Novel approach to control downy mildew in organic viticulture by innovative formulations – from the lab to the field

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MSc in Life Sciences – Agricultural Science – Sustainable Production Systems

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List of Abbreviations

FiBL	Research Institute of Organic Agriculture
PPP	Plant protection product
MW	Molecular weight in kDa
DDA	Degree of deacylation
YAN	Yeast assimilable nitrogen
EPPO	European and Mediterranean Plant Protection Organization
FOAG	Swiss Federal Office for Agriculture
SAGR	'Office de la viticulture et de l'agroécologie', Auvernier, Switzerland

Abstract

The replacement respectively the reduction of copper remains one of a main concern of research in organic wine farming. Copper is still unavoidable in protection of grapevine downy mildew caused by *Plasmopara viticola*. Copper accumulation in soil strongly affects the environment and biodiversity. A large proportion of agrochemicals - which also includes copper-containing products - are washed off without taking effect. This is also unsustainable in economic and social terms, as it requires more frequent application. Therefore, more precise delivery systems are currently under development. Chitosan binds and release active ingredients in a wide range of applications: environmental remediation, pharmaceuticals, and food technology. This polysaccharide is of natural origin (contained, e.g. in the exoskeleton of crustaceans, insects and fungal cell walls) and itself has an antimicrobial effect, albeit only partially. A combination of copper and chitosan is a promising approach to target antimicrobial pathogens while reducing copper dose through synergistic effects. But as bulk chitosan has low solubility, its further use is still limited. This is why a new and improved chitosan formulation should be investigated: Novochizol™ supplied by a Swiss company promises firstly easy handling and secondly a more precise release of the active ingredient [Cu^{2+}] ion. Four in plant protection used copper salts - the chemical form of [Cu^{2+}] - are formulated with Novochizol™ and tested in vitro, on grapevine seedlings and in the field. Finally, vinification with the treated berries should reveal whether the vine quality is affected.

The results show, that Novochizol™ alone is effective up to 75% againts an infestation with grapevine downy mildew. Further, depending on the different copper formulations used, very high efficiencies were achieved in the plant pathogen bioassays - especially with copper sulphate [$CuSO_4$] and copper oxychloride [$(ClCu_2H_3O_3)_2$]. Halving or in some cases even quartering the amount of reference copper doses (Kocide® Opti) did not significantly reduce the effectiveness against downy mildew infestation. At low concentrations of Novochizol™ (e.g. 0.0188%), the most interesting phenomenon of the work presented itself: an effect against grapevine downy mildew that was almost completely comparable to the full effect of the reference product (Kocide® Opti), by halving the dose of copper metal. The increase in efficacy of the copper-Novochizol™ compounds at low Novochizol™ concentrations, although the individual elements of the compounds lose efficacy linearly as they are diluted, is a strong indication of more effective delivery.

In field as well as in terms of wine quality, Novochizol™ in combination with copper oxychloride [$(ClCu_2H_3O_3)_2$] showed no negative effects. If the production technologies for Novochizol™ become marketable, the combination of Novochizol™ and copper could lead to potential copper savings in crop protection. This is also because the required quantity is affordable in terms of cost and volume.

1 Introduction

The grapevine is one of the most commercially valuable fruit crops in the world. However, grapevines have some devastating diseases and pests as hosts: downy mildew caused by *Plasmopara viticola* can potentially destroy the entire yield (Gessler et al. 2011). *P. viticola* it is a diploid, obligate biotrophic oomycete (Lafon and Clerjeau 1988) and was originally introduced to Europe by the importation of wild *Vitis* grafting rootstocks from North America (Farlow 1882).[1] Grapevine downy mildew is commonly managed by chemical or copper-based plant protection products (PPPs) and management intensifies during summers with high precipitation. In organic and biodynamic viticulture, copper-based PPPs emerge as the sole fully effective measure against grapevine downy mildew (La Torre and Iovin 2018). The following strategies, beyond chemical or copper-based PPP, should be considered for effectively managing grapevine downy mildew:

1. Integration of various field practices, such foliage removal for improved grape aeration, weed management beneath vine plants, adequate and balanced irrigation, and fertilization, and optimizing plant density, is essential for implementing good agricultural practices. These measures have been proven to help reduce inoculum (Howell et al. 1991; Bem et al. 2016).
2. The use of weather-based warning systems for more accurate application scheduling.
3. Piwi, short for 'Pilzwiderstandsfähige Rebsorte(n)' or fungus-resistant grape varieties, are interspecific grape hybrids bred to resist fungal diseases. Piwi(s) currently represent a small number of varieties used for wine production. The cultivation of such varieties in Europe is mainly limited to Germany and Switzerland, where wine produced from tolerant varieties has found a small market niche. In Switzerland, the area under cultivation of Piwi(s) is almost negligible: 3% (FOAG 2022a).

Viticulture in Switzerland is subsequently based on the traditional European grape varieties (*Vitis vinifera*) and their protection by copper-containing PPPs: the advantages of copper-containing PPPs and reasons for their widespread use both in organic and conventional agriculture are among its high efficacy and low costs. Copper-containing PPPs are also used to avoid the risk of resistance to synthetic PPPs - which is currently leading to copper-containing PPPs regaining popularity worldwide (Poggere et al. 2023). In contrast, copper-containing PPPs - which contain the heavy metal copper [Cu^{2+}] - have undeniable negative effects on the environment and human health at certain doses (La Torre and Iovin 2018). Repeated foliar application leads to its accumulation in the soil (Ballabio et al. 2018) and thus potentially to negative effects on soil fertility.
This reasons the replacement or reduction of copper as a major topic of research in organic viticulture (and related crops). And the Swiss Federal Office for Agriculture (FOAG), lists copper-containing PPPs among as '*PPPs with particular risk potential*' (FOAG 2022b). Copper-containing PPPs are also on the list of substances of concern in the European Union and are thought to be banned sooner or later. Various European countries have already banned PPP containing copper, like Denmark, Sweden, Finland, the Netherlands, and Estonia (Tamm et al. 2022). Although more recent studies relativise the negative effects (Karimi et al. 2021) and the current use of copper metal in Switzerland is already limited to 3 kg ha^{-1} y^{-1} in organic viticulture.

Personal experience shows further problems associated with copper applications, which comes in addition to the negative impact on soil and human health. So, the 'copper issue' can deter conventional winegrowers from converting to organic viticulture. A recently published study in 'Nature' by Madouas et al. (2023) explores learning, reflexivity, decision-making, and behavioural changes for sustainable viticulture through participatory action research, by involving conventional winegrowers which planning to convert to organic or biodynamic practices. A winegrower is quoted by the study: "considers that it is not only the immediate impact of the product that must be considered: it is better to spray once with a systemic (pesticide) than five times with an organic one when the rain washes it after each pass. This

[1] When phylloxera almost eliminated viticulture in Europe, phylloxera-resistant American rootstocks were imported in the 1870s for grafting. Phylloxera is an insect native to East and North America and primarily attacks the roots of grapevines, especially the Vitis vinifera species, and can ultimately lead to the death of the plant. That's why - as a consequence - almost exclusively all vines in Europe are grafted (Powell et al. 2013).

results in a much lower dose of product in the soil, not to mention the fuel saved, and the reduced soil compaction." It is therefore called into question if it's worth to higher the frequency of application by renewing copper-containing PPP applications after rainfall – what it's the practice in organic viticulture. Although conventional viticulture also uses copper-containing PPPs and other PPPs that are to be banned, the situation is somewhat different: synthetic PPP are used. Due to the systemic mode of action of these substances, the spraying intervals can be kept shorter regardless of the weather conditions and curative treatments are possible (Koledenkova et al. 2022).

Personally seen, it is important to reduce the application frequency in organic viticulture. This may reduce the copper metal applied, but also the time required for plant protection. Further, the economic risk associated with organic plant protection and the lack of predictability is more time-consuming and more resource-intensive - which is unsustainable. Not to forget the rising social pressures resulting from public awareness regarding the stress that agricultural fungal disease management put on the environment (Madouas et al. 2023).

There is thus a need to research more ecological and effective strategies to combat downy mildew in organic and conventional viticulture - other than copper and not of systemic nature. Some of these projects are on the agenda of national and international research and policy organisations: e.g. RELACS ('Replacement of Contentious Inputs in Organic Farming Systems') in the European Union (https://relacs-project.eu/) and COPPEREPLACE in France (https://coppereplace.com). Next to improving weather-based warning systems and mechanical manipulation methods, finding copper substitute products is a central point of these strategies. In Switzerland, the Research Institute of Organic Agriculture (FiBL) is the leading centre for screening and testing plant extracts as copper substitutes (Dagostin et al. 2011). As there is still no way of completely replacing copper - despite all attempts - reduction is in a first term the strategy of choice. The combination of copper with chitosan could be such a strategy: chitosan - a natural substance produced from chitin - has been shown to be effective against *P. viticola* (Garde-Cerdán et al. 2017) and has been used as a carrier of active (agro-)chemicals (Brunel et al. 2013). A lab-to-vineyard approach will be adopted to test copper in combination with an improved chitosan formulation (Novochizol™) from scratch: with the aim of copper reduction. The predicted controlled release of [Cu^{2+}] - the active ingredient of copper-based PPP - by Novochizol™ is attempted to be proven. The following question is tried to be answered: does the addition of Novochizol™ to the usual copper sources used to control *P. viticola* in organic viticulture allow a significant reduction in copper concentrations?

2 State of research

2.1 *Plasopara viticola* (Berk. and M.A. Curtis) and its control

2.1.1 Information about taxonomy, lifecycle, and infestation

2.1.1.1 Taxonomy of *P. viticola*

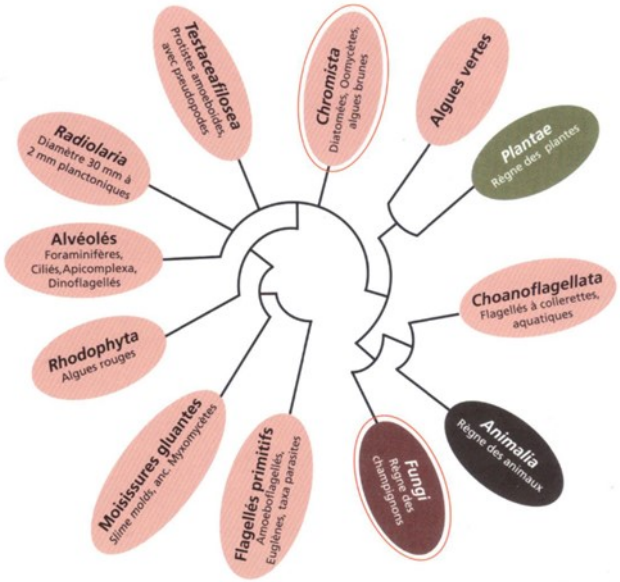


Figure 1: Schematic classification of eukaryotes. In black the kingdom of animals, in green the plants and in brown the fungi kingdom. The Protozoa - single-celled eukaryotes - kingdoms in light brown. The groups surrounded by red contains fungal pathogens.
(Source: Viret and Gindro 2014)

Plasmopara viticola, the causing agent of grapevine downy mildew, is commonly called a fungal pathogen – which is incorrect. Moreover, *P. viticola* is an oomycete. Oomycetes, also known as water molds, were traditionally treated in mycology and were formerly part of the fungal kingdom. They are eukaryotic organisms (see Fig. 1) that belong to the domain of chromista (former stramenophiles) together with heterokont algae, i.e., diatoms and brown algae (Baldauf et al. 2000; Cavalier-Smith and Chao 2006). Reclassification has revealed their origin as a common marine autotrophic precursor around 400 million years ago (Matari and Blair 2014). Over time, they lost the ability to photosynthesize. Oomycetes vary widely in their lifestyle, some being aquatic, others terrestrial. They also cover a broad spectrum of saprophytes or parasites of plants and animals (Judelson and Ah-Fong 2019; Gómez-Pérez and Kemen 2021). In contrast to fungi, oomycetes are characterized by cellulose-containing hyphae and typically lack chitin (Fawke et al. 2015). Most of the life cycle of oomycetes is diploid, unlike that of true fungi. Haploid nuclei occur only during sexual reproduction (Lévesque et al. 2010), which results in the production of oospore. Asexual reproduction occurs through the formation of sporangia (released by the oospore), which give rise to zoospores (Judelson and Ah-Fong 2019). *P. viticola* thrives exclusively on plant tissue, making it an obligate biotroph.
There is a distant relation to the true fungi despite apparent similarities, such as filamentous growth habit, heterotrophic lifestyle, and specialized infection structures. This is also due to multiple horizontal gene transfer events that enabled the oomycetes to acquire fungal genes (Lamour et al. 2007) - mostly related to pathogenic abilities of oomycetic diseases (Latijnhouwers et al. 2003). The downy mildew family (*Peronosporaceae*), which includes the genera *Plasmopara* and *Phytophthora*, poses a major threat in crop production. *Phytophthora infestans* is the most important and therefore best studied pathogen in this field (Yin et al. 2017). Important host species of *P. infestans* are potatoes and tomatoes (Baldauf et al. 2000). Potatoes' late blight - caused by *P. infestans* - led to famine in Ireland from 1845 to 1849 - and, like *P. viticola*, was imported from North America (Haas et al. 2009). In organic and

biodynamic farming, *P. infestans* is controlled with PPPs containing copper (La Torre et al. 2019) and is therefore confronted with the same issues as organic viticulture regarding the control of *P. viticola*.

2.1.1.2 Lifecycle of *P. viticola*

The life cycle of *P. viticola* can be divided into two parts: the primary and the secondary infection cycle. While the oospores represent the infection organs of the primary infection, the zoospores stand for the secondary infection.

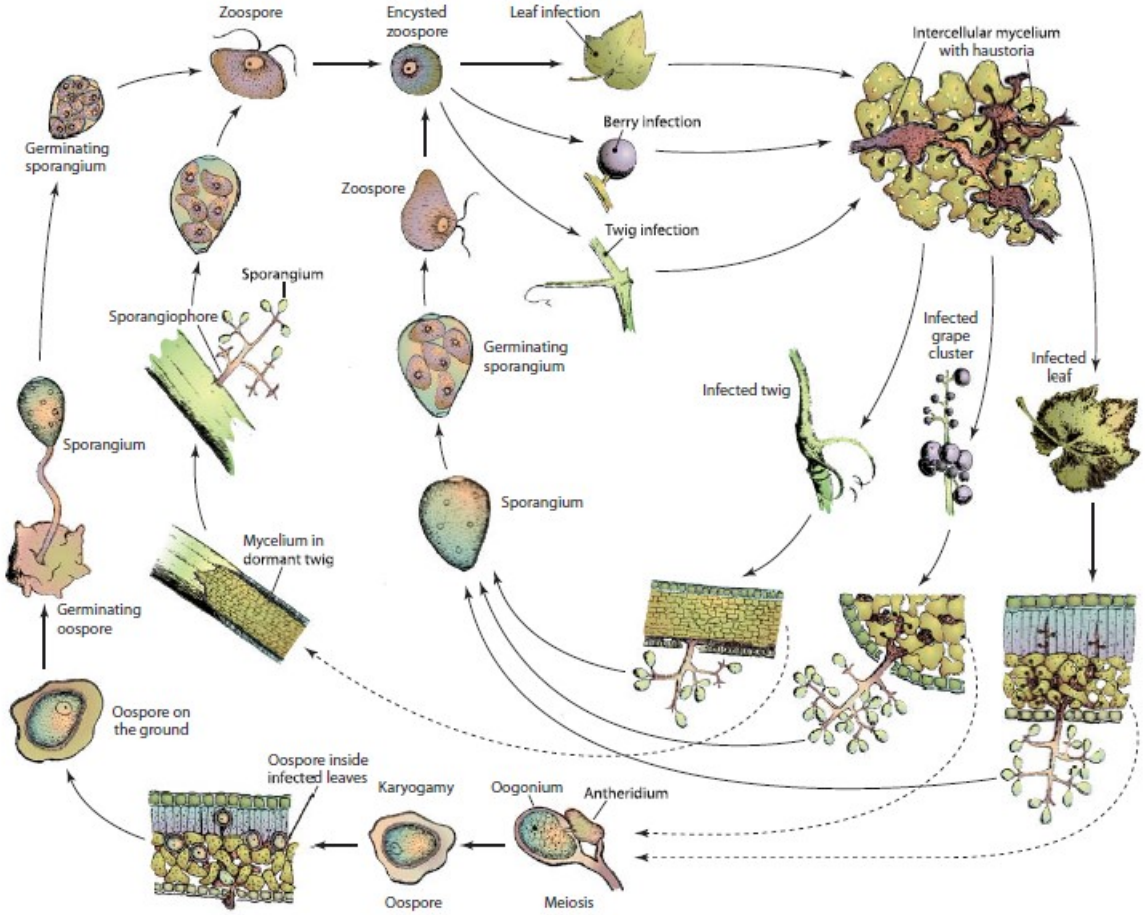


Figure 2: Disease cycle of downy mildew of grapes caused by *Plasmopara viticola*.
(Source: Agrios 2005)

The moment of the oospore formation - via sexual reproduction - mark the starting point of *P. viticola's* lifecycle. These oospores are formed in late fall in infested leaves and serve as a resistant survival stage, possibly able to survive several seasons (Viala 1887; Gehmann K. 1987). For sexual reproduction to occur, the hypha of two different mating types of *P. viticola* must interact in the host tissue: oogons (oospheres) and antheridia (Wong et al. 2001). Once the climatic conditions are favourable, an oospore can germinate via sporangia (see e.g. model of Gehmann (1987)[2] or www.rimpro.eu and www.agrometeo.ch).

As an originally aquatic organism, *P. viticola* - like all oomycetes that have transitioned to terrestrial life - is still dependent on water. Therefore, germination of the sporangia occurs only in contact with dripping water. The germination results in a hypha that ends in a primary piriform sporangium of varying size that can produce 30-60 infective zoospores. Germination time depends on the age of the sporangia

[2] The system developed by Gehmann K. (1987) to determine the time of germination, aims to predict when the oospores are mature. It is based on the summed temperatures above 8°C. To do this, all average temperatures above 8°C from the first of January are taken and summed. Once 140°C is reached, the oospores are ready to sprout and to form primary spores.

and temperature. The released heterokontic, flagellated zoospores rely on a film of water to migrate to the stomata of the host plant (Keil S. B. 2007). At the stomata, the zoospores cease their movements, retract the flagella, and build a cell wall instead (encystment). The encysted zoospore germinates with a germ tube growing through the stomata into the substomatal space, where it forms a substomatal vesicle. In the respiratory cavity, the substomatal vesicle develops into the primary hypha, which spreads intercellularly (Kiefer et al. 2002). Upon contact with parenchyma cells, haustoria are formed, which serve to take up nutrients from the plant cell and provide the hyphae with energy for its further growth. The pathogen's hyphaen branches and begins to grow in the intercellular spaces of the host tissue until the available space is almost filled. The plant tissue is severely damaged by the deprivation of nutrients. This can be seen in the early stages by the breakdown of colour pigments, which leads to the typical clinical picture of the so-called oil spots (Viala 1887; Müller K. et al. 1923) (see Fig. 3A). According to the curve of Müller K. et al. (1923), incubation lasts from four to twelve days, depending on the temperature. At the end of this period, sporangia appear on the lower leaf surface. This white fungal lawn on the underside of the leaves during sporulation is another typical symptoms of the disease (Rumbolz et al. 2002).
The conditions for sporulation of secondary infections are as follows: the leaves must be wet, or the relative humidity must be above 92%. In addition, the temperature at a height of two metres above the ground must be at least 12 °C at the start of four hours of leaf wetting. Additionally, these conditions must be met in the dark. Wind and rain splashes dispersing sporangia and zoospores for new infections (Gessler et al. 2011; Nogueira Júnior et al. 2019). If the leaf dries out beforehand, the zoospores die. According to Bläser and Weltzien (1979), at 15°C and 70-90% relative humidity the sporangia remain infective for 8 days. At 30°C and low relative humidity, the sporangia die after a few days. In the lower tissues, however, the fungus remains active and can form new sporangia on the same lesion when conditions become favourable.
The interplay of primary and secondary infections throughout the season is complex. Matasci et al. (2010) have summarised that the most common pattern of an epidemic consists of a random distribution of genetically different genotypes (resulting from primary infections) throughout the vineyard and some spatially limited, clustered lesions (secondary infections) originating from a single genotype. It can be assumed that there is a correlation between the number of zoospores of the secondary infection and the degree of infection due to a higher spore density, which explains the exponential development of the infection once the pathogen has established.
Understanding the principles of *P. viticola* infection is crucial for practical application. It helps to determine the right time for application. The use of weather-based forecasting models (www.rimpro.eu and www.agrometeo.ch), which take into account all known parameters of a *P. viticola* infection and development, simplifies but does not eliminate the need to determine the ideal time for treatment.

2.1.1.3 Susceptilitiy of *Vitis vinifera* and its infestation by *P. viticola*
Susceptibility to *P. viticola* varies among grape varieties. In Swiss vineyards, the main grape varieties - 'Chasselas' and 'Pinot noir' - are generally considered highly susceptible to *P. viticola*. To minimize disease impact, chemical or copper-based PPP together with accurate forecasting of downy mildew infections are crucial (Gindro et al. 2012). All known European grape varieties (*Vitis vinifera),* show moderate to high susceptibility (Boso and Kassemeyer 2015). Grapevines naturally possess defence mechanisms, but: they are insufficient to prevent downy mildew infestation in conventional European grapevine varieties (*Vitis v.*). These defence mechanisms are the first to be focused on in the development of resistant varieties (Yu et al. 2012).
Activation of plant defence mechanisms can also be achieved by treatment with specific compounds or microorganisms (e.g. elicitors) - a second focus in the pursuit of more sustainable grape production (Jacquens et al. 2022; Mian et al. 2023). Some such products are already available on the market, mainly to supplement the traditional spraying regime (Speiser et al. 2023). Because: for conventional European grapevine varieties (*Vitis v.*) in Swiss vineyards, the use of chemical or copper-based plant protection products (PPP) still remains indispensable to avert substantial yield losses (Wilcox et al. 2015).
Under favourable climatic conditions, *P. viticola* can possibly attack all green grapevine tissues, leading in the worst case to complete defoliation, berry desiccation, and stem damage (Gessler et al. 2011). Throughout the whole growing season - as soon the oospores are ready -, infection of all green

grapevine organs can occur. Infected or partially infected inflorescences dry out completely, which also count for the stems (see Fig. 3D and 3F).

