soprattutto nella fase d'idratazione prima dell'innesto, e possono causare imbrunimenti al legno sotto forma di punteggiature e strie bruno-nerastre. Gli imbrunimenti, nella maggior parte dei casi, non sono associati a sintomi esterni, rendendo impossibile distinguere una pianta in cui i patogeni sono presenti da una pianta esente da infezione. Tuttavia, sebbene numerosi studi indichino la presenza di patogeni nel legno delle viti alla fine del processo di propagazione, e questi siano stati dimostrati essere associati a fenomeni di deperimento nei nuovi impianti (Edwards e Pascoe, 2004) non è stato ancora dimostrato definitivamente il ruolo delle infezioni nel materiale di propagazione sulla performance delle piante in vigneto (Gramaje e Armengol, 2011).

Molte ricerche condotte nelle principali fasi di produzione delle barbatelle sono state finalizzate allo sviluppo di una strategia che consenta il contenimento delle infezioni da *Pch* e *Pal* e il miglioramento qualitativo della barbatella. I risultati più promettenti, sebbene non risolutivi, sono stati conseguiti utilizzando formulati a base di *Trichoderma*, con una riduzione della severità delle infezioni e un miglioramento dell'apparato radicale della barbatella (Di Marco e Osti, 2007; Fourie *et al.*, 2007; Gramaje e Di Marco, 2015; Pertot *et al*., 2016).

L'acqua acida elettrolizzata (EAW) è un disinfettante utilizzato prevalentemente su strumenti chirurgici e per la sterilizzazione di prodotti alimentari (Huang *et al*., 2008), ed è ottenuta per elettrolisi di soluzioni di NaCl o di KCl, eseguita in una speciale attrezzatura dove anodo e catodo sono separati da una membrana a scambio cationico. EAW è prodotta all'anodo ed è caratterizzata da pH 2,3-2,7, potenziale di ossido-riduzione (ORP)>1000 e una certa percentuale di cloro libero (Li *et al*., 2014).

Diversi studi hanno evidenziato effetti di acque acide elettrolizzate, utilizzate in vario modo, nei confronti di patogeni delle piante quali *Botrytis* spp., *Fusarium* spp., *Cladosporium* spp., *Helminthosporium* spp., *Colletotrichum* spp., *Epicoccum nigrum*, *Monilinia fructicola*, *Phomopsis longicolla***,** *Aspergillus flavus*, *Penicillium expansum* o *Tilletia indica* (Fujiwara et al., 2011; Xiong et al., 2014) e, preliminarmente, su *Pch* e *Pmi* (Di Marco e Osti, 2009).

Il presente lavoro riporta gli effetti di applicazioni di EAW in vivaio finalizzate al contenimento di infezioni di *Pch* provocate artificialmente su talee di alcuni portinnesti tra i più utilizzati su vite.

MATERIALI E METODI

Trattamento e verifica di piante vegetanti e certificabili

L'acqua acida elettrolizzata (EAW) è stata ottenuta da un generatore EAW-AG25 (CBC Europe, Nova Milanese) attraverso un processo di elettrolisi a membrana. L'EAW utilizzata nel presente studio aveva i seguenti valori: pH = 2,5–2,7; ORP = 1120–1129 mV, e 40–42 ppm di cloro libero.

Il processo di idratazione è stato effettuato secondo la consuetudine del vivaio, operando con mazzi di 650-700 talee di ciascuno dei 3 portinnesti 1103P, K5BB e SO4 oggetto di indagine. Le talee sono stati prelevate in marzo, dopo conservazione in cella frigorifera a 3-5°C, e idratate in EAW o acqua. L'idratazione è stata condotta per 7-8 o 10-12 ore, all'interno dei contenitori in PVC normalmente utilizzati in vivaio. Le talee sono state quindi adoperate come portinnesto per marze della cv Trebbiano Romagnolo. Dopo l'innesto a omega eseguito con innestatrice manuale, le piante sono state sottoposte a forzatura in segatura per circa 3 settimane e, dopo una settimana di acclimatamento in serra non riscaldata, sono state messe a dimora su terreno in vivaio. Per ogni tesi, sono state previste 3 parcelle randomizzate, ciascuna costituita da 150 piante e corrispondente a una ripetizione. Le barbatelle sono state periodicamente monitorate ed

è stato eseguito un rilievo alla raccolta atto a verificare la percentuale di piante vive rispetto a quelle inizialmente piantate in vivaio, e la percentuale di piante certificabili di prima scelta (Dir. 2002/11/CE) rispetto a quelle vive alla raccolta.

Per ogni portinnesto e tempo di idratazione realizzato utilizzando acqua o EAW, i dati ottenuti esprimono la percentuale media di barbatelle raccolte rispetto a quelle inizialmente piantate in terreno di vivaio, e la percentuale media di barbatelle certificabili rispetto a quelle raccolte. I dati sono stati analizzati statisticamente mediante ANOVA (P=0.05) per discriminare gli effetti dei diversi tipi di acqua utilizzati.

La prova è stata condotta nel 2015 e 2016 con analoghe modalità di esecuzione.

Inoculazione di *Pch* e trattamento

Ulteriori 100 talee per ciascuno dei 3 diversi portinnesti oggetto d'indagine sono state prelevate in febbraio durante la frigo-conservazione a 3-5°C e sono state inoculate mediante sospensione acquosa di conidi di *Pch*. L'inoculo è stato ottenuto da colonie di *Pch* (*Phaeomoniella chlamydospora*, CBS 229.95) di 15-20 giorni, cresciute su PDA e mantenute in frigo-termostato a 25°C e fotoperiodo di 12 ore di luce e 12 di buio. Dopo deposizione di acqua sterile nella piastra, la colonia è stata leggermente raschiata con ansa e la sospensione conidica è stata aggiustata alla concentrazione d'uso di $1x10^5$/ml, determinata in cella Thoma. L'inoculazione è stata condotta immergendo la base della talea (2 cm) per 2 ore, in recipienti di PVC contenenti la sospensione conidica, che è stata periodicamente agitata per evitare la deposizione dell'inoculo sul fondo del recipiente. Altre 50 talee per ogni portinnesto sono state immerse in acqua, secondo le stesse modalità, a rappresentare la tesi non inoculato e non trattato. Le talee sono state poi ricollocate in frigo-conservazione.

A 45 giorni dall'inoculazione, 50 talee inoculate sono state trattate per idratazione in EAW e 50 in acqua, per 7-8 ore. Parallelamente, le 50 talee non inoculate sono state idratate in acqua per la valutazione dell'infezione naturale.

La prova è stata condotta nel 2016.

Isolamento di *Pch* da talee e barbatelle

Gli isolamenti sono stati effettuati nel tessuto legnoso di talee prelevate prima dell'inoculazione e di barbatelle alla raccolta, focalizzando l'attenzione sulla presenza di *Pch*.

Venti talee prelevate a campione prima dell'inoculazione sono state decorticate, fiammate superficialmente e sezionate longitudinalmente in tutta la loro lunghezza. Frammenti di legno prelevati a diverse altezze e, sempre, da aree di legno imbrunito, sono stati posti in piastre Petri contenenti PDA + streptomicina. Le piastre sono state collocate in frigo-termostato alla temperatura e fotoperiodo sopra riportati per il mantenimento di colonie di *Pch* in crescita, verificando l'eventuale sviluppo del patogeno.

Dopo la raccolta, 20 barbatelle, ognuna delle quali rappresentante una ripetizione, di ogni combinazione innesto/portinnesto, inoculate e trattate in EAW o acqua (non inoculate e trattate in acqua per l'infezione naturale) sono state decorticate e fiammate superficialmente. Sono state poi ricavate dal tronco sezioni trasversali di legno, a diverse altezze a partire dal punto di inoculazione. Ciascuna sezione legnosa è stata suddivisa in 4-6 frammenti che sono stati collocati in piastre Petri contenenti PDA + streptomicina. Attraverso passaggi successivi in altre piastre con analogo substrato nutritivo, si è valutata la presenza di colonie di *Pch*. Per ogni tesi, portinnesto e distanza dalla base della barbatella i dati provenienti da tutte le piante sono stati mediati e analizzati statisticamente con Tukey MRT, $P = 0,05$.

Per ogni portinnesto, sono stati analizzati i dati relativi al reisolamento del fungo fino alla distanza dal luogo di inoculazione in cui la percentuale media di frammenti fertili per *Pch* ha

evidenziato valori statisticamente simili tra le talee idratate in acqua inoculate e non inoculate (infezione naturale).