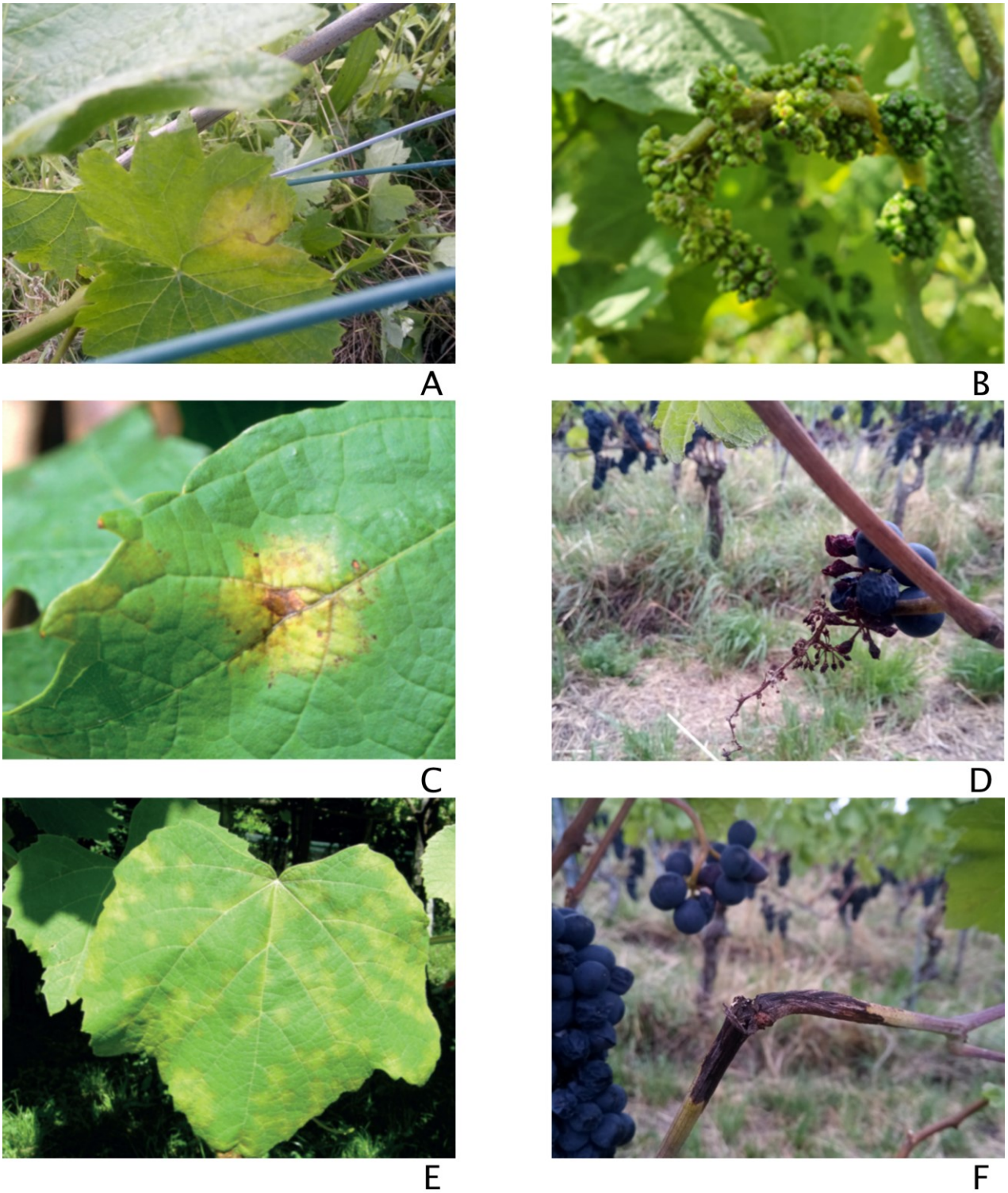


Figure 3: Symptoms of a downy mildew infection: (A) Fresh oil spot seen on 31 May 2023; **(B) freshly infected inflorescence, seen on 2 June 2023** (Source: 'Informations viticoles Neuchâtel, Fribourg et Berne 20230602 N°10', 2023); **(C) old oilspot with necrosis on the adaxial leaf surface** (Source: Gessler et al., 2011)**; (D) old infection of the inflorencences, which leads to partial death seen on 13 September 2023**; **(E) mosaic-like oilspots late in season** (Source: Gessler et al., 2011); **(F) partially infected and dried out stem seen on 13 September 2023**

Early infection of the leaves leads to the typical oil spots (see Fig. 3A). Depending on the disease pressure, these lesions can have serious consequences and lead to complete collapse of the foliage wall due to further - secondary - infections. Young leaves within leaf axils are particularly susceptible to downy mildew, serving as abundant sporulation sites. If the infection is manageable and the oil spots are hindered to sporulate, the lesions merely dry up (see Fig 3C). A disease attack and the resulting lesions have a more or less severe effect on photosynthetic activity (Nogueira Júnior et al. 2020). Late-season infestations on leaves result in mosaic-like punctures resembling wallpaper stitches (see Fig 3E). Later in the season grown leaves, are less important for the photosynthetic productivity and an infection is tolerated in practice.

Literature indicates that grape berries are theoretically protected after stage BBCH 69 - synonymous with the end of the flowering period - as the stomata are collapsed (Gindro et al., 2012). While the BBCH system serves to coding the phenological growth stages of plants, the numbers in the sixties describe the flowering stages (Meier et al. 2009). Before the end of the flowering period, functional, and thus infectious stomata were present on the calyptras (caps), rachis and pedicels of berries regardless of the grape variety. This also reflects that, in practice, increased protection is applied before and during flowering. Important work on this topic has shown that sporulation on the surface from stage BBCH 69 on may be from prior inflorescence infection (Gindro et al. 2012): thereafter, *P. viticola* can remain latent in the tissues, which could explain the occurrence of sporulation in late infected grapes ('rot gris') (Kennelly et al. 2005). Consequently, the pathogen can partially develop systemically in the tissues of the grapevine. Another possibility of later infection is sporulation by lenticels, as observed on the upper side of the leaves by Gindro et al. (2006). As the disease progresses, the stems of the grapes turn brown, the berries turn red and change colour from purple to brown ('rot brun') before drying out.

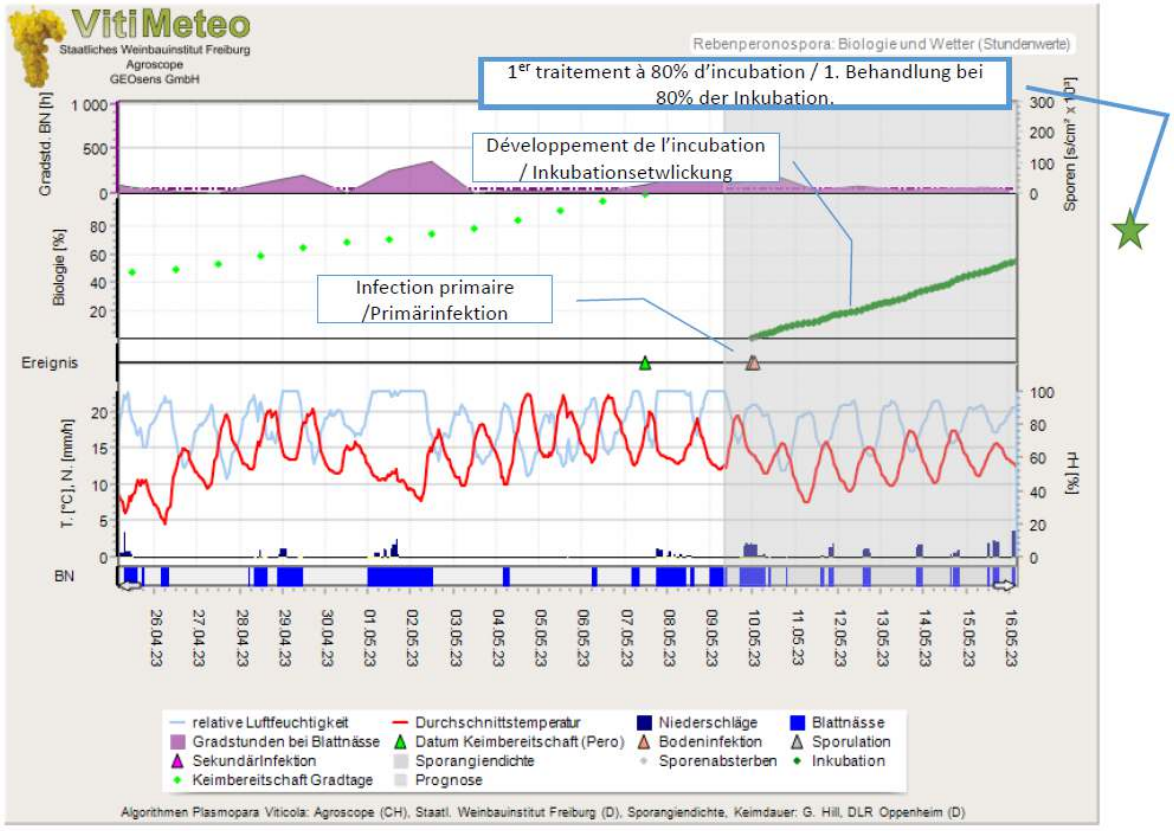


Figure 4: Scheme recommending the timing of the first treatment against downy mildew (green star) in the season 2023 depending on temperature, rainfall and thus progress of incubation. This information was sent Wednesday, 9 May 2023 by the 'Station viticole' in Auvernier to the winegrowers in the Drei-Seen-Region by newsletter.
(Source: SAGR (2023))

The delayed outbreak of grapevine downy mildew and the question of how it can be prevented is also a political issue and a point of contention, because: preventing the first primary infection with *P. viticola* does make the most sense from a theoretical point of view. This would ban definitively the risk of a spreading primary infection in the grape plant tissue, which could break out at a later point in season. But: in practice, a primary infection - tantamount to the possibility of a secondary infection - is often tolerated and suggested. Consequently, only the spread of the primary downy mildew infection needs to be prevented, which is justified by the (usually) low number of oo- and zoospores. This occurs in about 80% of the incubation period. The incubation period is calculated depending on the weather conditions using the available models (www.rimpro.eu and www.agrometeo.ch). The political will to reduce plant protection measures also provides for such a delay in the initial treatment as a possibility (SAGR 2023). Bleyer et al. (2020) also proposes this strategy, but points out that this applies to a rather weak infection.

The primary infection of downy mildew in various Swiss wine-growing regions in season 2023 showed that this can also backfire: Vaud and the 'Drei-Seen-Region' have recorded up to 20% infestation of the vines in some cases because the initial infection was not covered (Personal communication with several winegrowers in May and June 2023).

2.1.2 Downy mildew control by copper and why it must be replaces (or reduced)

Copper is a very old measure to prevent against all kind of microbial agents and are most frequently used in agriculture. Copper is known for its effect as: algicide, fungicide, nematocide, molluscicide and it is antibacterial. The biggest breakthrough for copper as fungicide came in the 1880s with the development of a lime-copper formulation by the French scientist Millardet (Borkow and Gabbay 2005). He showed that spraying grapes and vines with a mixture of copper sulphate [$CuSO_4$] (25% of [Cu^{2+}]), lime and water render them remarkably free of downy mildew. By 1885, his formulation was given the name of 'Bordeaux mixture'. In Europe, during the 1950s, copper metal ([Cu^{2+}]) in quantities of 20 to 30 kg ha^{-1} y^{-1}, and sometimes even more than 80 kg ha^{-1} y^{-1}, was applied (Coelho et al. 2020). Nowadays, copper usage is banned or limited to few kilos ha^{-1} y^{-1} in most European countries. E.g. in Switzerland actually it is limited to 3 kg ha^{-1} $year^{-1}$, within five consecutive years maximum 15 kg copper metal ([Cu^{2+}]) per ha (Speiser et al. 2023). The issue of copper is a very broad and much discussed topic in organic farming. The effects especially on the soil with its micro- and macroorganisms are highly problematic. The concrete issue: since copper is a heavy metal it cannot be degraded and its removal from the soil by leaching, runoff, or plant uptake is negligible, and it can remain in the environment as a pollutant for long periods of time, leading to bioaccumulation and thus potentially to negative effects on soil fertility (La Torre and Iovin 2018).

Additionally, the copper coming from the vineyard's treatments can also cause problems in winemaking. It is known that especially the period between the last treatment and the grape harvest is decisive for copper residues in must and wine. Significant differences between red and white wines exist, which can be explained due to the different winemaking process (i.e., different times at which the wine is clarified by yeast cells). The consequences of elevated copper levels can lead to retardation of fermentation, distorted aroma, and colour profile (Tromp and Klerk 1988). The maximum residue limits of copper in grape and wine is of 20 mg kg^{-1} and 1 mg l^{-1} (OIV 2023).

But there are several advantages of copper-containing PPPs and reasons for their widespread use in organic and conventional agriculture: its high efficacy under rainfed conditions, its multisite mode of action that minimises the risk of developing resistant pathogen strains, relatively low acute toxicity to terrestrial vertebrates and low cost (Speiser et al. 2018).

In both conventional and organic cultivation, protective and thus copper agents against *P. viticola* are used, which are only effective if they are present in sufficient quantity as a spray layer on the host plant during the infection event. Once the pathogen has established itself in the host tissue, these agents are ineffective. They act as contact fungicides, mainly against the sporangia and zoospores. In addition to copper-containing PPP, contact fungicides against *P. viticola* also include agents with active substances such as Folpet and Mancozeb. The latter of which has been banned in Switzerland since the 2022 season (Sauer et al. 2022). In contrast to the systemic acting PPP, contact fungicides cause no known resistance in *P. viticola*. In organic farming, (almost) exclusively copper-containing PPP are on the market against

P. viticola - Folpet is forbidden (Speiser et al. 2023). Kocide® Opti (copper hydroxide [H_2O_2Cu], 30% [Cu^{2+}], DuPont de Nemours, Wilmington, Delaware, USA) and Airone (copper oxychloride [$(ClCu_2H_3O_3)_2$], 14% [Cu^{2+}] + copper hydroxide [H_2O_2Cu], 14% [Cu^{2+}], Andermatt Biocontrol Suisse AG, Grossdietwil, Switzerland) are examples of conventional copper-containing PPP. Kocide® Opti and Airone will also be presented in the chapter 3 on 'Materials and methods', as these were used throughout the experiments. The main way copper containing products acts against oomycetes can be described as non-specifically (multisite). Basic active ingredient is the cupric ion [Cu^{2+}] which is released - by inorganic copper salts like e.g. copper oxide [Cu_2O], copper hydroxide [H_2O_2Cu], copper oxychloride [$(ClCu_2H_3O_3)_2$] or copper sulphate [$CuSO_4$]. The properties of inorganic copper salts differ in copper content and solubility, being copper sulphate [$CuSO_4$] the only salt which is soluble in water. Therefore, copper sulphate [$CuSO_4$] has the most immediate effect due to the fast release of Cu^{2+}, but is highly toxic to plants if it is not formulated. Kurnik et al. (2012) summarized the properties of different formulations containing copper salts, focusing on solubility and rain fastness. Although the type of formulation is decisive, it can be said generally that the more soluble a copper salt is, the less rainfast it is. In terms of solubility, the following applies: copper sulphate [$CuSO_4$] > copper oxychloride [$(ClCu_2H_3O_3)_2$] > copper hydroxide [H_2O_2Cu] > copper oxide [Cu_2O]. The interaction between copper salt and formulation is crucial, as the example in Fig. 5 shows.

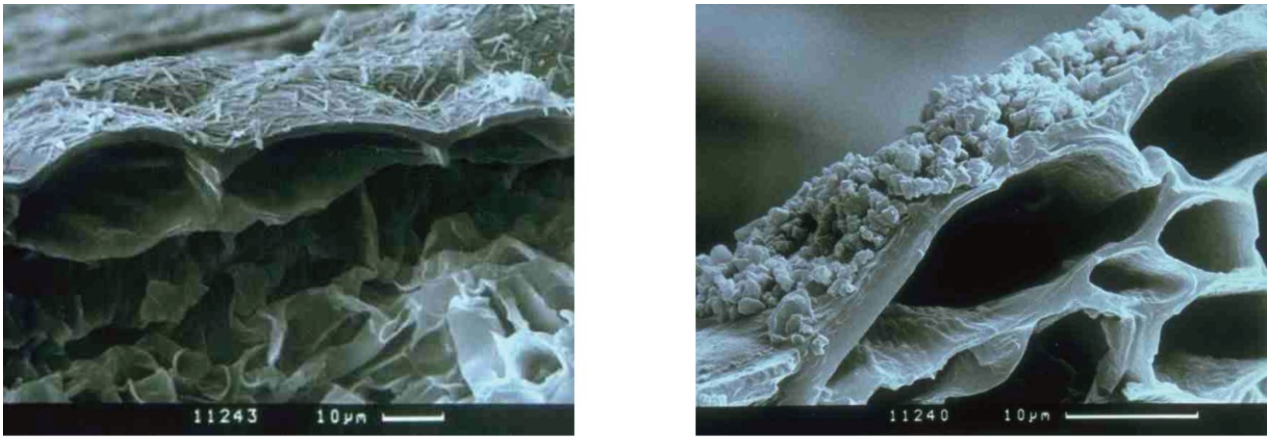


Figure 5: Various spray coatings due to copper salts as the basis of formulation of (A) copper hydroxide [H_2O_2Cu] (Cuprozin) and (B) copper oxychloride [$(ClCu_2H_3O_3)_2$] (Funguran) on a cut leaf. According to the manufacturer, the formulation with copper hydroxide [H_2O_2Cu], covers the leaf surface with less copper than the octahedron form of the formulation with copper oxychloride [$(ClCu_2H_3O_3)_2$] due to the shape of needle crystals. (Source: Diephaus (2010))

The initial site of action of copper is thought to be at the plasma membrane. It has been shown that exposure of fungi and yeasts to elevated copper concentrations can lead to a rapid decrease in membrane integrity (Ohsumi et al. 1988). Toxicity also results from changes in the conformational structure of nucleic acids and proteins (Borkow and Gabbay 2005). Specific studies on the effect of copper on oomycetes are lacking. Most studies have been conducted on the antibacterial properties of copper. This is also due to the ability of bacteria to resist copper. Oomycetes and fungi have shown no resistance so far to the various copper compounds, as reported by Fungicide Resistance Action Committee (FRAC 2023). The multisite effect and thus the difficulty of developing resistance against copper is one of the reasons why copper is once again gaining importance as a fungicide (Poggere et al. 2023).

If the use of copper is put into relation, the negative effects are defendable to a certain extent: applying copper at 4 kg ha^{-1} y^{-1} should not modify substantially soil biological quality and functions (Karimi et al. 2021). Despite, there is a consensus that it is necessary to find alternative and more ecological ways to replace or reduce the use of copper in organic farming and especially in (organic) viticulture. Because viticulture is one of the main consumers of copper in Europe (990 t y^{-1}, in second place with 30% after olive cultivation with 1'263 t y^{-1}, 39%) states Tamm et al. (2022). A way of reducing could be done by

copper alternatives from natural products - which is proving difficult due to the lack of suitable candidates. Only partial effectiveness could be shown yet (La Torre and Iovin 2018). Another promising approach seems to combine copper with (active) biomaterials as delivery systems. Because a large proportion of agrochemicals - which also includes copper-containing PPPs - are washed off without taking effect. Therefore, more precise delivery systems are currently under development (Mujtaba et al. 2020). One such biomaterial with a potential for synergistic interactions with fungicides is chitosan (Lemke et al. 2022).

2.2 Chitosan's antimicrobial activity

Chitosan - second most occurring polysaccharide in the world - gained raising attention in recent years and has demonstrated to be one of the most promising polymers for efficient delivery of agrochemicals and micronutrients (Kashyap et al. 2015). The biopolymer chitosan is the N-deacetylated form of chitin, found in the exoskeleton of crabs, shrimp, lobster and cell mass of many parasites (Kou et al. 2022). The high economic potential was attributed to the utilization of shells of marine crustaceans such as crabs and shrimps: a waste product of the food industry. Today, the knowledge about the sourcing and processing of chitosan forces the importance of a more standardized and therefore controlled way to produce - e.g. insect farming (Pellis et al. 2022). This leads to a chitosan with better properties, which can be easily and safely used and transformed. Also, because different molecular weight (MW) and degree of deacetylation (DDA) compared to chitin, have a great influence on the inherent properties (Joseph et al. 2021). Chitosan is non-toxic to humans and the environment, biodegradable and has very interesting biological properties such as antimicrobial activity. There is also evidence that chitosan stimulates plant defence (Choudhary et al. 2017; Adamuchio-Oliveira et al. 2020) and plant growth promotion (Abd El-Aziz et al. 2019; Kudasova et al. 2021).

Chitosan displays direct antifungal activity against certain oomycetes, demonstrated for example in in vitro experiments on *P. infestans*, the pathogen of potato late blight (Huang et al. 2021) remaining in the same family as *P. viticola*. Chitosan is also one of the most reported elicitors used to control *P. viticola* and other grapevine pathogens, such as *Botrytis cinerea* and *Erysiphe necator* (Garde-Cerdán et al. 2017). Reglinski et al. (2010) shows in their study that it is difficult to distinguish the suppression of *Botrytis c.* by chitosan into direct antifungal activity and indirect modes of action. Where the indirect mode of action stimulates the immune system (defence reaction) of the plants via various signalling processes or via regulatory molecules involved in signal transduction. This results in the case of grapevine in the production of phytoalexins (Rabea et al. 2003). Concretely, it has been shown via in vitro studies that chitosan oligomers of different MW and DDA triggered an accumulation of plant phytoalexins (e.g. trans- and cis-resveratrol, their derivatives ε-viniferin and piceid) in grapevine leaves (Aziz et al. 2006). While chitosan oligomers of low MW were shown to be more effective in inducing defence responses than those of higher MW. The main phytoalexins, ε-viniferin and trans-resveratrol, are considered to enhance grapevine plant resistance against *P. viticola* (Pezet et al. 2004). But the extent of the effectiveness of the plant defences triggered in European grape varieties (*Vitis v.)* is estimated to be low (Gindro et al. 2012).

The general (direct) mode of action of chitosan is by chelating minerals and nutrients from pathogens, leading to their death (Rubina et al. 2017). This is due to the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes, which can lead to the leakage of proteinaceous and other intracellular constituents altering cell permeability (Rabea et al. 2003). Chitosan has therefore also been used in several areas such as biomedical, pharmaceutical and biotechnological fields as well as in the food industry (Aranaz et al. 2021). Another property of chitosan's positively charged side groups is: it complexes active substances, which explains its ability to efficiently deliver agrochemicals and micronutrients. This is shown in Fig. 6 using copper as an example.

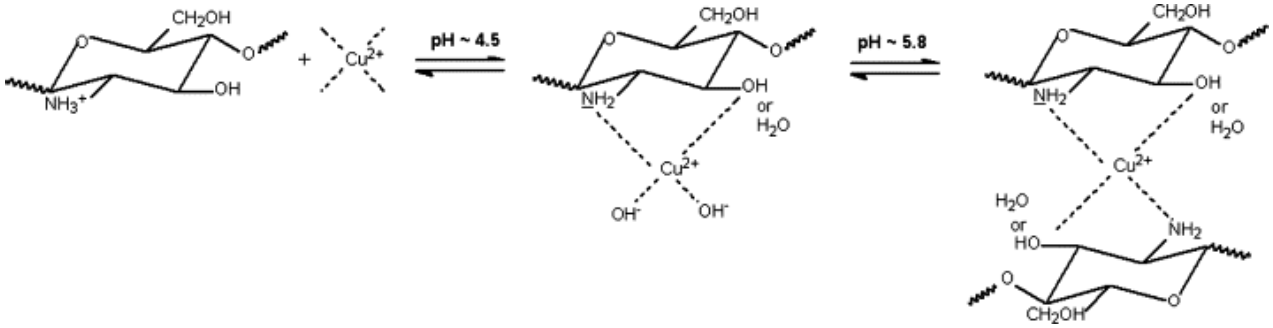


Figure 6: Proposed mechanisms of the Cu^{2+}- chitosan complexes.
(Source: Brunel et al. (2013))

Working with chitosan regardless complexation is often problematic because chitosan samples, like other polysaccharides and other polymers, are always mixtures of different molecules in terms of length and chemical structure. Further, the wide use of chitosan is limited by its low solubility in aqueous medium (Jiménez-Gómez and Cecilia 2020).