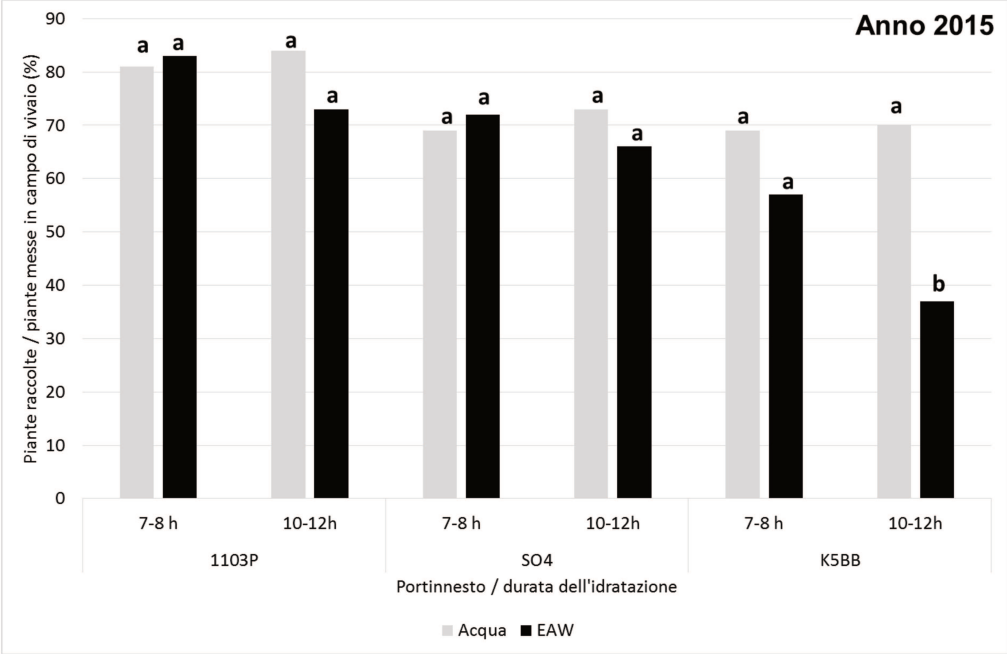
La prova è stata eseguita nel 2016.

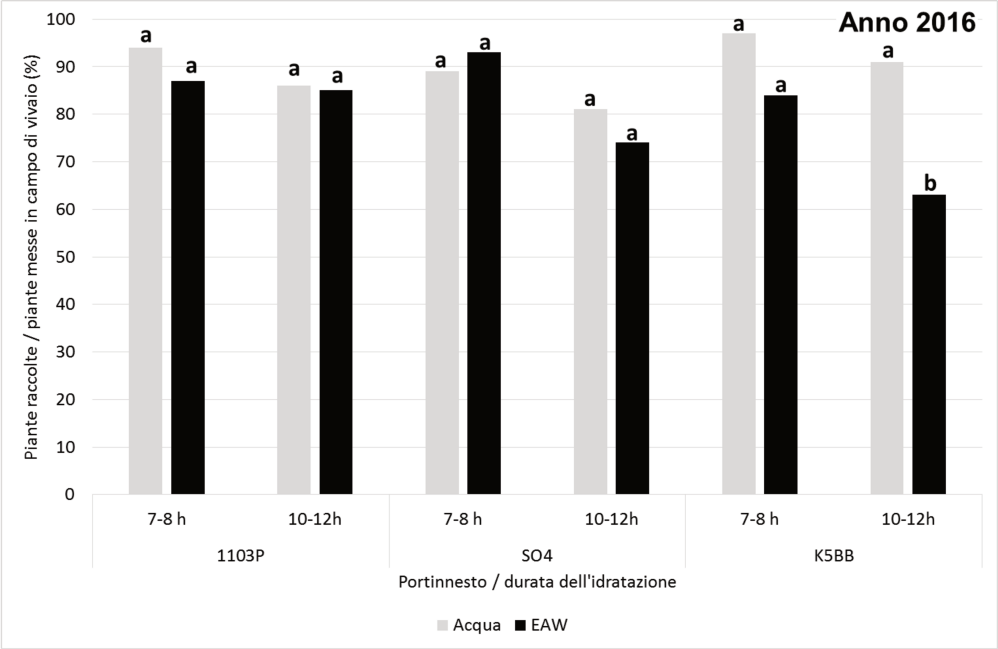
RISULTATI

Verifica di piante vegetanti e certificabili

L'idratazione in EAW per 10-12 ore ha evidenziato, in entrambi gli anni di prova, un decremento statisticamente significativo di piante vive alla raccolta per la sola combinazione K5BB/Trebbiano. Questo decremento non è stato rilevato in alcuna combinazione innesto/portinnesto idratando le talee per 7-8 ore (Figura 1).

Figura 1. Percentuale di piante raccolte a fine stagione vegetativa rispetto a quelle messe a dimora in campo di vivaio, nel 2015 e nel 2016

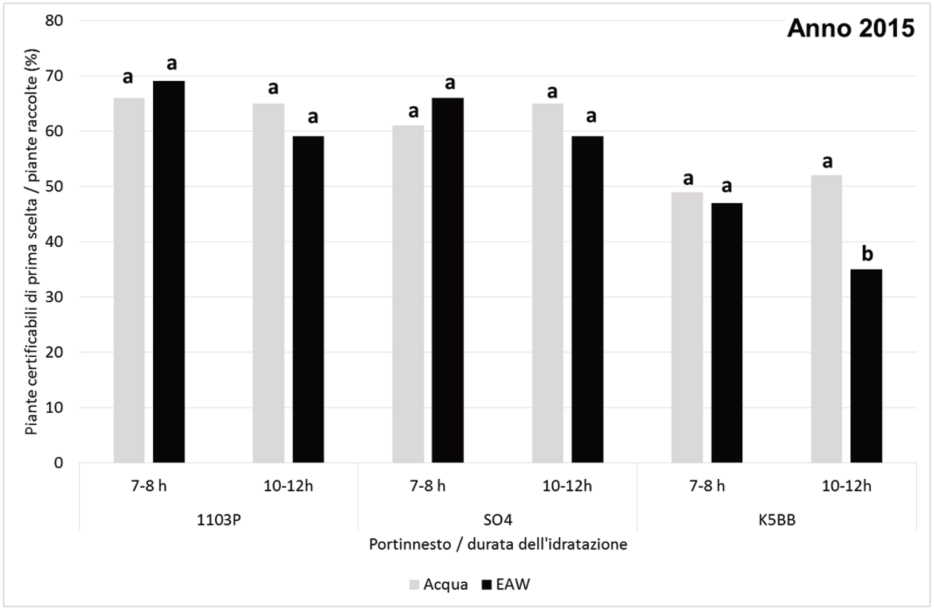


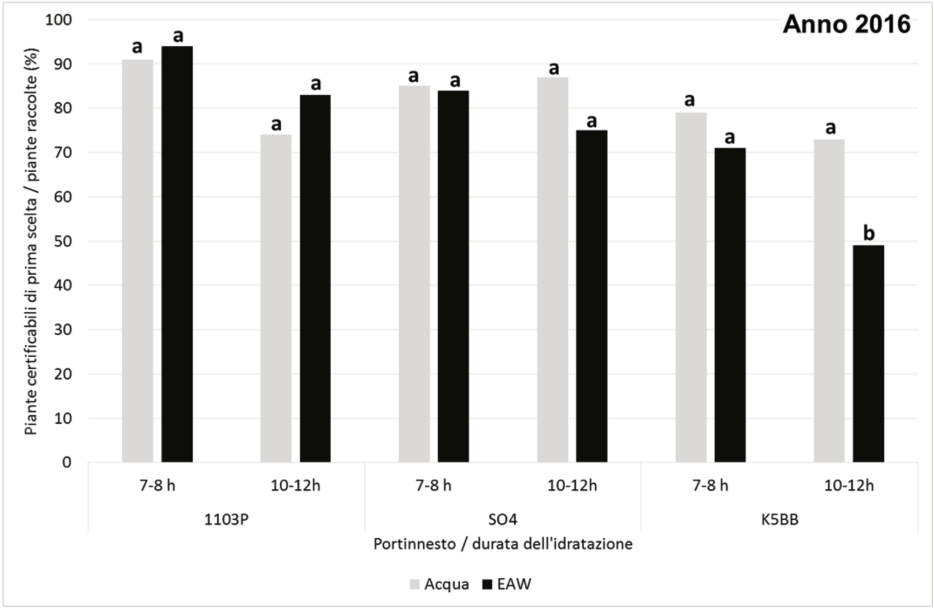


Per ogni tesi, i dati ottenuti si riferiscono alla percentuale media di barbatelle raccolte rispetto a quelle piantate in campo di vivaio. I dati sono stati analizzati statisticamente mediante ANOVA (P =0.05) per discriminare tra i diversi tipi di acqua utilizzati. Per ogni anno di prova, portinnesto e tempo di idratazione, lettere differenti indicano differenze statisticamente significative tra le tesi

La percentuale di barbatelle certificabili in relazione a quelle raccolte non è stata influenzata dal tipo di idratazione in EAW o in acqua. I dati ottenuti, infatti, hanno mostrato valori statisticamente simili per le 3 combinazioni innesto/portinnesto (Figura 2).