2.3 Copper-chitosan particles and their antimicrobial activity

When investigating the antimicrobial capacity of copper-containing chitosan particles - specifically against fungal and oomycotic plant pathogens and the associated plant defence mechanisms - the perspective must be broadened. In search of an additive or synergistic effect between copper and chitosan, it was looked at more plant pathogens interactions than just grapevine - *P. viticola*.
Meena et al. (2020) for example studied postharvest losses in tomato, where the causes were not attributed to a specific fungus. Damping-off - the death of young seedlings due to fungal infection favoured by moist conditions - was another subject, regarding antifungal properties of copper-containing chitosan particles: once for cotton (Abd-Elsalam et al. 2017) and once for chili, cowpea, and tomato plants (Vanti et al. 2020), respectively. The most studies addressed fungal pathogens associated with a particular crop, e.g. table grapes - *Botrytis cinerea* causing grey mold (Hashim et al. 2019), maize - *Curvularia lunata* causing curvularia leaf spot disease (Choudhary et al. 2017), chickpea - *Fusarium oxysporum f. sp. ciceri* causing fusarium wilt (Kaur et al. 2018), banana - *Fusarium oxysporum f. sp. cubense (Foc).* causing fusarium wilt (Kumar N. et al. 2022), wheat - *Fusarium graminearum* causing head blight (Lemke et al. 2022), tomatoes - *Alternaria solani* causing early blight (Saharan et al. 2015) or finger millet - *Pyricularia grisea* causing blast disease (Sathiyabama and Manikandan 2018).
Oomycetes were focused on studying *Pythium sp.* causing agent of rhizome rot of ginger (Vanti et al. 2020), *Pythium aphanidermatum* of chilli, cowpea and tomato plants (Ilnicka et al. 2015) and *P. infestans* of potato (Hadwiger and McBride 2006).
Most commonly, in vivo studies were conducted using a variety of assessment methods that examined the zone of colonization or percent rate of mycelial growth (e.g., on agar plates) and spore germination. In vivo studies in form of pot or greenhouse experiments were part only in few studies and field studies were rare, where also plant toxicity was extra tested. Compared to the negative control (if existing), there was an antifungal or anti-oomycotic effect in all studies - mostly significant. Saharan et al. (2015) and (Vanti et al. 2020) even show significantly better results than the positive control for the highest concentration of copper-chitosan (nano) particles. Whereby, in the case of Saharan et al. (2015) copper sulphate [$CuSO_4$] and Vanti et al. (2020) a commercial fungicide acted as positive control. Overall, the antifungal activity of the copper-chitosan material used was given, often with dose dependency, means higher concentration increased the inhibitory effect.
Looked at the defence and/or growth promoting properties as a 'side effect' of the combination of copper and chitosan, most of the above cited studies included field or pot trials, to complete the direct antifungal efficacy in the vivo studies. The responses are in a wide range, always related with specific defence enzymes, but clearly indicate the positive effect of improving plant defence and growth or yield. The results of testing the effect of copper-chitosan material against *Botrytis c.* on table grapes (Hashim et al. 2019), *F. graminearum* on wheat (Lemke et al. 2022), *A. solani* on tomatoes (Saharan et al. 2015) and *P. infestans* of potato (Hadwiger and McBride 2006) shows even higher effect with the combination of copper and chitosan than adding the effect of both compounds alone - this could indicate a

synergistic effect. Since synergy in relation to the combination of two PPPs is a narrow term according to Levy et al. (1986), who propose Abott's (1952) formula for testing synergy, the term additive effect is used instead.

Considering the properties of the starting materials - copper and chitosan - it is difficult to draw a general conclusion about the factors that are ultimately responsible for the antifungal and anti-oomycotic effect of their combination. The knowledge about chitosan and its properties - without being complexed - seems to be clear: MW and DDA (and their mixtures) influence mostly their effectiveness. Regarding copper sources, all the studies used exclusively one single copper salt ([$CuSO_4$]) and comparisons between different copper salts are lacking - although it can be assumed that differences in copper salts sources influences the antifungal and anti-oomycotic effect as well. In practice, different copper salts are considered to have different antifungal and anti-oomycotic effects (Kurnik et al. 2012). Copper sulphate [$CuSO_4$] was by far the most used in complexation with chitosan in the studies investigated. The release properties in combination with other nanomaterials possible to be combined with copper indicate different release properties depending on which copper salt was originally used (Jose et al. 2020) - with those obtained from a copper sulphate [$CuSO_4$] precursor, shows the lowest stability.

As for the size of the particles, the average chopper chitosan particle was in a wide range (2.75 to 500 nm). A nanomaterial is defined as at least half of the particles with a size distribution of 100 nm or less (Erickson 2009). It seems that the handling and encapsulation process of copper by chitosan is easier when working with nanoparticles due to their surface properties, as indicated by the various - more or less sophisticated - methods of complexing copper with chitosan (Gritsch et al. 2018; Akhtar et al. 2020; Lončarević et al. 2021).

The development of improved chitosan materials (with or without their combination with copper) for its use as PPP is necessary and involves some trade-offs: for example, it would be interesting to improve the solubility of chitosan by reducing its molecular weight (MW) (Du et al. 2009) but too small particles are critical. So, the use of particles below 100 nm - which then is called a nanoparticle - in agriculture in Switzerland is questioned. Because it has not been conclusively researched whether the use of nanoparticles could harm the environment (FOPH 2022). Other ways to circumvent the low solubility of chitosan is adapting the degree of deacetylation (DDA). When chitosan has a higher percentage of N-acetylglucosamine units, it is termed chitin and tends to be less soluble (Aranaz et al. 2021). But targeted and uniform deacetylation (Lemke et al. 2022) like other chemical modifications of chitosan to circumvent the low solubility of is mostly difficult and costly.

2.3.1 Novochizol™

Novochizol SA, Monthey, Switzerland (www.novochizol.ch) patented a cross-linked monomolecular chitosan. The patent invented by Kargapolov and Fomenko (2021) can be found here. Compared to normal chitosan, Novochizol™ is water-soluble while retaining the properties of normal chitosan (Teplyakova et al. 2016). It is also able to convert insoluble copper salts such as copper oxychloride [$(ClCu_2H_3O_3)_2$], copper hydroxide [H_2O_2Cu] and copper oxide [Cu_2O] into 'soluble' suspensions via Novochizol™ through a simple formulation process. The particle formation demonstrably bypasses the nanoparticle threshold of 100 nm. The upscaled industrial production of standardised chitosan based on Novochizol™ technology was secured by establishing a joint venture with Alpha-Chitin (www.alphachitin.ch, COMGRAF SAS, Lacq, France), where it is planned to produce 55 tons Novochizol™ per year. The information is based on personal communication with Novochizol SA (Loroch 2023, personal communication).

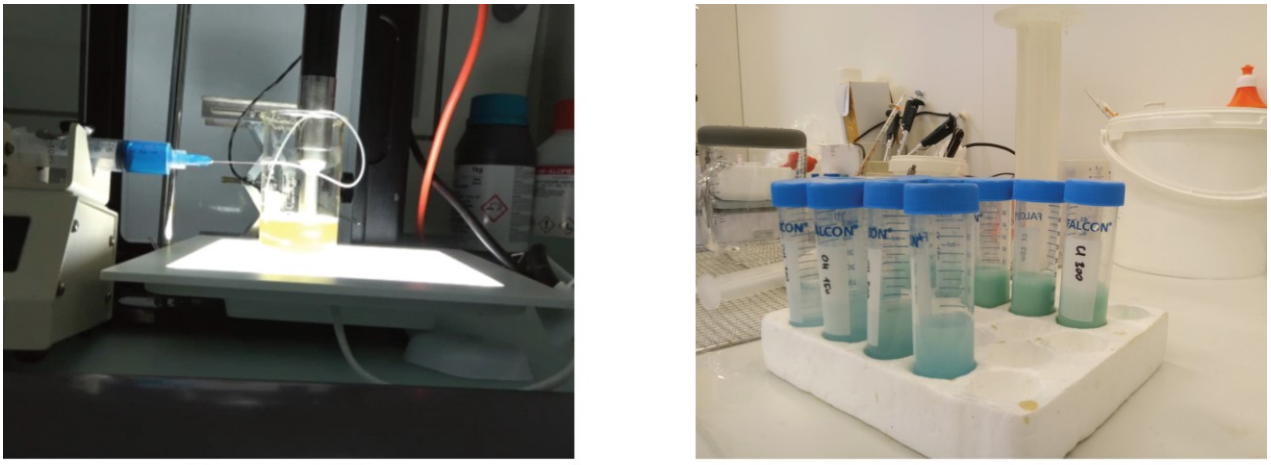
3 Materials and methods

3.1 Products

3.1.1 Formulation of copper loaded Novochizol™

Novochizol™ is a chitosan nanosphere whose formulation has been patented by Novochizol SA, located in Monthey, Switzerland. The product according to the manufacturer shows a DDA of less than 90%, and a MW of 500 kDa. Aqueous Novochizol™ solutions were prepared according to Loroch (2023), by dissolving succinic acid (500 mg per 100 ml of sterile water), gradually adding Novochizol™ (1000 mg per 100 ml of succinic acid solution) under sonication and sonicating the mixture for one hour with Sonicator model UZTA-0.4/22-OM (U-sonic, Biysk, Russia) at maximum power. Sterile water was added to compensate for evaporation caused by the long sonication. The solution was filtered with 0.45 µm apyrogenic acetate cellulose filters (Minisart®, Sartorius Stedim Biotech Göttingen, Germany).

Commercial copper oxide [Cu_2O, 88.6% copper metal], copper hydroxide [H_2O_2Cu, 65% copper metal], copper oxychloride [$(ClCu_2H_3O_3)_2$, 56.96% copper metal] and copper sulphate [$CuSO_4$, 39.81% copper metal], where used. Except for copper sulphate [$CuSO_4$], all copper salts were insoluble in water. For the sake of simplicity, the insoluble salts were also formulated with Novochizol™ using a heterogeneous aqueous suspension. The copper solution or suspension was added slowly and continuously to the aqueous Novochizol™ solution by hand using a syringe (see Fig. 7A) under constant sonication. This had to be done slowly enough to allow each portion of copper solution or suspension to dissolve. The small mobile sonicator had to be moved up and down in the meantime. This resulted in so-called single formulations. To produce double formulations, the single formulations were reformulated with Novochizol™ solutions. Usually, Novochizol SA provided the formulations or their concentrate.



A B

Figure 7: (A) Formulation of copper loaded Novochizol™, by adding a copper sulphate [$CuSO_4$] solution slowly and continuously to the aqueous Novochizol™ solution under sonication. (Source: Loroch V., Novochizol SA, 2022); **(B) Different gel-like concentrates of copper hydroxide [H_2O_2Cu] and copper oxychloride [$(ClCu_2H_3O_3)_2$] Novochizol™ mixtures for bioassays with plant pathogens.**

3.1.2 Commercial plant protection products

The reference copper products used during the assays were Kocide® Opti (copper hydroxide [H_2O_2Cu], 30% [Cu^{2+}], DuPont de Nemours, Wilmington, Delaware, USA) and Airone (copper oxychloride [$(ClCu_2H_3O_3)_2$], 14% [Cu^{2+}] + copper hydroxide [H_2O_2Cu], 14% [Cu^{2+}], Andermatt Biocontrol Suisse AG, Grossdietwil, Switzerland). Additionally, the sulphur Stulln (Andermatt Biocontrol Suisse AG, Grossdietwil, Switzerland) to protect against an infection of powdery mildew during the field assay was used. All three products are on the farm input list (Speiser et al. 2023) for organic farming in Switzerland and can all be used in the form of water dispersible granules (WG).

3.2 In vitro bioassays

To assess the concentrations needed to completely inhibit germination and/or activity of zoospores (MIC_{100}) of *P. viticola* were done in vitro in 96-well plates according to Thuerig et al. (2018). On formulated concentrates of Novochizol™-copper combinations with copper hydroxide [H_2O_2Cu] and copper oxychloride [$(ClCu_2H_3O_3)_2$] were focused. These concentrates were diluted for adjusting copper metal to 0.15 mg ml^{-1}. The ollowing controls were included: copper reference (Kocide® Opti) at 0.3 and 0.15 mg ml^{-1}, Novochizol™ at 3 and 1.25% and H_2O. The H_2O should ensure proper experimental design.
Sporangial suspensions of *P. viticola* were prepared by washing fresh, sporulating grapevine leaves with water (Volvic) and filtering the suspensions through cheesecloth. The concentration of the sporangial suspension was adjusted to 1 - 2 x 10^5 sporangia ml^{-1} before adding 50 µl to each well of the test plate. The sporangia adjustment was done by using a Thoma cell counting chamber. The total volume of the test plate was 100 µl, after adding 50 µl of test product. This was followed by a 1:1 dilution from the previous column, mixing well, i.e., up and down several times with a multichannel pipette, which had to be done slowly. The result was 10 concentrations.
The effects of the tested formulations were assessed 2-3 hours after setting up the experiment and storing the test plates at room temperature. All assessments were performed using a binocular microscope at magnifications of ×50-100. Five levels of inhibition were assessed: 0 (no germinated zoospores or no activity of zoospores), 0.5 (very few zoospores active), 1 (inhibition visible but still some activity), 1.5 (good activity but weaker than 2) and 2 (full activity, no inhibition). The test series was performed only once.

3.3 Plant-pathogen bioassays

The bioassays with downy mildew were carried out on grapevine seedlings in the experimental facilities at FiBL in Frick. The procedure of the trials have already been described in detail by the research group of FiBL (Thuerig et al. 2016). Each treatment was tested on six replicate plants. Totally, 10 x 24-26 treatments (inclusive controls) were tested over two years. The aim was to find in a 'straightforward' approach: 1. suitable copper sources, 2. accurate Novochizol™-copper ratios and 3. possible synergies between Novochizol™ and copper.

The experiments were carried out under semi-controlled conditions in greenhouse and growth chambers. Small grapevine (cv. 'Chasselas') seedlings were transplanted to individual pots (0.275 l) containing a standard substrate (Einheitserde Typ 0; Gebrüder Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) amended with 3 g l^{-1} of a mineral fertiliser (Tardit 3 M; Hauert Günther Düngerwerke GmbH, Erlangen, Germany) (see Fig. 8B). Plants were grown in the greenhouse at a temperature of 18–28°C under natural light (see Fig. 8C). The photoperiod was extended to 16 hours with mercury lamps. Plants were used for bioassays when they had 3–4 fully developed leaves (2–3 weeks after transplanting). Each experimental set included a non-treated non-inoculated control, a water-treated inoculated control, a standard treatment (Kocide® Opti; DuPont de Nemours, Wilmington, DE) at two concentrations (0.3 and 0.03 mg ml^{-1} of copper), and between 20-24 test treatments. All experiments included six replicate plants per treatment and were labelled accordingly.
Plants were sprayed with the test products using an automatic air-assisted spray cabinet (120 kPa at nozzle) until leaves (adaxial and abaxial side) were completely covered with a dense layer of small droplets. All replicates of a treatment were treated at the same time. Plants were subsequently left to dry at room temperature for approximately 2-3 hours before inoculation. *P. viticola* inocula were prepared from previously infected plants by washing freshly sporulating grapevine leaves with water and filtering through cheesecloth. The concentration of the sporangial suspensions was adjusted to 5–5.5 × 10^5 sporangia mL^{-1}, which was assessed using a Thoma cell counting chamber. Plants were randomly picked in pairs of two (same treatment) and spray inoculated using an air-assisted hand sprayer (Compact MINI HVLP touch-up spray gun; Devilbiss, Glendale Heights, IL) on the abaxial leaf side (see Fig. 8D). As soon as the plates of 18 plants were complete, they were brought into the humidity chamber for incubation at 20–21°C and 80–99% relative humidity in the light for 24 hours. Then, plants were maintained at 20°C, 60–80% relative humidity and a 16:8 hours day/night light regime. 5–6 days after inoculation, plants were incubated overnight in the dark at 20°C and 80–99% relative humidity to

promote sporulation. Disease incidence (percentage of leaves with disease symptoms) and disease severity (percentage of leaf area covered by lesions) were assessed 6–7 days after inoculation, as soon as the spores were clearly visible abaxial side of the leaf (see Fig. 8F). Disease assessments were made using continuous percentage values with the help of standard area diagrams (see Annex 1).

When necessary, a second assessment was carried out (in Exp. 6 and 7). Additionally, signs of phytotoxicity in the form of necrosis were documented. In the last trial (Exp. 10), a systematic evaluation of the sequence was performed to assess whether there was any bias due to inoculation sequence. The first and last plant was labelled on all plates of 18 plants (see Fig. 8C). Data that has been cleaned as a result is accordingly labelled when it comes to the results.

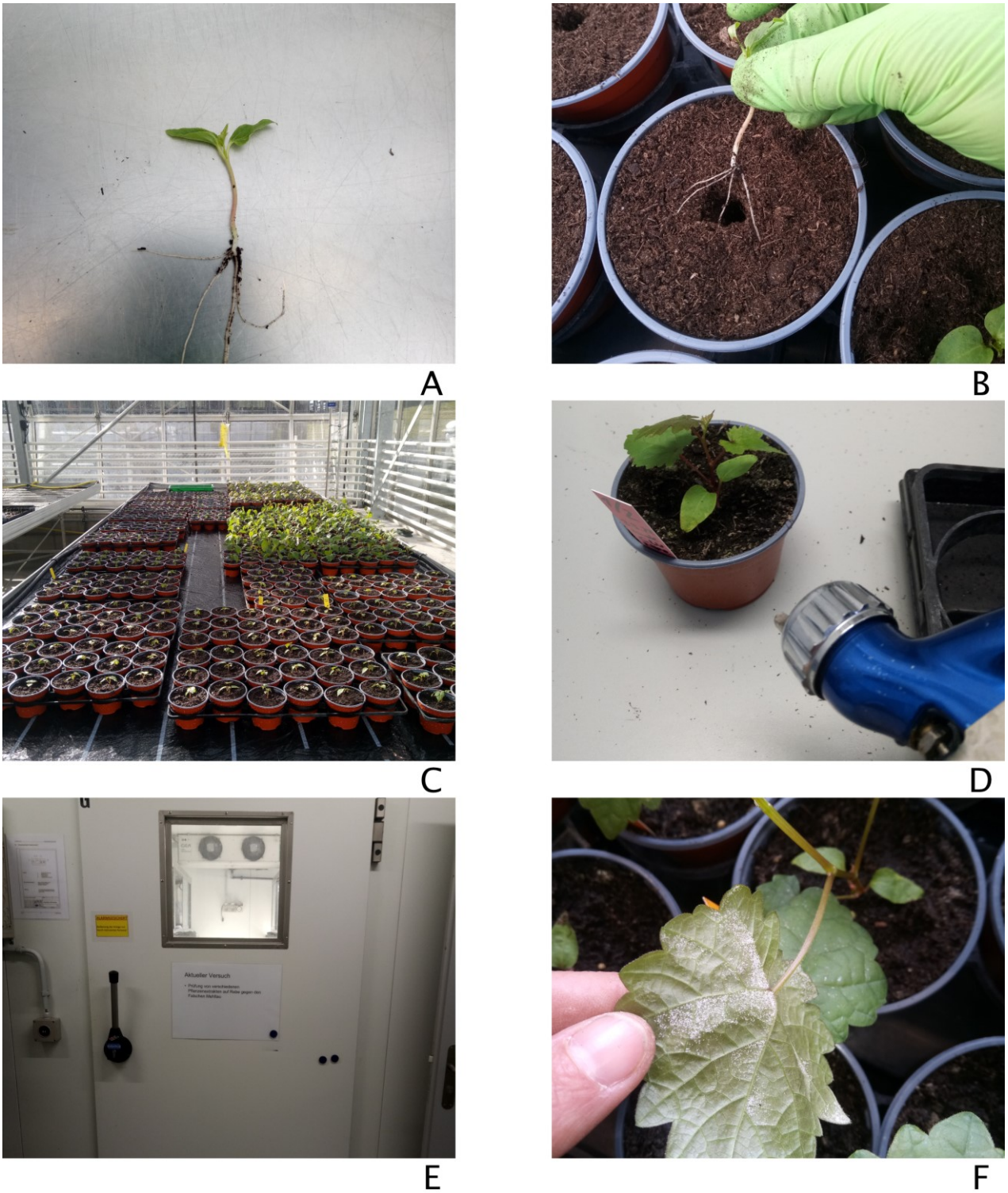


Figure 8: **Experimental execution of plant pathogens bioassays at FiBL facilities in Frick under semi-controlled conditions in the greenhouse and in growth chambers. (A) A single grapevine (cv. 'Chasselas') seedling was transplanted (B) into individual pots (0.275 litres) with standard substrate; (C) seedlings were grown in the greenhouse until they had 3-4 fully developed leaves (2-3 weeks after transplanting); (D) inoculation by hand using a touch-up spray gun 'Devilbiss' after the plants were treated and dried for three hours; (E) growth chamber and (F) sporulation on the abaxial side of the leaf.**

3.4 Field assay

3.4.1 Study site

The field assay was conducted in a commercial vineyard located in Ligerz on the family-owned winery 'Festiguet' of Michael Teutsch. The AOC Ligerz is part of the 'Drei-Seen-Region' and lies in the north-western part of Switzerland and is characterized by steep hills and calcareous soils. The assay took place during the 2022 and 2023 growing seasons. The altitude of the plot is 584 meters above sea level and belongs to the parcel 'Clos à l'Abbé' (2'577'030.000, 1'215'300.500). Pinot Noir (*V. vinifera*) vines are grafted onto 5C rootstock, planted in 2010 and in form of 'Direktzug', means that the lines of the vines are vertical to the slope and can be cultivated mechanically. The single plant was pruned in the system 'Guyot' with two reserves on both sides of the plant. In recent years, the yield per square meter was obtained between 600 - 800 grams. The cultivation has been officially certified organic since 2020, and even before, no herbicides were used. Planting density is 5'500 plants per hectare, with vine spacing between rows and within rows of 1.80 × 1.00 meters, respectively. The parcel is oriented towards the southeast, was intensively grassed due to the cultivation, and has shown to be highly heterogeneous in water and nutrient availability during the two relatively dry experimental seasons – also because the soil has little depth in the middle of the parcel and different inclinations. The average gradient over the entire plot is around 34%.