Figura 2. Percentuale di piante certificabili di prima scelta rispetto a quelle raccolte, nel 2015 e nel 2016





Per ogni tesi, i dati ottenuti si riferiscono alla percentuale media di barbatelle certificabili di prima scelta rispetto a quelle raccolte. I dati sono stati analizzati statisticamente mediante ANOVA (P=0.05) per

discriminare tra i diversi tipi di acqua utilizzati. Per ogni anno di prova, portinnesto e tempo di idratazione, lettere differenti indicano differenze statisticamente significative tra le tesi

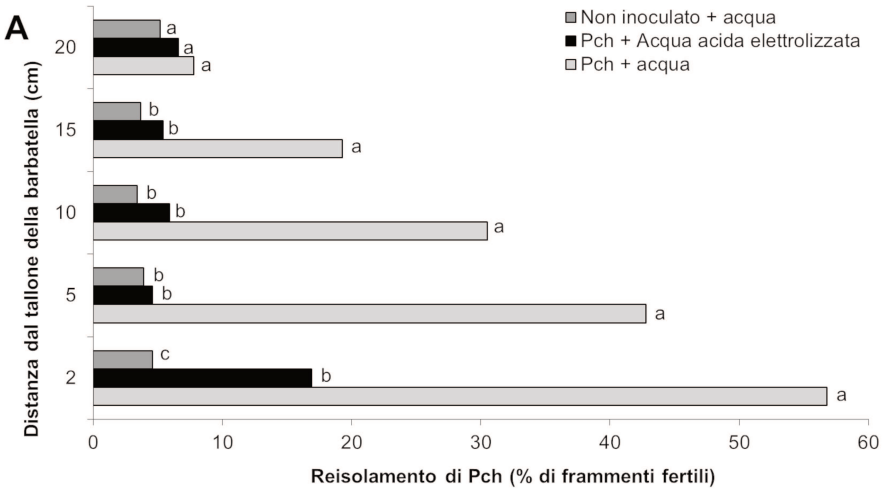
Isolamento di *Pch* da talee e da barbatelle

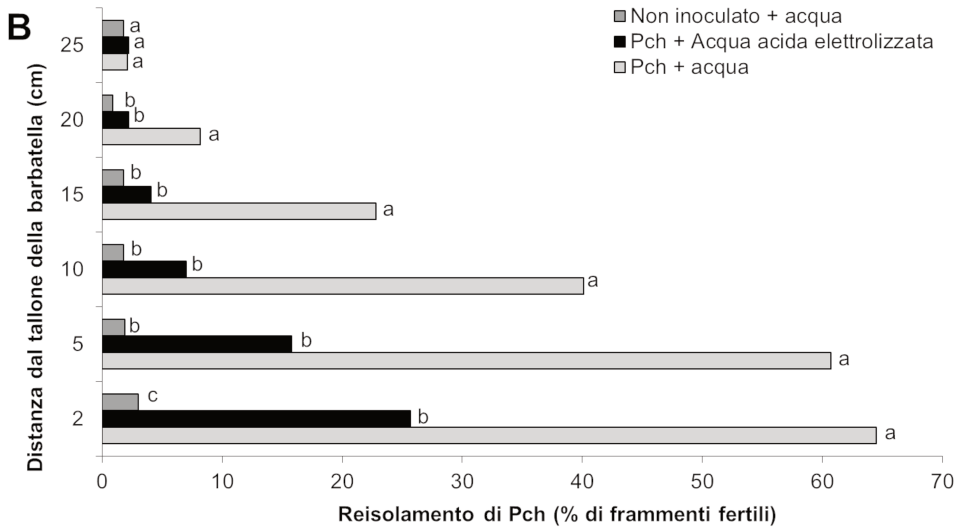
Gli isolamenti effettuati su talee prelevate a campione da magazzino prima dell'inoculazione hanno mostrato una scarsa presenza di *Pch* nei tessuti legnosi, con una frequenza media variabile tra 1 e 3% di frammenti fertili. Al contrario, la percentuale di talee in cui è stato rilevato il *Pch* è risultata del 65% (33 su 60) indipendentemente dal tipo di portinnesto.