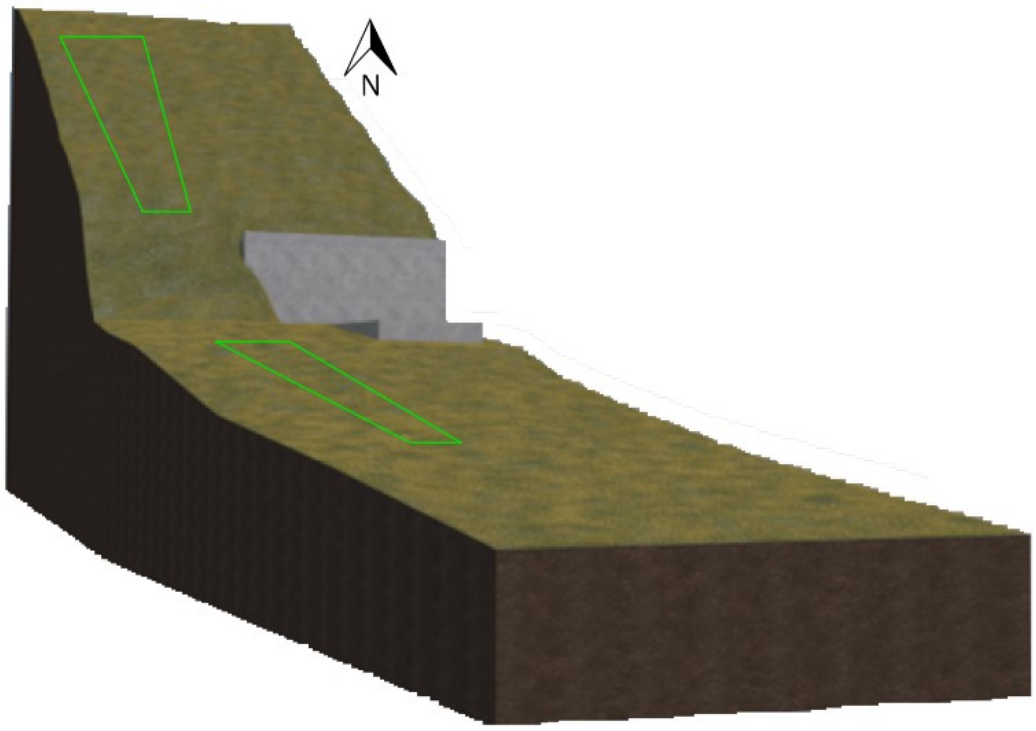


Figure 9: The terrain with the experimental field marked in green. There is rock in the ground at the transition from the steep to the flat part. The lower part is much flatter.

There was no fix installed irrigation system in the form of drip irrigation, although in the 2022 season the whole field (including the experimental field) was irrigated with a mobile sprinkler system at a rate of approximately 40 mm / m^2. In terms of management, the field was subject to the same steps as the rest of the farm and resembles a standardized method of cultivation of grapes in the region: primary pruning, removal of secondary shoots and mulching of last year's shoots and grass several times during the season. Only the foliage work - more precisely - the removal of leaves in the grape zone was slightly adapted to the timing of the disease assessment to avoid a removal of eventual infected leaves.

3.4.2 Climate and weather forecasting

Mean annual rainfall (2006-2020) at the closest weather station in Twann, next to Ligerz was 1'165.92 mm (www.agrometeo.ch), with being below average in 2022 with 987.3 mm and 1133.5 mm in 2023.

Weather data were recorded throughout the season with a campbell weather station. For complete weather data, see www.agrometeo.ch, weather station Twann. The average daily temperature and precipitation amounts can be found in Fig. 10 and 11.

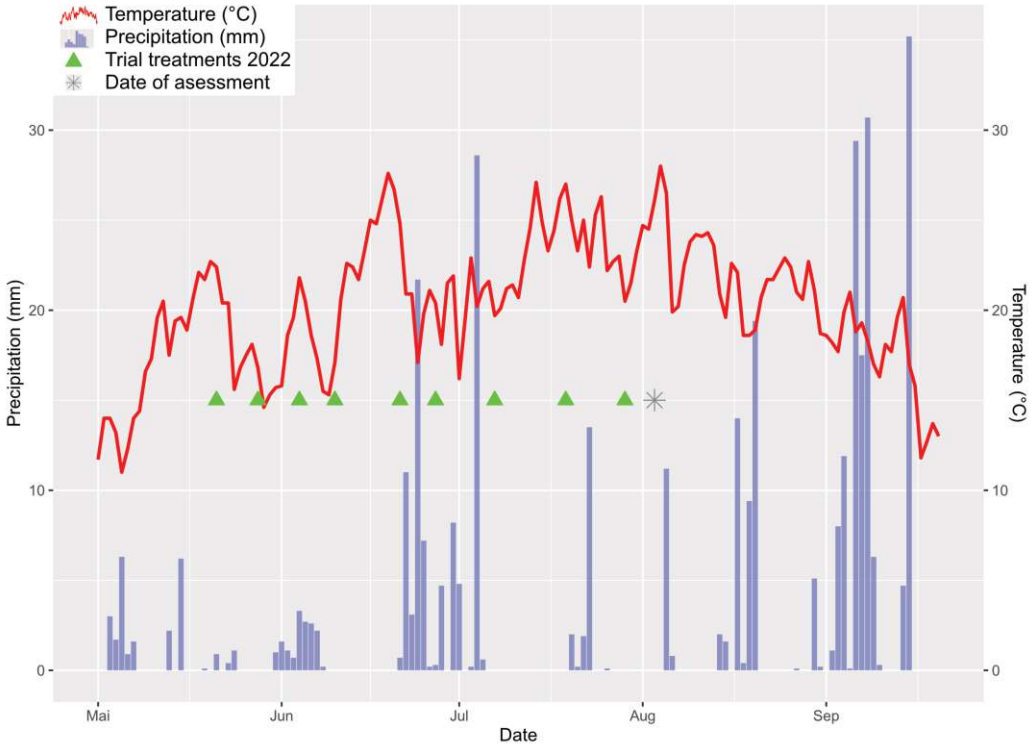


Figure 10: Mean daily temperature, rainfall, and dates (and amounts) of treatments and assessment during season 2022.
(Source: **www.agrometeo.ch**, 2022)

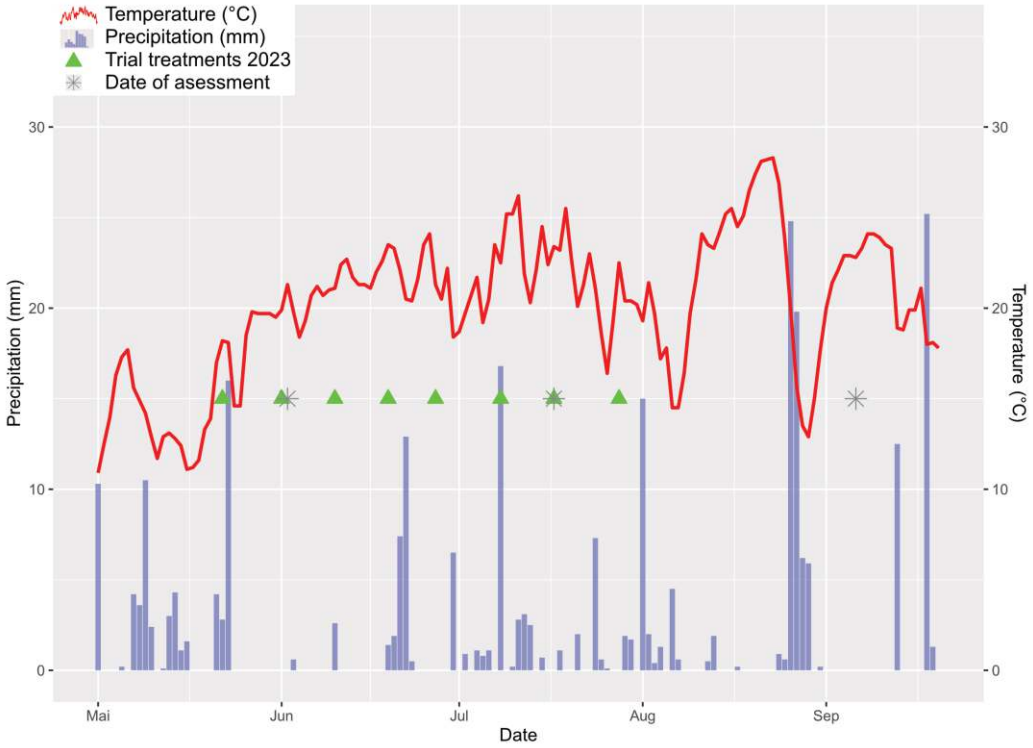


Figure 11: Mean daily temperature, rainfall, and dates (and amounts) of treatments and assessment during season 2023.
(Source: **www.agrometeo.ch**, 2023)

The climate in the 2022 and 2023 seasons was dry during the infection and treatment period, respectively, from mid-May to the end of July. Compared to the 2022 season, the budburst in 2023 season was two weeks delayed. Whereas the year 2022 with little rain during the winter and spring months meant a precipitation deficit for the start of the vegetation period. No important rain fell in the months of June and July 2022.
The 2023 season was characterized by a wet and cold late spring, with the last intense rainy period in mid-May - coinciding with the start of the vegetation period. The next important rain fell during July. Climate was tracked without recording the data. For weather forecasts, the MeteoSwiss application provided by the Swiss Federal Office of Meteorology and Climatology was consulted. Thunderstorms and rainfall with important amount and duration of precipitation - which would make an infection of *P. viticola* possible - were tracked several times a day during the relevant period.

3.4.3 Disease forecasting

To anticipate a possible infection with downy mildew, there are several decision support systems for the management of diseases of grapes and other crops. The models from RIMpro (www.rimpro.eu, Keizersgracht, Netherlands) and Agrometeo from Agroscope, the Research Institute of the Swiss Federal Office for Agriculture were used during the field assay. Both models use the data of the official weather stations of the Swiss Federation and meteoblue (meteoblue AG, Basel, Switzerland). The nearest weather station was located in the village of Twann, about three kilometres to the north-east direction and 150 m closer to sea level. While the use of Agrometeo data was free of charge, access to the RIM-pro model via FiBL was necessary as funding from the Canton of Bern had not yet been secured. Agrometeo provides a table in the form of a PDF file that predicts the date and severity of primary infections, as well as the time of onset of symptoms. RIMpro uses a graphical solution. The infection risk situation for the 2022 and 2023 seasons is shown in the RIMpro Figures 12 and 13.

The first treatment was planned and prepared as soon as the oospores were ready (see 'Active oospores' in Figs. 12 and 13) and the vine vegetation had reached the BBCH 11 stage (‘first leaf unfolded and spread away from the shoot’). The risk of initial infection was assessed using RIMpro and Agrometeo. In addition, a private chat 'Bio Spritzplan Bielersee' was participated in, in which the organic winegrowers of the region exchanged information about their treatment strategy.

The application needed to be completed at least two hours before precipitation. This also applied to all subsequent treatments. An application interval of 7-10 days is aimed and must be adapted according to the amount and duration of forecasted precipitation. Various other aspects, such as wind or soil conditions, musted be considered and, it was tried to avoid making an application on Sunday. Also, exceeding the spraying intervals to more than 10 days is not recommended: because the sulphur then must be renewed to combat powdery mildew. In addition to the technical means of tracking the disease, a visual inspection of the plot to monitor any symptoms that might occur had been done.

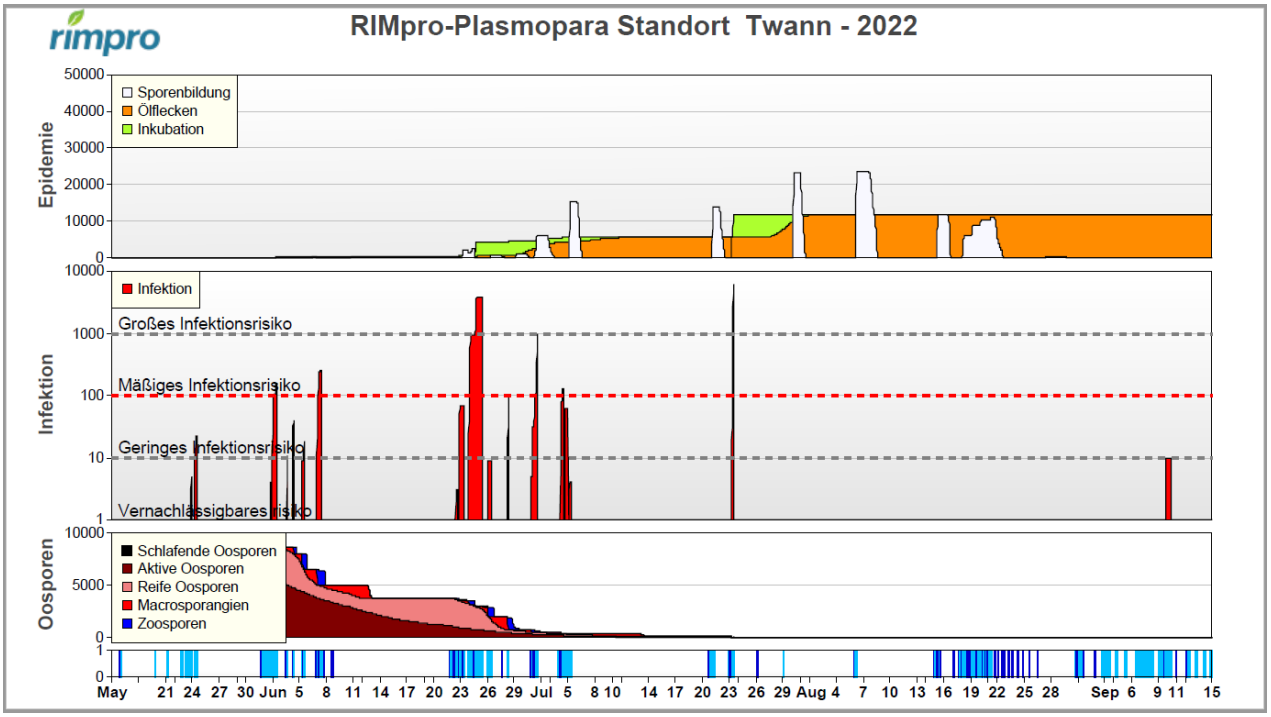


Figure 12: Results of the Plasmopara viticola infection simulation model 'RIMpro', based on the weather data for Twann 2022, during the efficacy trial.
(Source: **www.rimpro.eu**, 2022)

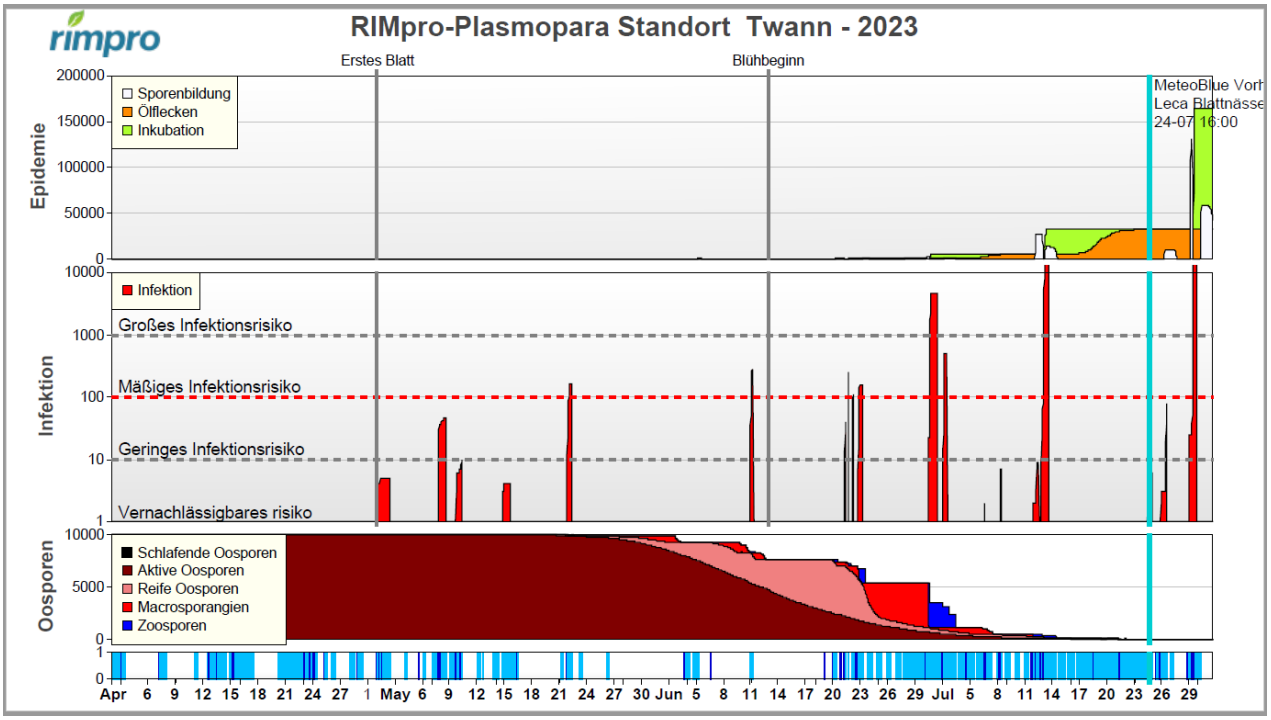


Figure 13: Results of the Plasmopara viticola infection simulation model 'RIMpro', based on the weather data for Twann 2023, during the efficacy trial.
(Source: **www.rimpro.eu**, 2023)

3.4.4 Experimental design

For the field assay, a randomized block design according to European and Mediterranean Plant Protection Organization (EPPO) guidelines was applied. All EPPO guidelines used during the experiments can be found in the Annex 1. There was a total of 4 blocks of 6 treatments each (see Fig. 14). Each treatment replication contained 5-6 plants, with only the treatment replication at the border counting 5 plants. The additional plants in the 'Ausläufer' lines were treated with the reference product. In total, there

were 148 plants. An individual colour code was chosen and attached to the metal bars between the plots. Before implementing the experimental design, it was discussed with a statistician from HAFL (Burren 2022, personal communication).

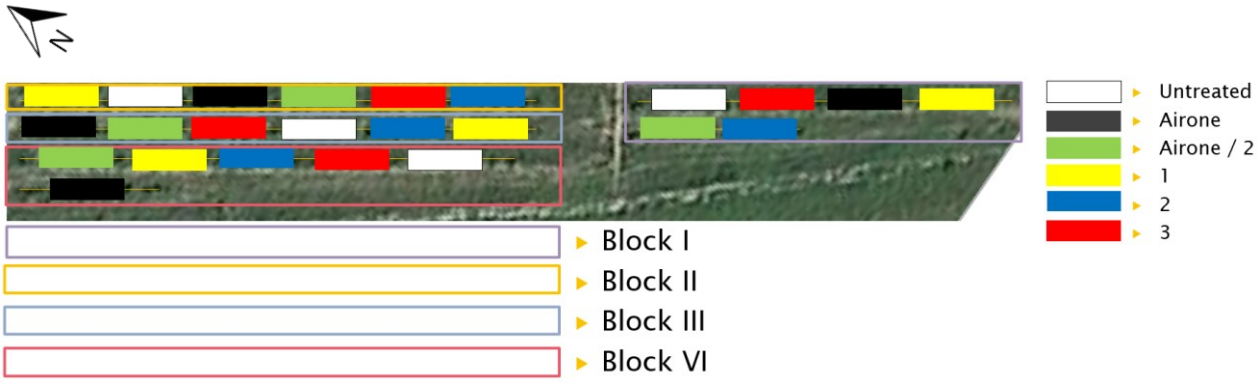


Figure 14: Plan of the experimental design for season 2022 and 2023.

The untreated control (=white) was treated with sulphur only to avoid powdery mildew infection. The reference product used was Airone (=black, copper oxychloride [$(ClCu_2H_3O_3)_2$], 14% [Cu^{2+}] + copper hydroxide [H_2O_2Cu], 14% [Cu^{2+}], Andermatt Biocontrol Suisse AG, Grossdietwil, Switzerland) in normal dosage. Halving the Airone dosage (=green) referred to the theses' objective of halving the copper metal concentration. Products 1 (=yellow) and 2 (=blue) were the actual experimental products resulting from the previously conducted plant pathogen bioassays and differed from the 2022 to 2023 season. Product 3 (=red) was the Novochizol™ (0.15%) alone. For the details of the products used, see Table 3 and 4.
The experimental field was part of a bigger parcel called 'Clos à l'Abbé' and was located at the western end of this parcel. To ensure that there was no drift due to the treatment of the rest of the field, the adjacent lines were treated only in the eastern direction. In direction west there was a biodiversity field that is managing by 'Festiguet' and that belongs to the municipality. The test field consisted of an upper and a lower part, with a small house in the centre surrounded by a square. In this place, the treatments were prepared. Therefore, a table and access to water were installed.

3.4.5 Grapevine treatments

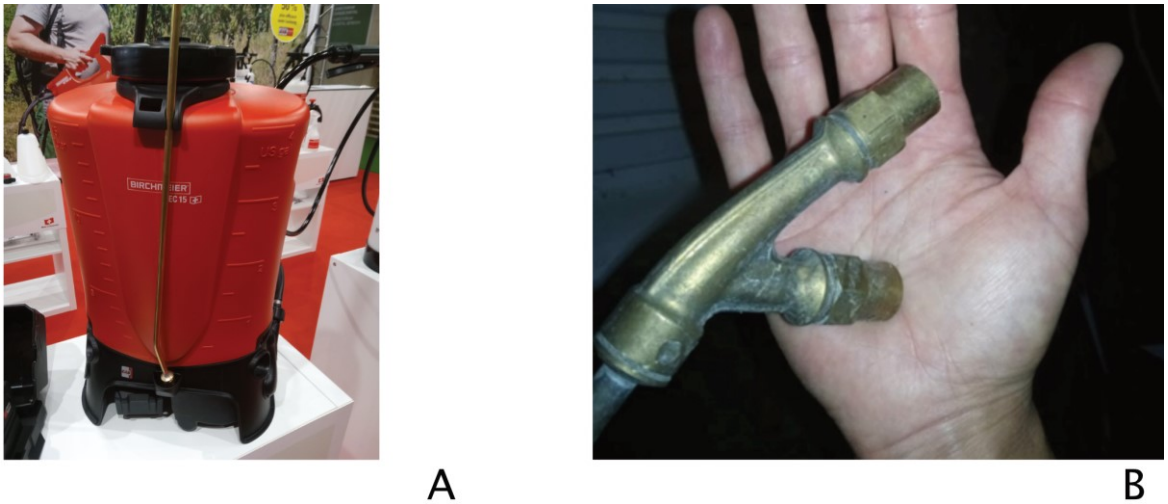


Figure 15: (A) REC 15 AC1 electric driven hand sprayer by Birchmeier; (B) Y-form double nozzle

The treatments were conducted by an electric driven handsprayer by Birchmeier (Birchmeier Sprühtechnik AG, Stetten, Switzerland). The model used was the REC 15 AC1, a pressure-regulated battery back sprayer (see Fig. 15A). It had a maximum capacity of 15 l and could be regulated from 0.5 - 6 bar, which meant a flow rate of 0.2 - 1.4 l per minute. Together with the technician of Birchmeier, it was tested the various options at the FiBL in spring 2022 to achieve the best possible spray pattern. Hydrosensitive

papers were used for this purpose. By personal choice, a short lance (slightly bent in front of the spray head) with a Y-shaped double nozzle was used (see Fig. 15B).
A pressure of 4 bar was set for the regulation. The required amount of liquid was determined on site, and the flow rate per time and the optimum running speed were tested. Someone helped to measure and record the times. This information was used to create an Excel spreadsheet (Microsoft, Redmond, Washington) for the calculations (see Digital Annex). The litres of liquid consumed had to be constantly adjusted to the amount of vegetation, using the data in the 'Index Phytosanitaire pour la viticulture' from Agridea (Dubuis et al. 2022) as a reference.

Table 1: Treatment plan 2022, including the targeted amount of copper (in grams) and sulphur (in kilograms) per hectare, number, and date of effective treatments, with the liquid used per plot (4x6 = 24 grapevine plants) and hectare.

Nr.	Date	BBCH	Copper per ha (g)	Sulphur per ha (kg)	Liter per treatment and plot	Liter per treatment per ha
1.	21.5.	14	150	2.4	2.5	579
2.	28.5.	55	180	2.4	3.8	741
3.	4.6.	61	300	4	4.5	880
4.	11.6.	67-69	300	4	4.5	1042
5.	21.6.	73	150	5	4.5	1042
6.	28.6.	75	150	5	4.4	1042
7.	8.7.	77	150	5	4.7	1088
8.	19.7.	81	150	5	4.5	1042
9.	29.07.	83	150	5	4.5	1042
Total	-	-	1750	37	37.1	8498

Table 2: Treatment plan 2023, including the targeted amount of copper (in grams) and sulphur (in kilograms) per hectare, number, and date of effective treatments, with the liquid used per plot (4x6 = 24 grapevine plants) and hectare.

Nr.	Date	BBCH	Copper per ha (g)	Sulphur per ha (kg)	Liter per treatment and plot	Liter per treatment per ha
1.	22.5.	14	150	2.4	2.5	579
2.	1.6.	57	180	2.4	3.2	741
3.	10.6.	67	300	4	4.5	1042
4.	19.6.	73	300	4	4.5	1042
5.	27.6.	75	150	5	4.5	1042
6.	8.7.	77	150	5	4.5	1042
7.	11.7.	81	150	5	4.2	972
8.	28.7.	83	150	5	4.2	972
Total	-	-	1680	32.8	36.6	7432

The specifications in the practical viticulture documents, including those on the recommended amount of copper, refer to the specification per hectare. The quantities and dosages therefore had to be adjusted based on one hectare. It was assumed that there were 5'500 vines per hectare, which means an area per vine of 1.8 m^2. The maximum amount of copper metal allowed in organic viticulture is 3 kg ha^{-1} y^{-1} (over five consecutive years). The amounts per application can be adjusted individually, but usually a maximum of copper is used around the flowering stage (BBCH stage 67-69). The number of applications can also vary and depends primarily on the disease pressure of the season. With the help of some experts (personal communication with Bioline, Biocontrol Andermatt and FiBL) it was worked out a

treatment plan before the start of the season. Targeted amounts of copper and sulphur applied are shown in Table 1 and 2 together with the effective treatment date and liquids used per plot and hectare. The sulphur treatments to control powdery mildew were applied on the same day and right before the assay treatments. Using the commercial sulphur 'Stulln' (Andermatt Biocontrol Suisse AG, Grossdietwil, Switzerland), the entire surface of the experimental field was covered. When the 15 l became insufficient for the entire surface, the treatment was divided into two parts. The calculation of the amount of sulphur needed in grams was as follows for the total of 148 grapevine plants, which were covering 0.02264 hectares:

$$= 0.02264\ ha \times \text{ amount of sulphur required [kg } ha^{-1}\text{]} \times 1000$$

Fixed the amount of copper needed in the relevant stage, it had to measure the reference product as well as the copper containing concentrates. The calculations for the correct dosage (in grams or millilitres) were as follows to treat all replicates of 4x6 grapevine plants (= 24 vines), correspond to 0.00432 hectares:

$$= \frac{(\text{amount of pure copper metal required [g } ha^{-1}] \times 0.00432\ ha)}{share\ of\ pure\ copper\ metal\ in\ the\ product}$$

To calculate the amount of concentrated Novochizol™ in millilitres for a concentration of 0.15%, it was proceeded as follows to treat all replicates (= 24 vines):

$$= \left(\frac{\text{litres required} \times 0.0015}{\text{Novochizol concentration in the concentrates}}\right) \times 1000$$

To measure the commercially available copper and sulphur product, a Domo pocket scale (Linea 2000, Herentals, Belgium) weighing in 0.01-gram increments was utilized. Sulphur and Airone wettable granulates and the liquid Novochizol™ copper concentrates were directly measured in the field. Prior to application, a thorough shaking of the liquid concentrates was ensured. For accuracy, a 100-milliliter measuring cylinder was utilized. Furthermore, a 2-liter measuring beaker served the dual purpose of dissolving the product before introducing it to the backpack sprayer and measuring the precise amount of required water. To maintain environmental standards, all containers were promptly rinsed in the vineyard, preventing any pollutants from entering wastewater.

The copper reference product and Novochizol™ formulations were applied per treatment across four replicates. Variations in the application pattern were introduced for each application, covering both sides of the vine rows, and emphasizing the underside of the leaves to ensure effective protection. Post-application, any remaining product was documented, and special events, such as heavy morning rain or inadvertent treatment errors, were noted. These observations, along with dates and planned/applied product quantities, were recorded in a handwritten crop protection journal. Periodically, the product quantities were transferred to a Microsoft Excel spreadsheet for comprehensive end-of-season traceability (see Digital Annex).

The entire measurement, treatment, and washing process prioritized human safety, necessitating the use of an overall, gloves, masks, and goggles (see front cover image).

Table 3: Details of the products used in 2022

Code	Treatment	Products	Formulation type	Supplier	Active incredient (a.i.)	Copper formulation	Plant-Pathogen assays	Concentration in field (eff.)	Amount of a.i. per y ha^{-1} (eff.)
White	Control (Sulphur only)	-	-	-	-	-	-	-	-
Black	Cu Ref.	Airone	WG	Biocontrol Ander-matt	copper hydroxide	$[Cu(OH)_2]$	0.3 mg ml^{-1}	0.15-0.30 mg ml^{-1}	1.702 kg
					copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$			
Green	Cu Ref./2	Airone	WG	Biocontrol Ander-matt	copper hydroxide	$[Cu(OH)_2]$	0.15 mg ml^{-1}	0.073-0.137 mg ml^{-1}	0.802 kg
					copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$			
Blue	Novo-1	Novo-1	SL	Novochizol™ SA	copper sulphate	$[CuSO_4]$	0.15 mg ml^{-1}	0.067-0.146 mg ml^{-1}	0.841 kg
					Novochizol™	-	0.3%	1.46-2.92%	17.5 kg
Yellow	Novo-2	Novo-2	SL	Novochizol™ SA	copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$	0.15 mg ml^{-1}	0.068-0.15 mg ml^{-1}	0.832 kg
					Novochizol™	-	0.15%	0.68-1.55%	8.75 kg
Red	Novo-3	Novo-3	SL	Novochizol™ SA	Novochizol™	-	0.15%	0.144-0.15%	12.71 kg

Table 4: Details of the products used in 2023

Code	Treatment	Products	Formulation type	Supplier	Active incredient (a.i.)	Copper formulation	Plant-Pathogen assays	Concentration in field (eff.)	Amount of a.i. per y ha^{-1} (eff.)
White	Control (Sulphur only)	-	-	-	-	-	-	-	-
Black	Cu Ref.	Airone	WG	Biocontrol Ander-matt	copper hydroxide	$[Cu(OH)_2]$	0.3 mg ml^{-1}	0.137-0.3 mg ml^{-1}	1.502 kg
					copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$			
Green	Cu Ref./2	Airone	WG	Biocontrol Ander-matt	copper hydroxide	$[Cu(OH)_2]$	0.15 mg ml^{-1}	0.069-0.15 mg ml^{-1}	0.743 kg
					copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$			
Blue	Novo-1	Novo-1	SL	Novochizol™ SA	copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$	0.15 mg ml^{-1}	0.071-0.143 mg ml^{-1}	0.746 kg
					Novochizol™	-	0.125%	0.108-0.12%	7.65 kg
Yellow	Novo-2	Novo-2	SL	Novochizol™ SA	copper oxychlo-ride	$[(ClCu_2H_3O_3)_2]$	0.15 mg ml^{-1}	0.66-0.15 mg ml^{-1}	0.738 kg
					Novochizol™	-	0.0188%	0.016-0.018%	0.96 kg
Red	Novo-3	Novo-3	SL	Novochizol™ SA	Novochizol™	-	0.15%	0.144-0.15%	11.15 kg

3.4.6 Disease assessment

During the growing season, leaves were assessed based on disease appearance. A visual inspection, conducted several times a week, aimed to identify oil spots - the initial signs of downy mildew infection on leaves. Once infection occurred, disease severity (proportion of leaf area infected) was recorded for downy mildew on leaves. Depending on the vine's stage, 20 to 100 leaves per replicate underwent visual inspection to assess infection levels. As the infection primarily occurs on the underside of leaves, a manual turnover was necessary, inspecting each leaf individually. Leaves were randomly selected but needed to be in the relevant zone of the plant: the fourth to tenth leaf of the main shoot. Higher leaves and secondary shoots, being less crucial for wine production, were less protected and naturally more sensitive. Data per leaf were systematically recorded in a purpose-prepared table (see Digital Annex).
In the 2022 season, the assessment was conducted personally, as no infection occurred, resulting in a final evaluation during ripening on 3rd August 2022. In the subsequent 2023 season, the initial infection (not covered by treatments) was recorded on 20 leaves on 2nd June 2023. In a second assessment on 17th July 2023, assistance was employed, recording 100 leaves per replicate. Assessments of downy mildew on grapes (severity) involved 50 grapes. All disease ratings adhered to continuous percentages based on the EPPO standard scale (see Annex 1). Additionally, necrosis was considered.

3.4.7 Must oenological parameters

At the end of the 2022 growing season on the 12th and 13th of September 2023, the quality parameters of the grape berries were analysed. Because must oenological parameters are crucial for a proper vinification process. Therefore, 100 berries were collected per replicate both on the day before and on the day of harvest. For capacity reasons of the laboratory, the analysis had to be performed on two separate days. Each sampling included twelve samples out of the total 24 samples. The berries were randomly selected, taking care also to include berries from the shoulder and the end of the bunch.

The laboratory responsible for the analysis of the samples was the 'Office de la viticulture et de l'agroécologie' in Auvernier, Switzerland. Immediately after collection, the berries were taken to the laboratory in Auvernier, which was done within 30 minutes.

The analysis package chosen was called 'Analysis suivi de maturité' and included the following preparations and analyses:

- Preparation of grape sample; the berries are weighed and pressed
- Measurement of total acidity (g l^{-1}) and pH
- Analysis of assimilable nitrogen (YAN), inorganic, and organic nitrogen by enzymatic analyse

3.5 Wine production

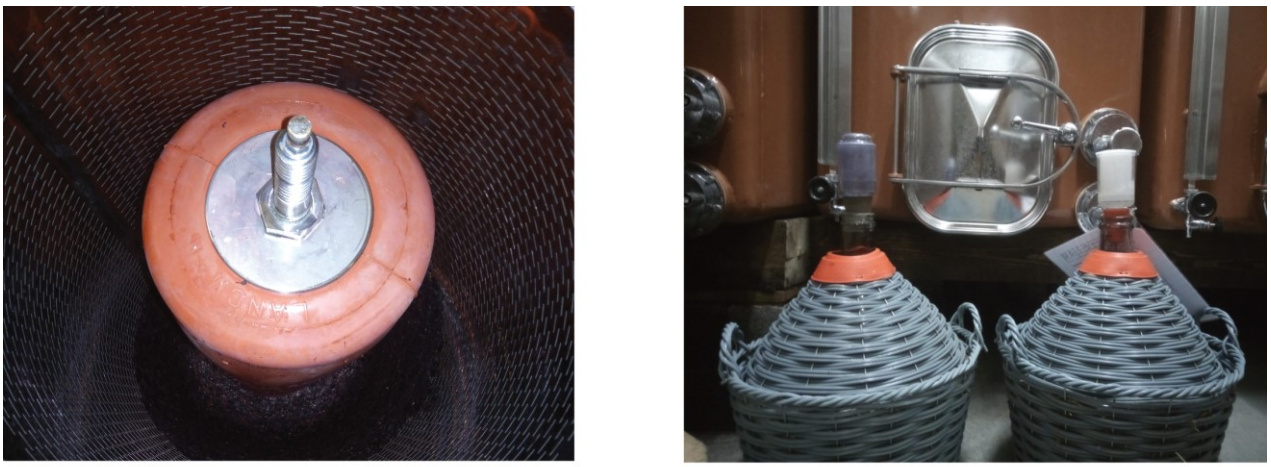
3.5.1 Grape harvest and processing

On the day of harvest in 2022 season (13th of September 2022) the experimental field was harvested with two instructed persons. The treatments were divided based on the presence of Novochizol™. The harvest followed a standard procedure, and due to the absence of infected berries, minimal selection was required. The collected berries were placed into two separate containers, each with a capacity of 610 l. These containers were prepared in advance, placed at the top of the rows, and numbered according to a protocol. Personally, transporting the berries from the vineyard in 15-liter green grape buckets (Landi, Switzerland) ensured they went into the correct container. Subsequently, the containers were transported with a vineyard caterpillar to the processing area, one floor higher than the cellar.
The destemming and light crushing of the berries were performed by machine. Gravity then facilitated the transportation of the processed berries into two separates cylindrical inox containers, one with a capacity of 110 l and the other 200 l (Speidel, Germany), prepared in advance at cellar level. During the grape destemming process, mash samples were taken at different times to ensure even distribution. Using these samples, the degree of Oechsle (°Oe) was determined with a refractometer, and pH and total acidity were assessed through titration to pH 7 using a 5% [NaOH]-solution. After levelling, measuring, and noting the amount of grape mash, a standard vinification process was initiated.

3.5.2 Vinification process

The grape mash in the two inox containers received 30 g hl^{-1} of 'Be-Red' yeast, a commercial red wine yeast from Erbslöh, Germany. The yeast was prepared by adding yeast powder to 1/10-part 37°C water in decilitres and adding must in two steps to maintain the tolerance ΔT < 10 °C. After 20 minutes, foaming yeast was introduced to the grape mash. Using a wooden mash pestle, the must-yeast blend was superficially incorporated to enhance yeast-must contact. Both containers were situated in the cellar's red wine fermentation area, characterized by a constant, windless temperature. Daily tamping of the grape mash occurred from the day after maceration until half the sugar conversion, then every other day until pressing. This was done by gently pressing the wooden pestle into the grape mash. The entire surface of the floating grape skins should at least be covered by the liquid again - without being crushed at the bottom of the container. Oechsle (°Oe) and temperature were measured using a must scale with a thermometer (0-140°Oe / 0-32 % Brix division 1°Oe, Baldinger, Switzerland). Depending on fermentation speed, this process occurred once to twice daily, even post-pressing.

As alcoholic fermentation proceeded well, there was no need to cool or heat the mash. Completion of alcoholic fermentation was determined using EasyDens (Anton Paar, Graz, Austria). The time of pressing was determined by tasting and happened at -2 and 0 remaining Oechsle (°Oe) respectively. On the day of pressing (27th and 28th of September 2022) the liquid was extracted from the containers first using a mash sieve (handmade) and then pressed the remaining grape berries using a hydropress (250 l, Lancman, Slovenia) (see Fig. 16A).

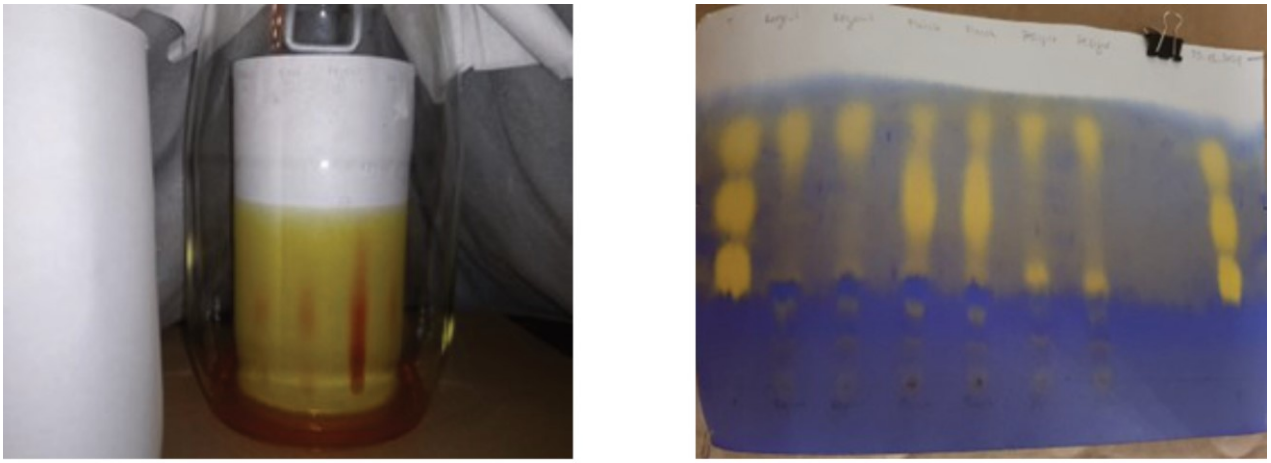


A B

Figure 16:(A) Hydropress 250 l, Lancman, Slovenia; (B) 25-liter balloon bottles.

After multiple tastings, the coarse yeast was removed from the two wines on 14th of October 2022 to prevent wine reduction. On 10th of November 2022, it followed the transfer to 25-liter balloon bottles before physical stabilization (see Fig. 16B). The wines were separated from the yeast once again at this point. The remaining portion was incorporated into the wine produced from the remaining plot 'Clos à l'Abbé'.

To ensure malolactic fermentation had occurred - a crucial step before the physical stabilisation - paper chromatography was employed (see Fig. 17). For this, small wine samples were applied to chromatograph paper using capillary tubes. The paper was then rolled up and placed for several hours in a vessel containing a butanol solution and the indicator dye bromocresol green. After the paper was pulled out and dried, the distance of the yellow-coloured spots from the baseline indicated the presence of various acids, with tartaric acid closest to the baseline, followed by citric, malic, and lactic acids near the top of the paper (Yair 1996).



A B

Figure 17: Performance of paper chromatography to determine the completion of malolactic fermentation; (A) Paper with the wine sample placed for several hours in a vessel containing a solution of butanol and the indicator dye bromocresol green; (B) Result after drying; the calibration solution on the far left and right, where the dot in the centre is malic acid and the dot on the top is lactic acid. Only single dots on the top indicate whether malolactic fermentation is complete (no quantitative figure).

After completion of malolactic fermentation, the two 25-liter samples were physically stabilized in the appropriately refrigerated cellar at 4-6°C for two months. The step of filtration was not performed due to the lack of equipment for such small volumes. Bottling was done on 6th of April 2023 whereby the 25 liters of Novochizol™ wine sample were treated with 40 mg l^{-1} SO_2 beforehand. The bottles were kept in a cool and dark place. The 25 liters of the control plots were poured back to the rest of the plot of wine without bottling it separately.

3.5.3 Wine sampling

After the step of physical stabilization by cooling, two 0.5 l wine samples were delivered to the laboratory of the 'Office de la viticulture et de l'agroécologie' in Auvernier, Switzerland. For this purpose, glass sample bottles were filled with Novochizol™ sample and the control sample, respectively. The samples were labelled accordingly and sent by mail together with the analysis order. Following the list of the commissioned analyses:

- Fourier transform infrared spectroscopy analysis (incl. acetic acid ('volatile acidity') [g l^{-1}], alcohol [%vol.], citric acid [g l^{-1}], density [Oe°], fructose [g l^{-1}], glucose [g l^{-1}], glycerol [g l^{-1}], lactic acid [g l^{-1}], malic acid [g l^{-1}], sucrose [g l^{-1}], tartaric acid [g l^{-1}], total acidity [g l^{-1}], total sugars [g l^{-1}])
- Standard 'classique' analyses (incl. free SO_2 [mg l^{-1}], total acidity [g l^{-1}], pH, residual sugars by enzymatic method [g l^{-1}], Turbidity [NTU]
- Analyse 'malolactic fermentation' by enzymatic method
- Tasting

The Fourier transform infrared spectroscopy analysis (Griffiths and Haseth 2007) was intended to provide a broader characterization of the wines. The standard analyses determined the most important oenological indicators and the tasting served as an objective, qualified sensory evaluation.

3.6 Statistical analyses and presentation

Statistical analyses were carried out in RStudio (Posit PBC, Boston, Massachusetts). The analyses of the plant pathogen bioassay were performed as a single variable design. Values of disease severity were converted using the square root of arcsine, followed by the calculation of the efficiency according to Abbott (1925), as:

$$[1 - (A \times B^{-1})] \times 100$$

where A is the disease severity on an individual plant and B is the mean disease severity of control plants treated with H_2O. A one-way ANOVA was performed in case of normal distribution of residues (Shapiro-Wilk normality test p-value > 0.05) and equal variance (Levene test p-value > 0.05). The Tukey post hoc test was used for multiple comparisons. In the case of non-homoscedasticity, a Welch two-sample t-test was performed (p-value < 0.05) to compare the mean efficacy of two different treatments on both sides.
Correlations were tested according to Pearson after the assumption had been checked and by providing the correlation coefficient.
The functions implemented in RStudio ‘geom_smooth(method = "lm")’ and ‘geom_smooth(method = “loess”)’ were used for the graphical representation using ggplot. Where "lm" stands for ‘Linear Regression’ and "loess" for ‘Locally Weighted Least Squares Regression’, which was labelled accordingly.
The field assay analyse was performed as randomised complete block design, where treatment (six levels) was a fixed factor and block (four levels) was a random factor. Field severity data was not transformed as homoscedasticity was met before performing a one-way ANOVA. The same applied to the analysis of the oenological must parameters. The usual significance level of $p < 0.05$ was applied in all cases. No statistical analyses were carried out for the data obtained from in vitro testing and wine sampling.

Where necessary, Affinitiy Publisher (Serif (Europe) Ldt, Nottingham, United Kingdom) was used to provide the graphics from RStudio with additional legends. The illustration of the plot was also graphically processed using Affitiy Publisher. The plot was drawn in ArchiCAD (Nemetschek SE, Munich, Germany) according to a hand-drawn sketch by a family member.

4 Results and discussion

4.1 In vitro activity of a Novochizol™-copper combinations

All combinations with Novochizol™ maintained stable in copper concentration (0.15 mg ml^{-1}) examined comparable oomycotic activities (MIC_{100} 6.25-25µgmL^{-1}) against *P. viticola* spores. In combination with copper oxychloride [$(ClCu_2H_3O_3)_2$], Novochizol™ at a concentration of 0.75 mg ml^{-1} showed the highest activity throughout the experiment with a MIC_{100} of 6.25 µg ml^{-1}.