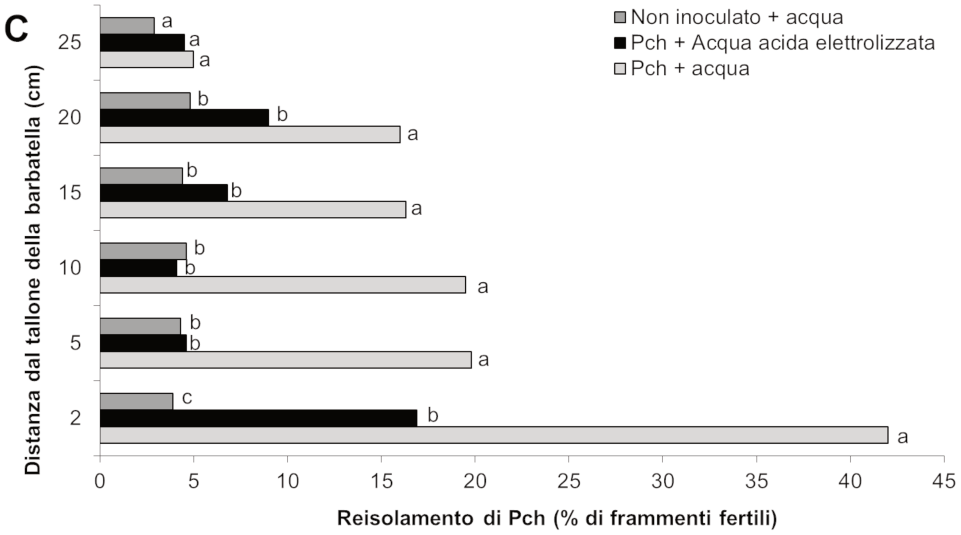
Relativamente ai risultati ottenuti sulle barbatelle derivate da talee inoculate artificialmente con *Pch*, la dinamica di reisolamento del patogeno lungo il tronco verificata alla raccolta è risultata uguale nelle 3 combinazioni innesto-portinnesto. Una riduzione costante della colonizzazione in seguito all'infezione artificiale (frammenti fertili per *Pch*) è stata osservata dal tallone della pianta andando verso il punto di innesto (Figura 3).

Le percentuali rilevate di frammenti fertili per *Pch* evidenziano una minore colonizzazione da parte del patogeno nella barbatelle con portinnesto K5BB, rispetto a quelle con 1103P e a quelle con SO4, dove si è registrata la massima percentuale di colonizzazione. Gli isolamenti effettuati nei primi 15 cm dal tallone per 1103P e nei primi 20 cm dal tallone per SO4 e K5BB, hanno evidenziato una percentuale media di frammenti di legno colonizzati da *Pch* sempre statisticamente inferiore nelle piante inoculate e idratate in EAW, rispetto alle corrispondenti piante inoculate e idratate in acqua (Figura 3).

Figura 3. Reisolamento di *Pch* effettuato dopo la raccolta in barbatelle innestate su portainnesti 1103P (A), SO4 (B) o K5BB (C)







Per ogni tesi, portinnesto e distanza dalla base della barbatella i dati provenienti da tutte le piante sono stati mediati e analizzati statisticamente con Tukey MRT, a $P = 0,05$. Per ogni distanza dal tallone, lettere differenti indicano differenze statisticamente significative tra le tesi

Ad eccezione di quanto rilevato nei primi 2 cm dal luogo di inoculazione (fino a 5 cm per SO4), le piante inoculate e idratate in EAW hanno mostrato una percentuale di frammenti fertili per *Pch* statisticamente simile a quanto rilevato nelle piante non inoculate e trattate in acqua. Le piante inoculate artificialmente e idratate con acqua hanno mostrato una percentuale media di

frammenti fertili di *Pch* simile a quella rilevata nelle piante non inoculate e idratate in acqua solo a una distanza dal tallone di 20 cm per 1103P e 25 cm per SO4 e K5BB.