Table 5: Minimal inhibitory concentrations (MIC_{100}) of Novochizol™-copper combinations, copper (Kocide® Opti) controls and Novochizol™ against *Plasmopara viticola* in in vitro experiments

Product	Copper [Cu^2] mg ml^{-1}	Novochizol™ mg ml^{-1}	MIC_{100} µg ml^{-1}
Kocide® Opti	0.3	-	12.5
Kocide® Opti	0.15	-	12.5
copper hydroxide [H_2O_2Cu]	0.15	0.75	12.5
copper hydroxide [H_2O_2Cu]	0.15	0.5	12.5
copper hydroxide [H_2O_2Cu]	0.15	0.375	12.5
copper hydroxide [H_2O_2Cu]	0.15	0.25	12.5
copper hydroxide [H_2O_2Cu]	0.15	0.0188	12.5
copper oxychloride [$(ClCu_2H_3O_3)_2$]	0.15	0.75	6.25
copper oxychlride [$(ClCu_2H_3O_3)_2$]	0.15	0.5	12.5
copper oxychloride [$(ClCu_2H_3O_3)_2$]	0.15	0.375	12.5
copper oxychloride [$(ClCu_2H_3O_3)_2$]	0.15	0.25	25
copper oxychloride [$(ClCu_2H_3O_3)_2$]	0.15	0.0188	25
Novochizol™ 3%	-	3	0.1
Novochizol™ 1.25%	-	1.25	0.1
H_2O	-	-	-

The minimum concentration required to completely inhibit zoospore germination and/or activity ranged from 6.25 to 25 µg ml^{-1} for the copper-containing products, including the positive Kocide® Opti controls (see Tab. 5). There were no differences in germination inhibition of copper hydroxide [H_2O_2Cu], regardless of the Novochizol™ added. For copper oxychloride [$(ClCu_2H_3O_3)_2$], at the highest concentration of Novochizol™ added resulted in the lowest inhibitory concentration of 6.25 µg ml^{-1}. In contrast, copper oxychloride [$(ClCu_2H_3O_3)_2$] with the lowest concentration of Novochizol™ added resulted in a lower MIC_{100} of 25 µg ml^{-1}. Novochizol at 3% respectively at 1.25% inhibited at 0.1 µg ml^{-1}. The blank formulation H_2O did not inhibit *P. viticola* in vitro. Due to the lack of repetitions, no statistical analyses were carried out.

Consider the relatively balanced picture related to the results of the combination of Novochizol™ and copper hydroxide [H_2O_2Cu], copper seemed to be the determining factor for the direct anti-oomycotic effect. Changing the Novochizol™ concentration added did not change its behaviour in terms of inhabitation of zoospore germination and/or activity. Slightly greater inhibition was observed with the highest Novochizol™-copper oxychloride [$(ClCu_2H_3O_3)_2$] combination than with the positive Kocide® Opti controls. In contrast, at the two lowest Novochizol™ concentrations in combination with copper oxychloride [$(ClCu_2H_3O_3)_2$], inhibition was lower. This may indicate dose-dependent interactions between copper oxychloride [$(ClCu_2H_3O_3)_2$] and Novochizol™.

Novochizol™ at high concentrations (10 to 20 times higher than the concentration used in the seedling and field experiments) showed very high inhibitory activity at low concentrations, which did not differ between both evaluated concentration of 3% and 1.25% respectively. In the absence of a lower concentration as a control, this led to the conclusion that Novochizol™ alone can act at high concentrations in vitro better than copper at normal doses (or in its absence). Whether this is also the case at low concentrations, the experiment should be repeated with the Novochizol™ exact controls (0.75 - 0.0188%). The use of such a high concentration of chitosan or Novochizol™ in vitro and in field is unusual. Firstly, in terms of handling and costs: at nine treatments in 2022 - a low number of treatments by Swiss standards - it would require 174.98 kg ha^{-1} according to personal calculations when a concentration of 3% of Novochizol™ is aimed. Secondly because the risk of causing phytotoxicity is very high, which has been shown in other experiment i.e. on oil palm seedlings (Maluin et al. 2020) and potatoes (Elsahhar et al. 2022).
The highest concentration used within the plant pathogen assays on grapevines did not exceed 0.7% of Novochizol™ with having no phytotoxicity and a low efficacy in terms of disease severity (14.5% in Exp. 2). Only the combination of copper sulphate [$CuSO_4$] at 0.75 mg ml^{-1} and Novochizol™ at 0.3% showed slight symptoms of leaf necrosis in the plant pathogen bioassays and in the field assay (see Fig. 21).

Given the objective to assess Novochizol™'s direct anti-oomycotic effect, one may infer that in the absence of plant pathogen interactions, efficacy increases with dosage. In view of the proven anti-oomycotic effect of chitosan (Huang et al. 2021), this property can be transferred to Novochizol™. There is no information in the literature on the specific in vitro activity of chitosan or Novochizol™ against *P. viticola* spores tested using the 'Minimum Inhibitory Concentration (MIC_{100})'.
Further, it proves challenging to compare the in vitro bioassay results of Novochizol™-copper combinations to in planta outcomes. This due to the interplay of plant and disease metabolism. Numerous pathways, influenced directly or indirectly via secondary metabolites, contribute to this complex interplay between triggered plant defence of grapevine plants and *P. viticola* (Jacquens et al. 2022). Additionally, the limited repetition of the test series, making the interpretation of results uncertain and drawing conclusions difficult.
But evidence for a linear dose dependence - manifested only in the combination of Novochizol™ and copper oxychloride [$(ClCu_2H_3O_3)_2$] - is absent in any other results of the experiments performed in planta on grapevine seedlings. Nor, that the higher the dosage of Novochizol™ alone (up to 0.7%), the higher its effect. Despite inconsistent results regarding the efficiency against downy mildew infection, the opposite can be seen: there is a non-linear relationship between the concentration of Novochizol™ (alone or when its added to any copper salt) and its effect. This is also reflected in literature dealing with chitosan. Where for example, in the context of enhancing artemisinin production, higher doses did not necessarily result in higher effectiveness; instead, there was a non-linear response, where certain low doses maintained similar levels of effectiveness as higher doses (García-García et al. 2023). Both, the inconsistent results regarding the efficiency against downy mildew infection of Novochizol ™ and its combination with copper and the non-linear nature of it's in planta efficiency will be discussed in the next chapters.

4.2 Plant-pathogen bioassays: Efficacy of Novochizol™-copper against downy mildew infestation

4.2.1 General observations of disease pressure and infection pattern

4.2.1.1 Disease pressure and related SD

The efficacy of Novochizol™ and its combination with copper was measured in ten independent experiments (Exp. 1 - Exp. 10) over two years in the same facilities at FiBL in Frick under the same semi-controlled conditions. Mean disease severity in control plants, treated with H_2O and infested with *P. viticola* ranged from 60.0±17.18% (Exp. 9) to 94.3±2.13% (Exp. 3).

Table 6: Experimental code, date (of inoculation), number of treatments, mean severity and SD of H_2O controls of all plant pathogen experiments.

Exp	Date (Inoculation)	Nr. of treatments	Mean severitiy of H_2O controls (%)	SD (%)
1	10.03.2022	24	86.57	±11.82
2	31.03.2022	24	85.21	±9.84
3	21.04.2022	25	94.33	±2.13
4	28.04.2022	26	83.54	±10.68
5	05.05.2022	25	91.58	±11.23
6	16.02.2023	25	63.13	±13.55
7	07.03.2023	27	90.01	±12.40
8	16.03.2023	26	66.18	±31.06
9	30.03.2023	24	60.00	±17.18
10	20.04.2023	24	79.82	±20.50

In the comparison of mean disease severity in control plants per experimental round using ANOVA, a p-value of 0.0856 was obtained. It can be stated - with low evidence - that there was no difference of disease pressure between the experiments. Comparing the mean disease severity in control plants and its mean standard deviations (see Tab. 6) a slight but significant negative correlation according to Pearson (p-value 0.04168) was given. The correlation coefficient was -0.65.

The interpretation of the results suggests that the standard deviation decreased with increasing disease pressure and vice versa. Even if the existing literature does not mention a relationship between the severity and its standard deviation, such a relationship can be assumed. This was also confirmed by the experts at FiBL (Schärer 2023, personal communication). According to FiBL, the exclusion of the two plants with the highest and lowest infestation rates were in this case been discussed as a valid way to reduce the standard deviation.

Since there was no proofed (significant) difference in mean severity between the ten experiments, no results were excluded from the outset systematically. The purpose of reporting the disease severity and standard deviation of the corresponding experiment was to avoid potential bias. Where it deemed useful (e.g. Fig. 23 and 24), the highest and lowest infected plants were excluded and accordingly described.

4.2.1.2 Correlation between the order of inoculation and disease severity in Exp. 10

To get to the reason for the high standard deviations, the order of inoculation was reported in Exp. 10. Examination of the data from Exp. 10 identified a slight correlation of disease severity and the sequence of inoculation. It revealed additionally a significant difference of the mean severity between the plants inoculated in the 9th and 10th position respectively and the 18th position.

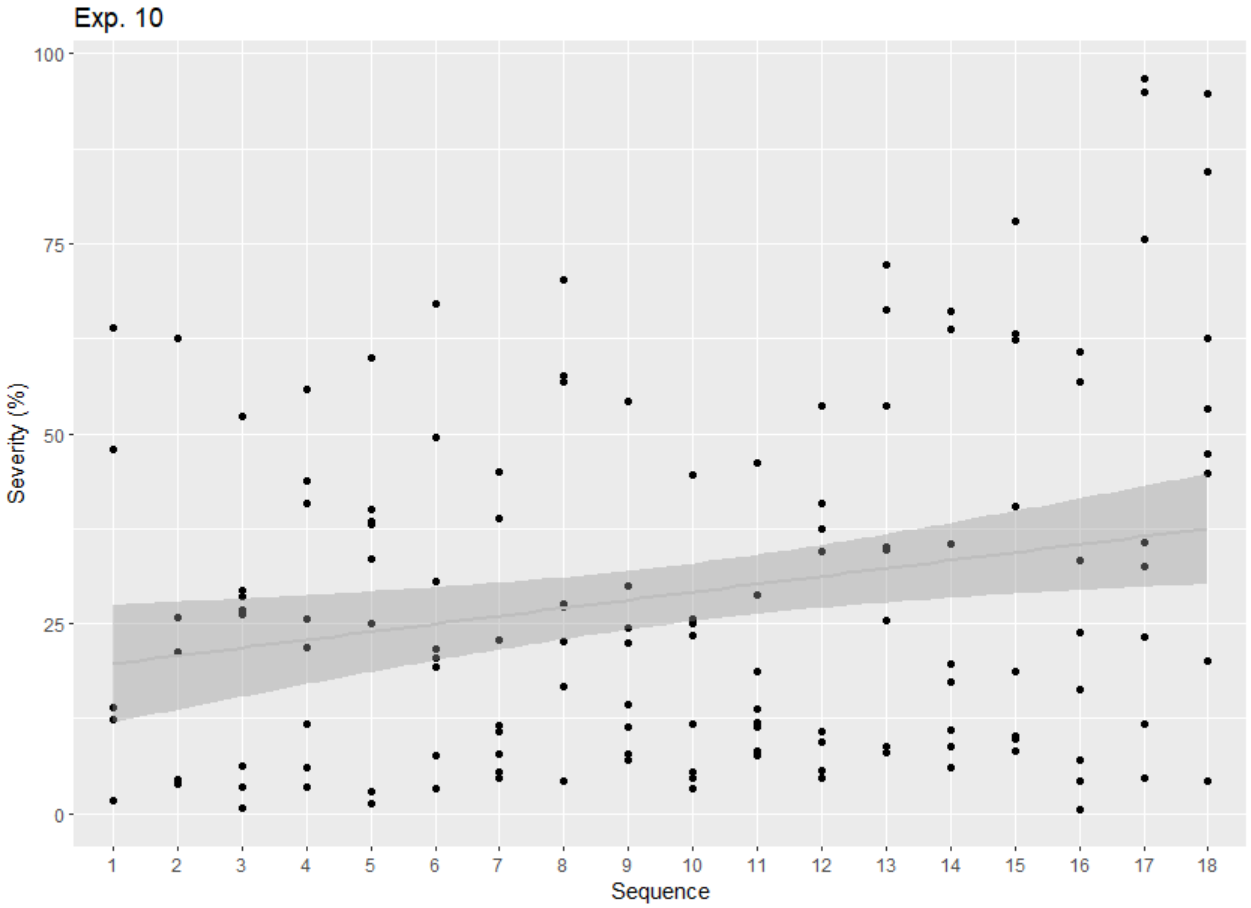


Figure 18: Grapevine downy mildew disease severity and treatment sequence of grapevine seedlings by plant (excluding non-inoculated plants) tested within Exp. 10. Mean disease severity of non-treated controls was 79.82±20.50 (mean±SD). The figure shows mean, standard deviations per plant (n= 5-8), and a linear fitted regression model (correlation coefficient 0.227).

Examination of the data from Exp. 10, it was identified a correlation (correlation coefficient 0.227) suggesting a positive relationship between the order and its mean disease severity by plant (according to Pearson, p-value 0.007434,). When comparing the other positions via Welch Two Sample t-test, mean severity between the plants in the 10th (p-value 0.01779) and 9th (p-value 0.03083) position did significantly differ from the 18th position. When comparing the other positions, no significant differences were observed. Plants in the 10th position exhibited, on average, the lowest severity level (17.98%), whereas the plants in in the 18th position displayed the highest severity level (51.4%). According to the analysis, the order of the sets itself did not have any effect on the severity (t-test, p-value > 0.05). For no other experiment, a similar analysis was conducted.

Although the plants are randomly inoculated in pairs (manually picked) to prevent potential bias in the results due to the order of inoculation, the error must be found there. Based on the given results, it is unlikely that any previous steps in cultivation, storage, and treatment have influenced the results. The correlation between the increasing order (1-18) and the mean disease severity of the plants can be explained as follows: the inoculum present on the plants in earlier positions has had time to dry out during the timespan in which the remaining plants were inoculated. Considering that inoculation took a maximum of 45 minutes for 24 treatments of 6 plants each (144 plants in total) and the plants were

arranged in sets of 18 sets, it took about 5 minutes to inoculate and place a set of 18 plants in the humidity chamber, which was located 10-15 m away. 5 minutes are expected to be sufficient to diminish the activity of the inoculated spores.
Other errors in the inoculation step can be ruled out, such as irregular or insufficient inoculum pattern on leaves, spores settling down in the pistol, spores dying in the reserve inoculum on the magnetic stirrer, or variations in conditions within the humidity chamber. Because the sufficient inoculum drop layer is relatively easy to visually maintain, and the pistol is re-loaded maximum 3-4 times during the inoculation process, what wouldn't lead to such a distribution (compare Fig. 18). To exclude spores dying in the reserve inoculum on the magnetic stirrer, the spore density was re-measured several times after inoculation for fitting the density at the start. Problems in the humidity chamber would have led to set dependent severity distribution. The relatively high standard deviation (and low disease pressure) could also be accounted for within this explanation, which was explained in the previous chapter (4.2.1.1 about 'Disease pressure at related SD').
This does not explain why exactly the plants inoculated at the 9th and 10th position of the 18 plants showed the lowest average infestation. No explanation was found for this.

4.2.2 Efficacy of Novochizol™ alone

In Exp. 1-4, the dose-response of different Novochizol™ concentration were tested, where systematic testing was lacking (see Fig. 19, showing a selection of the data from Exp. 1-4). Novochizol™ concentrations of 0.125 - 0.175% expressed the highest effect. Higher the concentration to 0.3% Novochizol™ (7.4% in Exp. 2) and 0.7% of Novochizol™ (14.52% in Exp. 2) did not increase the efficacy.

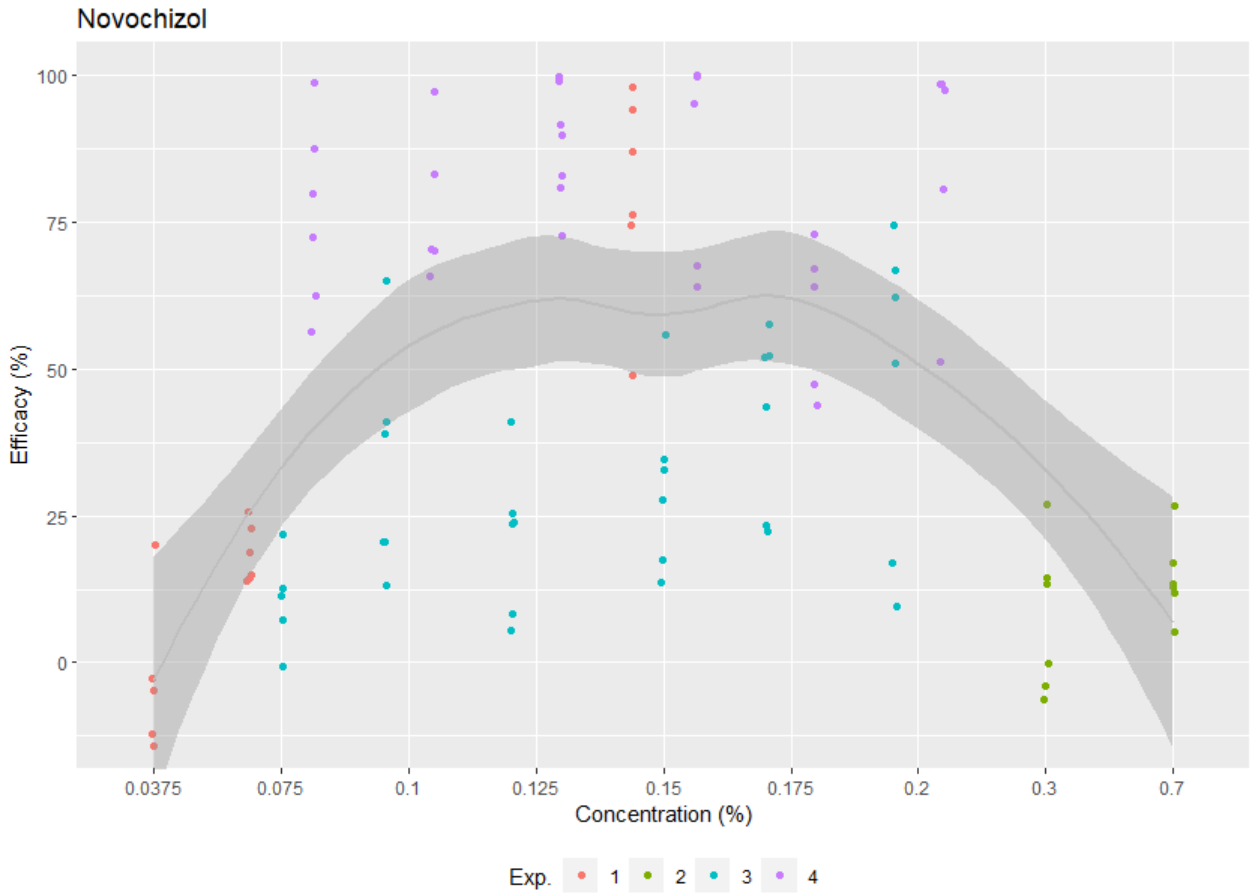


Figure 19: Dose-response curves of Novochizol™ tested in Exp. 1-4 with concentrations of 0.0375-0.7%. Mean disease severity of non-treated controls was for Exp. 1 86.57±11.82%, Exp. 2 85.21±9.84%, Exp. 3 94.33±2.13% and Exp. 4 83.54±10.68 (mean±SD). The figure shows mean per plant (n=6) within a locally weighted least squares regression model.

Exp. 1 included Novochizol™ concentration at 0.0375%, 0.075% and 0.15%, with the latest concentration performing best with 79.64% efficacy. Exp. 2 included the highest Novochizol™ concentration tested:

0.3% Novochizol™ showed efficacy of 7.4% and 0.7% of Novochizol™ of 14.52%, respectively. Exp. 3 and 4 were replicates. Thereby, the infestation of the control plants in Exp. 3 was the highest with 94.33±2.13% and the Kocide® Opti reference at 0.3 mg ml^{-1} copper metal performed worse than usual with an efficiency of 58.19% (data not shown). In Exp. 4, the protection of the Kocide® Opti reference at 0.3 mg ml^{-1} copper metal was with 100% complete (data not shown) and the Novochizol™ treatments were on average higher (except for the Novochizol™ concentration of 0.175%) than in Exp. 3. In Exp. 3, the Novochizol™ concentrations of 0.175% and 0.2% performed best with efficiencies of 41.84% and 46.79% respectively. A further reduction of the Novochizol™ concentration below 0.1% and 0.075% respectively resulted in a loss of protection again, not significantly different from the untreated controls (t-test, p-value 0.89). Very small Novochizol™ concentrations (0.0375 with an efficiency of -5.89% in Exp. 1) did not provide protection. The same was shown by testing Novochizol™ concentration of 0.0188% in Exp. 9 (data shown in chapter 4.2.4). The protection reached there was -15.97% with a severity of the control plants of 60.31±7.32% after adjustment of the data (n=4).

Data are limited, but in the concentration range of Novochizol™ tested, dose and efficacy did not follow a linear relationship. Rather, the maximum effect in this tested range peaked at 0.125-0.175%. Higher the concentration to 0.3% Novochizol™ and 0.7% of Novochizol™ respectively did not increase the efficacy. Decreasing the concentration, on the other hand, resulted in an increase in efficacy. As Exp. 3 and 4 were replicates, the pattern was only confirmed fully in Exp. 4.
The reasons for this are difficult to compare with the existing literature. Novochizol™ has not been tested in any other study on plant pathogens. To find an explanation, it will be referred to the literature on chitosan - knowing that this may not be accurate.
The studies, in which a non-linear relationship between dose and effect of chitosan has been proven, mostly refer to growth parameters and/or plant hormones. For example, Asgari-Targhi et al. (2018) showed that bulk chitosan (MW of 100 kDa and 85-90% DDA) resulted in cessation of plant growth and development of *Capsicum annuum* when the concentration was increased from 0.02 to 0.1 mg ml^{-1}. The concentrations of elicited compounds involved in plant defence were also differentially related to the amounts of chitosan used, with 0.01 mg ml^{-1} appearing to increase the activity of peroxidase and catalase in leaf and root. When increased to 0.02 mg ml^{-1}, a decrease in activity was observed. For secondary metabolites (proline, soluble phenolics, alkaloid, etc.), both concentrations 0.01 and 0.02 mg ml^{-1} appeared to have a significantly better effect than 0.001 mg ml^{-1}. Suarez-Fernandez et al. (2020) tested chitosan (MW of 70 kDa and 80.5% DDA) for plant hormones and defences in tomato root exudates. The amount of salicylic acid, jasmonic acid and abscisic acid produced by the roots peaked at the treatment with 1 mg ml^{-1} of chitosan. 0.1 and 2 mg ml^{-1} of chitosan had a much lower effect. Considering the peak around 1.25 - 1.75 mg ml^{-1} of the results, the latter case seems more consistent with the values obtained. It can be assumed: the elicitation effect of Novochizol™ is present - although to a lesser extent in *V. vinifera* than other plants due to its high susceptibility - and at the same time, the direct anti-oomycotic effects of Novochizol™ comes into play. It is certain that Novochizol™ has a strong anti-oomycotic effect against *P. viticola* at high concentrations of 1.25 and 3%, which means at 3 and 1.25 mg ml^{-1} respectively. At the same time, - as already discussed - too high concentrations negatively affect plant physiology (e.g. phytotoxicity and plant growth). At best, at low concentrations, Novochizol™ can be digested by the fungus and therefore leading to higher infestations compared to the control (0.0375 and 0.0188%) - which is not supported by any existing literature.