DISCUSSIONE E CONCLUSIONI

Il presente studio è stato condotto a completamento e conferma di una più ampia ricerca atta a valutare caratteristiche, modalità d'azione e interazioni con i patogeni e la pianta ospite dell'EAW, e finalizzata allo sviluppo di una strategia di contenimento delle infezioni di patogeni fungini del legno di vite in vivaio (Di Marco *et al*., 2017).

I dati qui riportati mostrano che l'idratazione con EAW non ha mai interferito sulla percentuale di piante certificabili rispetto a quelle raccolte.

Tuttavia si è rilevata una riduzione di piante vegetanti a seguito dell'idratazione in EAW ma solo in una combinazione, K5BB/Trebbiano Romagnolo, e solo se sottoposta ad una prolungata idratazione (10-12 ore). Tale effetto fitotossico potrebbe costituire un limite dell'applicazione e dovrebbe essere valutato preliminarmente nelle diverse cultivar e combinazioni nesto/portainnesto, ma va ricordato che rilievi precedentemente condotti sull'assorbimento e sull'idratazione delle talee per i portinnesti investigati e le condizioni della prova, hanno mostrato che un'idratazione di 7-8 ore in acqua o EAW era adeguata a garantire una corretta idratazione della talea (Di Marco e Osti, 2009).

Considerando globalmente i risultati ottenuti, è stato possibile osservare una consistente riduzione della colonizzazione in seguito all'infezione artificiale di *Pch* nelle barbatelle derivanti da talee idratate in EAW, rispetto a quelle idratate in acqua.

Nel materiale proveniente da magazzino prelevato prima dell'idratazione, si è notata una scarsa presenza di legno naturalmente infetto da *Pch*, ovvero una bassa percentuale di frammenti risultati fertili. Su barbatelle alla raccolta, dopo innesto, forzatura e radicazione, l'incidenza delle infezioni naturali, era maggiore rispetto a quanto rilevato sulle talee in magazzino, pur sempre a fronte di una limitata colonizzazione ovvero limitata frequenza di isolamento. Tale fatto conferma che il rischio d'infezione è crescente durante le diverse fasi di lavorazione del materiale di propagazione (Gramaje e Armengol, 2011).

L'incremento di infezione rilevato anche in questo lavoro, potrebbe attribuire un ulteriore valore all'azione di contenimento dell'infezione da *Pch* da parte dell'EAW. Infatti, la percentuale di frammenti fertili nelle piante inoculate e idratate in EAW, rilevata per le 3 combinazioni innesto/portinnesto già a 5 cm dal punto di inoculazione e a distanze superiori, è generalmente allineata a una naturale presenza di *Pch* nella pianta. In altre parole l'idratazione in EAW ha ridotto un'infezione artificiale di gravità elevatissima, a livelli di contaminazione della barbatella "fisiologici" per le condizioni in cui si è operato e la partita di materiale legnoso utilizzata.

Ulteriori verifiche potranno evidenziare l'eventuale effetto di riduzione da parte di EAW delle infezioni che si verificano naturalmente in vivaio, in un contesto in cui non sono attualmente disponibili strategie di difesa che consentano la produzione di materiale di propagazione esente da patogeni del legno.

In conclusione, i risultati acquisiti in questo lavoro e le recenti evidenze sull'efficacia di EAW nel contenimento della germinazione conidica di *Pch* e *Pmi*, e sul mantenimento di favorevoli valori di pH e ORP anche dopo il contatto con i tessuti della pianta ospite (Di Marco e Osti 2009; Di Marco *et al*., 2017), suggeriscono un potenziale utilizzo dell'acqua acida elettrolizzata per la riduzione del livello di contaminazione delle viti da patogeni del legno nel processo di propagazione delle barbatelle in vivaio.

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Annex IV – Curriculum Vitae

Curriculum Vitae

PERSONAL DATA

Name	Enrico
Surname	Battiston
Date of birth	11.05.1988
Place of birth	San Donà di Piave, Venezia (Italy)
Nationality	Italian
Mail address	battiston.enrico@gmail.com

EDUCATION

2014 -2018 Doctorate (Ph.D.) in Plant Physiology and Plant Pathology

i. Doctoral program in Physiology and Biology of Organisms-Populations-Interactions
Université de Reims Champagne-Ardenne (France)

ii. Doctoral program in Agricultural and Environmental Sciences
Università degli Studi di Firenze (Italy)

Thesis: "Developing an innovative tool to enhance the biological activity of active substances for the sustainable control of fungal diseases in *Vitis vinifera* L."

2012 - 2013 Master of Science (M.Sc.) in Viticulture and Enology
Hochschule Geisenheim University (Germany)
Thesis: "Developing a guideline for organic grafted vine production"

2008 - 2011 Bachelor of Science (B.Sc.) in Viticulture and Enology
Università degli Studi di Udine (Italy)
Thesis: "Composting of wood from pruned vine and pomace and potential infection of *Phomopsis viticola*"

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Di Gennaro F.S., **Battiston E.**, Di Marco S., Facini O., Matese A., Nocentini M., Palliotti P. and Mugnai L. (2016). Unmanned Aerial Vehicle (UAV)-based remote sensing to monitor grapevine leaf stripe disease within a vineyard affected by esca complex. Phytopathologia Mediterranea 55, 2, 262−275. DOI: 10.14601/Phytopathol_Mediterr-18312

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SCIENTIFIC TRAINING

Mar. - Aug. 2014 Training in the test procedures on grapevine pests and diseases | Institut für Phytomedizin, Hochschule Geisenheim University (Germany).

Feb. 2015 Training School "Biological methods to study the microbial communities colonizing the wood of vines" | COST Action FA1303 "Sustainable Control of Grapevine Trunk Diseases" | UMR Santé et Agroécologie du Vignoble, INRA Centre de Bordeaux (France).

June 2016 Training School "Isolation and identification of grapevine fungal trunk pathogens" | COST Action FA1303 "Sustainable Control of Grapevine Trunk Diseases" | Instituto Agroforestal Mediterráneo, Universitat Politècnica de València (Spain).

Oct. - Nov. 2016 Short Term Scientific Mission "Imaging approach to evaluate the control of GTD related pathogens" | COST Action FA1303 "Sustainable Control of Grapevine Trunk Diseases" | Bioresources Unit, Department of Health & Environment, Austrian Institute of Technology (Austria).

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Poster: *"Composting of grapevine pruning residues and survival of Diaporthe neoviticola*".

May 2015 COST Action FA1303 Working Group 3 on "Host-Pathogen interactions" | Agricultural University of Athens | May 12-13, 2015, Athens (Greece).

June 2015 COST Action FA1303 Workshop on "Grapevine trunk diseases: current state and future prospects" | Jas Hennessy & Co | June 23-24, 2015, Cognac (France).

Oct. 2015 COST Action FA1303 Working Group 4 on "Management of grapevine trunk diseases in nurseries and in vineyards cultural practices" | Bordeaux Science Agro | October 8-9, 2015, Gradignan (France).

Nov. 2015 2nd World Congress on "The use of biostimulants in agriculture" | Florence Convention Center | November 16-19, 2015, Firenze (Italy).

Sept. 2016 Conference & Exhibition "Nano Innovation 2016" | Faculty of Civil and Industrial Engineering of Sapienza University of Rome | September 20-23, 2016, Roma (Italy).

Poster: *"Development of a drug-delivery system for the control of plant diseases: in situ ESEM and TEM observation"*

June 2017 — 15th Congress of the Mediterranean Phytopathological Union on "Plant health sustaining Mediterranean ecosystem" | University of Córdoba | June 20-33, 2017, Córdoba (Spain).
Poster: "*Grapevine trunk diseases: the relevance of disinfection of propagation material*"

June 2017 — 1st International Symposium on "Plant bioprotection sciences & technologies" | University of Reims Champagne-Ardenne | June 27-30, 2017, Reims (France).

July 2017 — 10th IWGTD Workshop on "Grapevine trunk diseases" | University of Reims Champagne-Ardenne | July 4-7, 2017, Reims (France).
Oral Presentation: *"Imaging methods to evaluate rootstock colonization by a GTD related pathogen and its control in grapevine nursery process"*
Poster: "*Developing a delivery system for the control of plant diseases: from leaf pathogens control to grapevine trunk diseases control in the nursery*"

Sept. 2017 — COST Action FA1303 Working Group 4 on "Management of grapevine trunk diseases in nurseries and in vineyards cultural practices" | CNR – IBIMET of Bologna | September 20-21, 2017, Bologna (Italy).
Oral Presentation: *"In planta biostimulant properties of copper-based formulations against the esca-associated fungus Phaeoacremonium minimum: potential application in nursery to protect the propagating material"*