4.2.3 Efficacies depending on copper origin

Copper or the [Cu^{2+}] ions can be delivered in the form of various copper salts and thus formulated with Novochizol™. For this purpose, copper oxide [Cu_2O], copper hydroxide [H_2O_2Cu], oxychloride [$(ClCu_2H_3O_3)_2$] and copper sulphate [$CuSO_4$] were - in presence of more or less Novochizol™ - diluted and tested to find the most valuable candidate in terms of copper origin. Two different test methods were chosen: In chapter 4.2.3.1, the added concentration of 0.3% Novochizol™ was diluted too (single formulation); in chapter 4.2.3.2, on the other hand, the Novochizol™ was kept at constant concentration of 0.15% in a reformulation step (double formulation). For the sake of simplicity, in the result section mostly, only the copper metal is referred to.

Table 7: Copper-Novochizol™ combination tested within Exp. 2

Chapter	Treatment	Copper [mg ml^{-1}]			Novochizol™ [%]		
		1	2	3	1	2	3
4.2.3.1	Copper oxide [Cu_2O]	0.15	0.075	0.03	0.3	0.15	0.06
	Copper hydroxide [H_2O_2Cu]	0.15	0.075	0.03	0.3	0.15	0.06
	Copper oxychloride [$(ClCu_2H_3O_3)_2$]	0.15	0.075	0.03	0.3	0.15	0.06
	Coppers sulphate [$CuSO_4$]	0.15	0.075	0.03	0.3	0.15	0.06
4.2.3.2	Copper hydroxide [H_2O_2Cu]	0.15	0.075	0.03		0.15	
	Coppers sulphate [$CuSO_4$]	0.15	0.075	0.03		0.15	

At the end of the present chapter, the results of both - single and double formulation will be discussed.

4.2.3.1 Efficacies of single formulation with 0.15 mg ml^{-1} copper - 0.3% Novochizol™ - dilutions

In Exp. 2, the dose response of four copper salts were tested at 0.15 mg ml^{-1} copper metal in combination with 0.3% Novochizol™ (see Fig. 20), whereby both have been diluted (2-, 4- and 10-fold in relation to the Kocide® Opti reference at 0.3 mg ml^{-1} copper metal). Except for copper oxide [Cu_2O], all copper salts performed very well in combination with simply formulated Novochizol™ at an initial concentration of 0.3%. The combination of copper oxychloride [$(ClCu_2H_3O_3)_2$] and copper sulphate [$CuSO_4$] with Novochizol™ performed almost similar to the positive Kocide® Opti reference at 0.3 mg ml^{-1} copper metal, even at a fourfold dilution (corresponds to 0.075 mg ml^{-1} copper metal and 0.15% Novochizol™), while only copper oxychloride [$(ClCu_2H_3O_3)_2$] performed significantly the same.

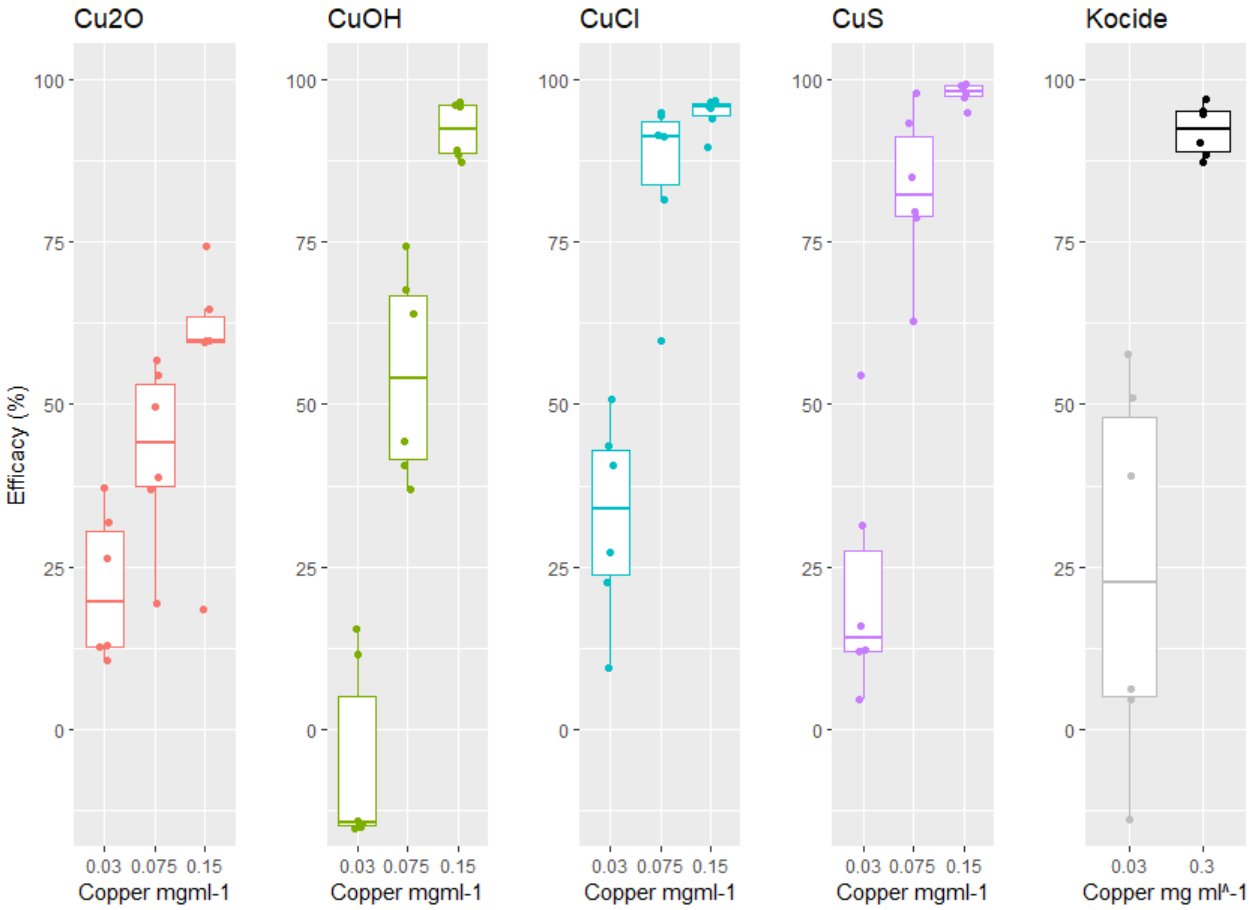


Figure 20: Dose-response curves of single formulations of copper oxide [Cu_2O] (Cu2O), copper hydroxide [H_2O_2Cu] (CuOH), copper oxychloride [$(ClCu_2H_3O_3)_2$] (CuCl) and coppers sulphate [$CuSO_4$] (CuS) with an initial concentration of 0.3% of Novochizol tested on grapevine seedlings against *P. viticola*. Compounds were tested within Exp. 2. with two concentrations of a copper reference (Kocide® Opti) at 0.3 and 0.03 mg ml^{-1} included. Mean disease severity of non-treated controls was 85.21±9.84% (mean±SD). The figure shows mean, standard deviations of the treatment and mean per plant (n= 6).

Efficiencies of Novochizol™ at 0.3% in combination with copper oxide [Cu_2O], copper hydroxide [H_2O_2Cu], oxychloride [$(ClCu_2H_3O_3)_2$] and coppers sulphate [$CuSO_4$] were diluted and tested, where Fig. 20, shows only a selection of the data from Exp. 2. At the copper dose of 0.15 mg ml^{-1} - which is halfening the copper reference (Kocide® Opti 0.3 mg ml^{-1}) - there was no significant difference to the positive copper control apart from copper oxide [Cu_2O] (ANOVA, p-value 1.0). At a 4-fold dilution of the copper reference dose, both copper oxychloride [$(ClCu_2H_3O_3)_2$] (85.57%) and copper sulphate [$CuSO_4$] (82.92%) remained effective, with copper oxychloride [$(ClCu_2H_3O_3)_2$] didn't perform significantly different comparing to the copper reference (Kocide® Opti 30 mg ml^{-1}) (ANOVA, p-value 0.89). Copper oxide [Cu_2O] performed in single formulation with 0.3% of Novochizol™ only with an effectiveness of 56.14% at a copper dose of 0.15 mg ml^{-1} and was also less effective than the other copper salts in the 4- and

10-fold diluted formulations. Only copper hydroxide [H_2O_2Cu] at 10-fold dilution formulation performed even worse. Being with -12.32% even significantly worse than the untreated control (ANOVA, p-value 0.0086).

According to the results of Exp. 2 copper sulphate [$CuSO_4$] and copper oxychloride [$(ClCu_2H_3O_3)_2$] performed better. The option of copper hydroxide [H_2O_2Cu] as the third most effective formulation remained viable, with an efficacy of 92.27% at 0.15 mg ml^{-1} copper dose and single formulated with Novochizol™. The fact that copper oxychloride [$(ClCu_2H_3O_3)_2$] tends being more effective than copper hydroxide [H_2O_2Cu] was shown in experiments conducted later (chapter 4.2.4 'Efficacies depending on Novochizol™ concentration'), whereby a formulation with both copper oxychloride [$(ClCu_2H_3O_3)_2$] and copper hydroxide [H_2O_2Cu] was performing weaker (data not shown). No further experiments were done with copper oxide [Cu_2O] due to its weak performance. The two highest dosed copper sulphate [$CuSO_4$] Novochizol™ combinations in Exp. 2 caused light symptoms of phytotoxicity (Fig. 21A). The same applied to the field assay (see Fig. 21B). This was the reasons why copper sulphate [$CuSO_4$] was subsequently excluded from the further tests.

Figure 21: Slight leaf necrosis (red circles) in Exp. 2 of the highest dosed copper sulphate [$CuSO_4$] at 0.15 mg ml^{-1} copper metal and 0.3% Novochizol™ formulation in (A) and the same in the field assay (B).

4.2.3.2 Efficacies of double formulation with 15 mg ml^{-1} copper dilutions with 0.15% Novochizol™

Exp. 2 tested among the dose response of two copper salts in combination while keeping a concentration of 0.15 % Novochizol™ constant (double formulation). Copper sulphate [$CuSO_4$] proved to be more effective than copper hydroxide [H_2O_2Cu] (see Fig. 22, showing only a selection of the data from Exp. 2). Copper sulphate [$CuSO_4$] still retains 77.69% efficacy at 0.03 mg ml^{-1} - which corresponds to a 10-fold dilution of the copper reference dose of 0.3 mg ml^{-1} (Kocide® Opti).

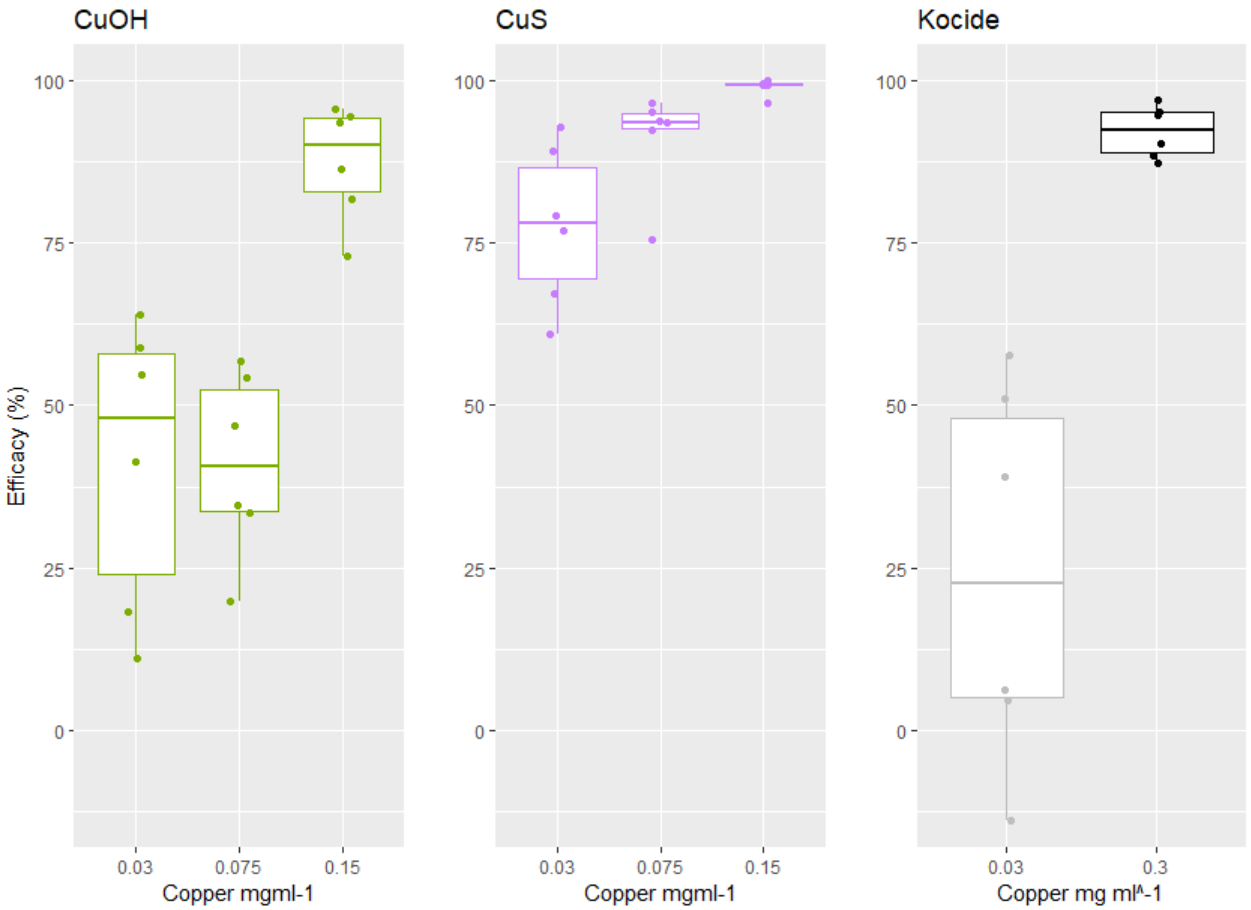


Figure 22: Dose-response curves of double formulations of copper oxide [Cu_2O] (Cu2O) and copper sulphate [$CuSO_4$] (CuS) with 0.15% of Novochizol™ tested on grapevine seedlings against P.viticola. Compounds were tested within Exp. 2. with two concentrations of a copper reference (Kocide® Opti) at 30 and 3 mg ml^{-1} included. Mean disease severity of non-treated controls was 85.21±9.84% (mean±SD). The figure shows mean, standard deviations of the treatment and mean per plant (n= 6).

According to the results of Exp. 2, copper hydroxide [H_2O_2Cu] showed an efficiency of 87.48% at 0.15 mg ml^{-1} copper metal. When copper metal within the copper hydroxide [H_2O_2Cu]-Novochizol™ combination was reduced to 0.075 mg ml^{-1} or 0.03 mg ml^{-1}, the efficiency dropped to below 50% of efficacy, whereby - although considerably more diluted - the efficiency could be kept constant at 0.03 mg ml^{-1} copper metal. Copper sulphate [$CuSO_4$] with 0.15 mg ml^{-1} and 0.075 mg ml^{-1} performed significantly the same (ANOVA, p-value 1) as the copper reference (Kocide® Opti with 0.3 mg ml^{-1} copper metal). Copper sulphate [$CuSO_4$] still retains a 77.69% efficacy at 0.03 mg ml^{-1}.

4.2.3.3 Interpretation and Discussion: Efficacies depending on copper origin

The results concerning the efficiency behaviour of Novochizol™ alone were discussed previously: in Exp. 1 it was seen that 0.15% Novochizol™ elicits protection to a much higher degree (79.64%) than 0.3% Novochizol™ (7.4%). It can therefore be assumed that the efficacy observed in the single formulations was mainly due to the active ingredient copper. The various properties of copper salts, which are also used in commercial formulations, seemed to apply. Copper sulphate [$CuSO_4$] is highly soluble in water, forming a blue solution due to its high solubility and the release of copper ions. This solubility allows for immediate and strong effects. In contrast, copper oxychloride [$(ClCu_2H_3O_3)_2$], copper hydroxide [H_2O_2Cu], and copper oxide [Cu_2O] are considered more stable compounds, with decreasing solubility (Kurnik et al. 2012). This decreased solubility might result in a slower release of copper ions, leading to a less immediate and intense effect compared to copper sulphate [$CuSO_4$]. Also, it is assumed that with copper oxide [Cu_2O] there should no chemical reaction with Novochizol™ happen (unlike the other copper compounds). The work of Kurnik et al. (2012) indicates that the various copper salts largely retain their properties even in formulations. In case of the single formulation with 0.3% Novochizol™ the pattern of the efficiencies of the tested copper-Novochizol™ combination was given, except for copper oxychloride [$(ClCu_2H_3O_3)_2$], where unexpected high efficacies were obtained at 4- (85.57%) and even 10-fold (32.37%) copper concentrations. The same accounts for the efficacies obtained at 4-fold dilution of copper sulphate [$CuSO_4$] (82.92%). An additional effect of Novochizol™ can be assumed in these three cases, whereby this mechanism is known and discussed in connection with copper-chitosan formulation (Saharan et al. 2015; Hashim et al. 2019; Lemke et al. 2022). For the exact mechanism, Lemke et al. (2022) propose: chitosan interacts with the fungal cell wall, disrupting enzymes and nucleic acids and makes therefore copper ions get into the cell easier.

When analysing the results of the double formulations, an irregularity becomes more apparent compared to when using copper alone as active ingredient. Since Novochizol™ alone elicited for 79.64% effectiveness (in Exp. 1) the effectiveness of 91.20% in the 4-fold dilution of copper sulphate [$CuSO_4$] double formulated with 0.15% Novochizol™ can be best explained by both activities of copper and Novochizol™. Because the results for copper hydroxide [H_2O_2Cu] are different to what was obtained with copper sulphate [$CuSO_4$] at its single formulation and the emergence of a plateau between 0.075 and 0.03 mg ml^{-1} of copper metal suggests that perhaps the copper ions are released slowly, i.e. that the double formulation may work well for sustained release. This is to be contrasted with copper sulphate [$CuSO_4$] single formulations, which may be more rapid release. Which would be consistent with the fact that copper sulphate [$CuSO_4$] is soluble and copper hydroxide [H_2O_2Cu] is not. Also, it was seen: the release properties in combination with other nanomaterials possible to be combined with copper indicate different release properties depending on which copper salt was originally used (Jose et al. 2020) - with those obtained from a copper sulphate [$CuSO_4$] precursor, shows the lowest stability.

The finding supports further the idea that the higher efficacies which were seen for copper sulphate [$CuSO_4$] double formulations could be due not to Novochizol™ elicitor effects but to the formulation itself, i.e. dose reduction through an additive or even synergistic effect. The results were discussed in personal communication with Loroch V., from Novochizol SA (2023).

4.2.4 Efficacies depending on Novochizol™ concentration

In comparison to the previous experiments, Exp. 7 and Exp. 8 were used to evaluate the effect of the added Novochizol™ concentration. Copper was therefore kept constant at 0.075 mg ml^{-1} and 0.15 mg ml^{-1} respectively, once with copper oxychloride [$(ClCu_2H_3O_3)_2$] and once with copper hydroxide [H_2O_2Cu]. All combinations showed the same - wave-like - pattern, depending on the Novochizol™ concentration. This was more pronounced at 0.075 mg ml^{-1} than at 0.15 mg ml^{-1} of copper metal, irrespective of the copper origin.

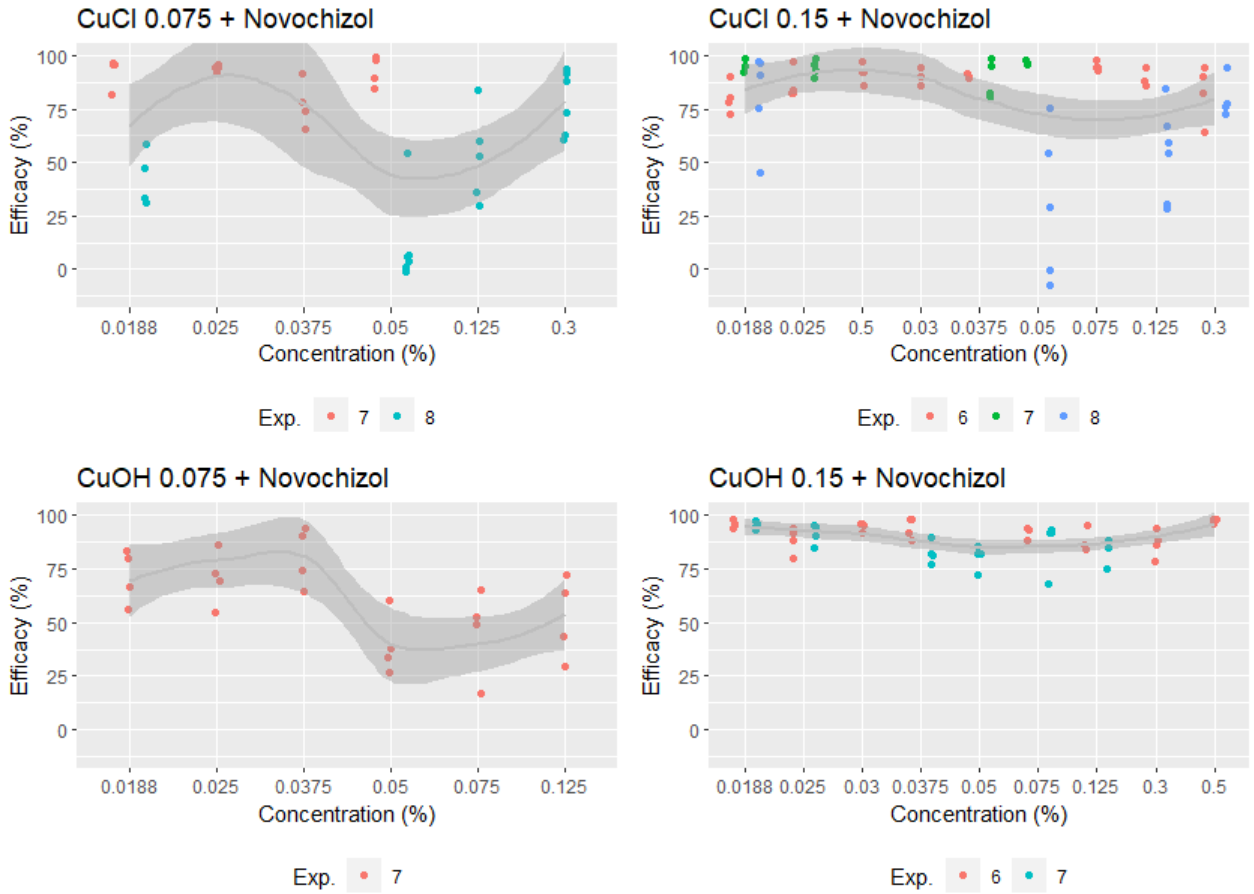


Figure 23: Dose-response curves of Novochizol™ in combination of copper hydroxide [H_2O_2Cu] (CuOH) and copper oxychloride [$(ClCu_2H_3O_3)_2$] (CuCl) at eighter 0.075 or 015 mg ml^{-1} in Exp. 6-8 with Novochizol™ concentrations of 0.0188-0.0.5%. Mean disease severity of non-treated controls after cleaning the data was for Exp. 6 62.92±7.11%, Exp. 7 92.33±9.60% and Exp. 8 71.78±21.94% and Exp. 4 83.54±10.68 (mean±SD). The figures show mean per plant (n= 3-4) within a locally weighted least squares regression model.

At the lower copper dose of 0.75 mg ml^{-1}, a peak in terms of its efficacy can be surmised for copper hydroxide [H_2O_2Cu] with 0.125% of Novochizol™ and for copper oxychloride [$(ClCu_2H_3O_3)_2$] with 0.3% of Novochizol™ added. Whereby, these were the highest Novochizol™ concentration tested. For both combinations, the lowest efficacy can be seen at 0.05% of Novochizol™. Where only copper oxychloride [$(ClCu_2H_3O_3)_2$] at 0.75 mg ml^{-1} combined with 0.3% of Novochizol™ (78.18%) performed significantly better (t-test, p-value 0.04772) than the same combination with 0.05% of Novochizol™ (11.77%) in Exp. 8. The results for copper oxychloride [$(ClCu_2H_3O_3)_2$] at 0.75 mg ml^{-1} copper metal in combination with 0.05% of Novochizol™ were different in Exp. 7: a non-significantly (t-test, p-value 0.05061) increase from 77.46% of efficacy to 92.74% of efficacy when the Novochizol™ concentration increase from 0.0375% to 0.05%.

A second peak was again reached at a copper dose of 0.75 mg ml^{-1} at 0.025% of Novochizol™ combined with copper oxychloride [$(ClCu_2H_3O_3)_2$] and 0.0375% of Novochizol™ combined with copper hydroxide [H_2O_2Cu] respectively. A further decrease in efficacy at Novochizol™ concentration of 0.0188% happened for both copper oxychloride [$(ClCu_2H_3O_3)_2$] and copper hydroxide [H_2O_2Cu] at 0.75 mg ml^{-1}.

At higher copper doses of 1.5 mg ml^{-1}, the efficiencies achieved on average were higher. Hereby it should be noted: the disease pressure in Exp. 6 was lower. The mean severity of the control plants in Exp. 6 was the lowest with 62.92±7.11%. Thus, the efficiencies were all not significantly different from 100% efficacy (t-tests, p-value > 0.05). In Exp. 8, it was achieved a higher result with 1.5 mg ml^{-1} copper hydroxide [H_2O_2Cu] at a concentration of 0.3% (92.40%) and 0.0188% (67.52%) added Novochizol™ than with 0.05% (31.77%) added Novochizol™. In Exp. 7 both copper oxychloride [$(ClCu_2H_3O_3)_2$] and copper hydroxide [H_2O_2Cu] performed well, with no significant differences in Novochizol™ concentrations added to copper oxychloride (t-tests , p-value > 0.05). Within Exp. 7, the infestation pressure was relatively high, with a mean disease severity of non-treated controls of 92.33±9.60%. For copper hydroxide [H_2O_2Cu], the only difference appeared between 0.05% and 0.0188% added Novochizol™, although this was also non-significant (t-test, p-value 0.01301).

The added Novochizol™ seemed to lead to a bimodal effect in all four different combinations: the efficacy reaches two peaks. The layering of the different experiment - more precisely their results - allows the assumption of such a bimodal effect, as it is manifested in all four different combinations.
The only confirmed evidence of the drop between 'higher' and 'lower' concentrations of Novochizol™ added to copper (hydroxide [H_2O_2Cu] and oxychloride [$(ClCu_2H_3O_3)_2$]) in terms of efficacy is provided by the experiments with 0.75 mg ml^{-1} with Novochizol™ added. The evidence for a decrease in the efficacy at Novochizol™ concentrations of 0.05% (0.0375% in the case of copper oxychloride [$(ClCu_2H_3O_3)_2$] in Exp. 7) at copper doses of 0.75 mg ml^{-1} was more distinct than in the case of 1.5 mg ml^{-1}. Regarding the copper doses used, it is relatively obvious to explain how the weaker performance on average comes about: while a copper dose of 3 mg ml^{-1} - regardless of the copper source - fully protected against downy mildew infestation, the protection becomes weaker at its half dilution (1.5 mg ml^{-1}) or at the latest at its 4-fold dilution (0.75 mg ml^{-1}). An improvement in the formulation by adding Novochizol™ to 1.5 mg ml^{-1} of copper was therefore more effective. And this is what must have happened: by adding Novochizol™ to copper at a concentration either higher or lower than 0.05%, its effect can be increased. In the case of copper hydroxide [H_2O_2Cu] 1.5 mg ml^{-1} in combination with Novochizol™ the variation is very small. Given the fact, this was the case at both high (in Exp. 7) and relatively low (in Exp. 6) disease pressure, bias can be excluded for this reason. The effects are likely to be smaller due to the higher efficacy of the copper itself at a dose of 1.5 mg ml^{-1} compared to 0.75 mg ml^{-1}. Looking at the dose-response curve of Novochizol™ alone, a kind of inverted pattern of efficacy emerged: at eighter high or low concentration Novochizol™ alone lost its efficacy and this was where Novochizol™ in combination with copper had an additional effect. Since nothing can be found in the literature about such - wave-like - pattern when chitosan is combined with copper or any other heavy metal, it can again only be speculated about the reasons for such a behaviour. The answer must be found in the different mode of action of the compound of the copper-Novochizol™ formulations:

1. Direct anti-oomycotic effect of Novochizol™
2. Direct anti-oomycotic effect of copper
3. Indirect anti-oomycotic effect through plant elicitation of Novochizol™
4. Mechanism of copper delivery by Novochizol™

Depending on the concentration of Novochizol™ added, the mode of action of the individual substances - copper and Novochizol™ - may come into play differently.

4.2.5 Additive effect of Novochizol™ in combination with copper

To test whether Novochizol™ has an additional effect when added to copper salts within a simple formulation compared to a commercial copper-containing PPP, Airone - also chosen in the case of 'Festiguet's' vineyard - was included. Although the comparison is difficult, it was shown that the addition of 0.0188% Novochizol™ to 0.15 mg ml^{-1} copper oxychloride [$(ClCu_2H_3O_3)_2$] achieved a significantly better effect (89.63% of efficacy) than Airone at a copper content of 0.15 mg^{-1} (50% of efficacy).

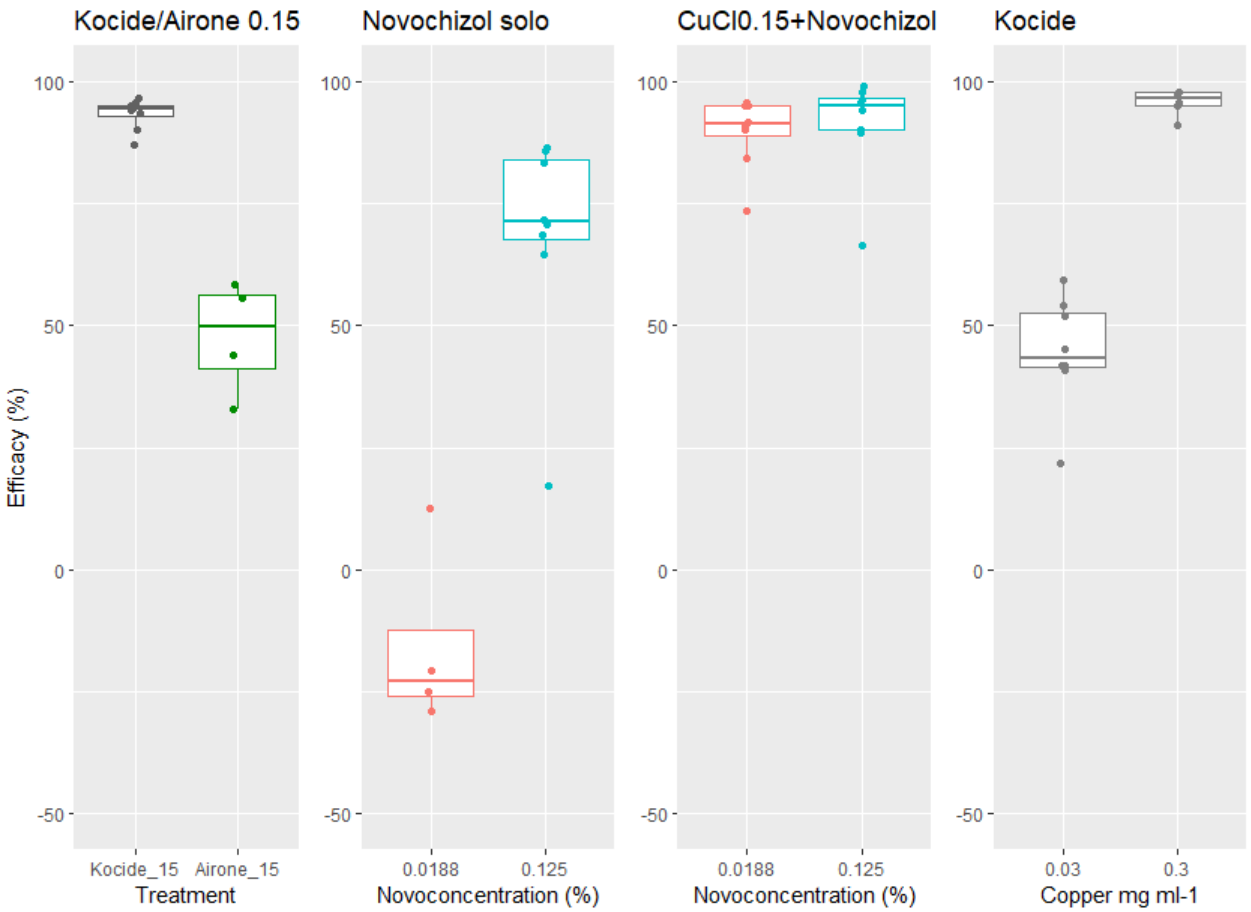


Figure 24: Selected results of Exp. 9-10 with two different commercial copper products - Kocide® Opti and Airone - included at 0.15 mg ml^{-1}, Novochizol™ in low (0.0188%) and high (0.125%) concentration and finally the combined copper and Novochizol™ formulation, with copper oxychloride [$(ClCu_2H_3O_3)_2$] (CuCl) being the copper compound and 0.15 mg ml^{-1} its dosage. Two concentrations of a copper reference (Kocide® Opti) of 0.3 and .03 mg ml^{-1} were included. Mean disease severity of non-treated controls after cleaning the data was for Exp. 9 60.00±17.18% and for Exp. 10 79.82±20 (mean±SD). The figure shows mean, standard deviations of the treatment and mean per plant (n=8). Novochizol™ at 0.0188% and Airone were included only in one of the experiments (n= 4).

The efficacy of Novochizol™ in combination with copper oxychloride [$(ClCu_2H_3O_3)_2$] against downy mildew was very high in both independent experiments, averaging 89.63% and 91.23% disease control in Exp. 9 and 10 at Novochizol™ concentrations of 0.125 and 0.0188%, respectively. This at lower disease pressure in Exp. 9 than in Exp. 10 (60.00 and 79.82% disease severity in control plants). The two concentrations of Novochizol™ used as controls behaved as expected, with efficacy higher at a concentration of 0.125% (68.63%) than at a concentration of 0.0188% (tested only in Exp. 9), where efficacy was at -15.63% weaker than the untreated control. Considering the copper controls represented by Kocide® Opti - which is also always included with 0.3 and 0.03 mg ml^{-1} as references - and Airone, the results differ strongly, although they contain the same amount of copper (15 mg ml^{-1}). Kocide® Opti contains 30% copper hydroxide [H_2O_2Cu] where Airone contains 14% copper oxychloride [$(ClCu_2H_3O_3)_2$] and 14% copper hydroxide [H_2O_2Cu]. The poor performance of Airone of around 50% efficacy was confirmed by the fact that in Exp. 10, where Airone was included, pre- and rain reatment were also carried out. There

the results between Kocide® Opti and Airone were different from a minimum of 31.48% to a maximum of 53% in terms of efficiency (data not shown). Also compared to Novochizol™ at 0.0188% in combination with copper oxychloride [H_2O_2Cu] at 0.15 mg ml^{-1} copper metal, Airone performed consistently weaker in the pre- and rain-treated tests, minimal efficiency from 16.9% up to 37.16%, again with copper dose being the same.

Halving the reference dose of 0.3 mg ml^{-1} while maintaining full protection, is what was targeted - along with other goals - with respect to the field assay. Exp. 9 and 10 reflected the final treatment plan for 2023. Kocide® Opti has again be proven very effective in all trials, and the results confirmed that it is the commercial product offering the highest protection. FiBL, which has carried out systematic screening on seedling of commercial products in previous trials, confirmed this. In contrast, Airone, the commercial product also chosen in the case of the winery's plant protection strategy against grapevine downy mildew, performed significantly worse in FiBL's systematic screening, although field screening in Frick showed no such difference (Schärer 2023, personal communication).
In terms to the field conditions, copper doses were adapted to the actual disease pressure and growth stages, means that a range of 0.144-0.288 mg ml^{-1} copper metal was applied per treatment. At the highest dose, this corresponded to the copper metal dose applied to the seedling. Airone was used as reference for the field treatments. Although a direct comparison with field conditions seems difficult, and the differences in the semi-controlled environment lack to reflect them accurately (see differences between Kocide® Opti and Airone), some synergy can be suspected that the combination of Novochizol™ and copper oxychloride [$(ClCu_2H_3O_3)_2$] provides. This was evident, especially when the reference dose was halved from 30 mg ml^{-1} to 15 mg ml^{-1}. The formulation which was proposed together with 0.0188% Novochizol™, had not gone through any formulation optimization process, seems to outperform Airone in all modes of testing. The fact that both work with the same amount of copper leads to ask the question about synergies. Levy et al. (1986) suggest the formula by Abott (1952) for testing synergy:

$$E_{(exp)} = a + b - \left(\frac{a+b}{100}\right)$$

in which $E_{(exp)}$ is the expected control efficacy of a mixture, and a and b represent the proportion of the efficacy in percent fungicides A and B, respectively. The ratio (SF, synergy factor) between the observed experimental efficacy of the mixture $E_{(obs)}$ and the expected efficacy of the mixture is computed:

$$SF = \frac{E_{(obs)}}{E_{(exp)}}.$$

A ratio $E_{(exp)}$ / $E_{(obs)}$ greater or smaller than 1 would indicate a deviation from the hypothesis of independent action, which means that there is biological interaction between the fungicides. If SF > 1, there is synergism; if SF < 1, there is antagonism. There could therefore be a synergistic effect compared to Airone. This is by no means a valid method for calculating a possible interaction. Because Airone should be combined with Novochizol™ in the case of synergy evaluation. This was not done as this was neither the approach of the thesis nor the supplier was informed. Testing copper oxychloride [$(ClCu_2H_3O_3)_2$] as an isolated control was not possible due to the lack of solubility.
This interaction - whether synergistic or simply additive in character - is more pronounced at 0.0188% than at 0.125% Novochizol™ and 0.15 mg ml^{-1} copper oxychloride [$(ClCu_2H_3O_3)_2$]. The reasons for this have already been discussed in the previous subsection. However, in terms of practical application, this seems to be a very interesting approach. Calculations thus show that better protection can be achieved with around 1 kg Novochizol™ per ha y^{-1} in combination with copper oxychloride [$(ClCu_2H_3O_3)_2$] than with Airone and this with a halving of the copper metal dose compared to the use of Airone.
The other goals, such as longer-lasting protection and rainfastness due to Novochizol™, could not be demonstrated by the plant pathogen bioassay in any of the trials. It can also be assumed that there is still room for optimisation in this respect with an improved formulation.

4.3 Field assay

4.3.1 Disease levels

In 2022, a disease severity level of 0% resulted on leaves and grapes during the sampling period from May to the end of July. A single disease sampling was carried out on 3rd August 2022.

In 2023, primary infection with *P. viticola* occurred due to a wet and cold late spring, with the last intensive rain period in mid to late May - coinciding with the start of the growing season. The subsequent spread of the disease proved to be more site-dependent rather than treatment-dependent (see Fig. 25). Block 1 was the most severely affected, with an average disease level of 5.37%.

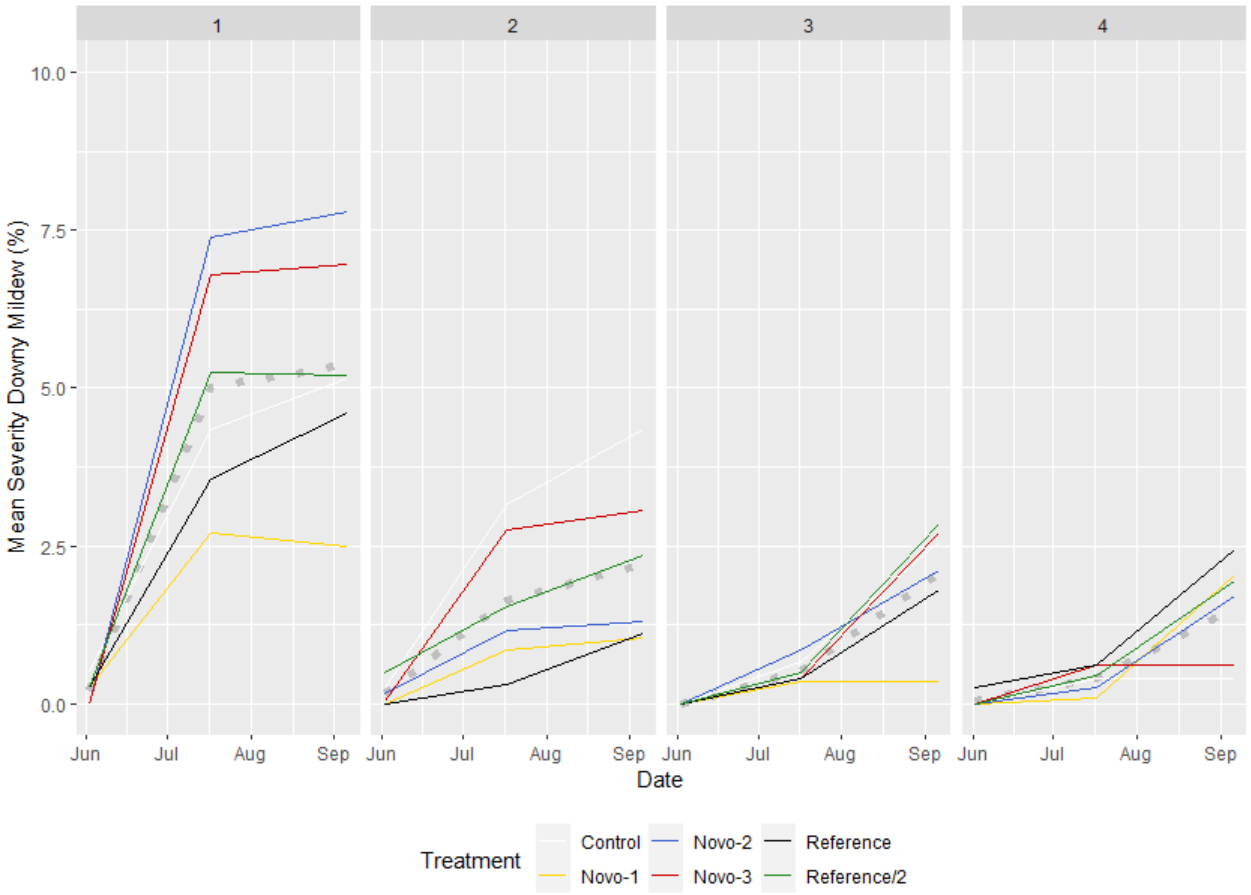


Figure 25: Mean disease severity (%) of downy mildew by treatment and block in the 2023 season, with the dotted grey line indicating the mean disease severity (%) per block in the 2023 season.

Rainfall on 9th May 2023 of 10.5 mm at an average daily temperature of 14.2°C resulted in 1-2 oil spots (or infected bunches) per replicate, not exceeding 10% of the leaf surface. The symptoms were sighted on 31 May 2023 (see Fig. 3A) and confirmed on 2nd June by the 'Station viticole' in Auvernier (see Fig. 3B). On the 2nd of June, a disease assessment was carried out. The symptoms appeared independently of the treatments, as no treatment had been carried out at this time and were statistically not relevant. The effect of the treatments on the secondary infection (and other primary infections) was assessed by further disease screenings on 17th July and 6th September 2023. Whereby, the last screening also examined the disease severity with powdery mildew (data not shown). Both symptoms of downy mildew (17th June and 6th September 2023) and symptoms of powdery mildew (6th September 2023) were visible. Disease severity of both, downy and powdery mildew was significantly affected by the location in the plot (ANOVA, p-values > 0.05) and not by the treatments. The average disease severity of downy mildew on leaves (see Fig. 25) ranged from 1.53% in Block 4 to 5.37% in Block 1 on 6th September 2023. Previous downy mildew infections on grapes were no longer visible. Powdery mildew severity of grapes was higher in Block 1, averaging 15.41%, than in Blocks 2 - 4, where disease severity averaged 2-3.9% (data not shown). Powdery mildew infestation on leaves was negligible, averaging less than 1% for all blocks.

Under the given conditions of the field assay, which extended over two seasons, nothing can be said about the effectiveness of the treatment tested. In both 2022 and 2023, the climatic conditions were overall unfavourable for the development of downy mildew disease - thus an important infection with *P. viticola*. The conditions in the 2022 season were not even sufficient for a primary infection. Although this was the case in the 2023 season and a very low primary infection occurred, the progression of the disease was not affected by what treatment was used. The almost complete absence of rainfall after the first symptoms appeared (on 31 May 2023) and the high temperatures largely prevented sporulation. To favour secondary infection, certain conditions must be met: the leaves must remain moistened, or the relative humidity must be above 92% and persist for at least four hours. According to Bläser M. and Weltzien H. C. (1979) the sporangia, at 15°C and 70-90% relative humidity retain their effectiveness for 8 days. At 30°C and low relative humidity, however, the sporangia die within a few days. Considering the rainfall during the period when the vines were susceptible, it is likely that the necessary conditions for secondary infection were generally lacking. Downy mildew infestation was significant, especially in Block 1, although still at a low level of 5.37% percent on average and hardly evidenced as distinct oil spots. The fact that powdery mildew infestation was also significantly higher in Block 1 indicates that the different properties in terms of slope and soil depth (and other aspects related to the topography) of the plot could be the determining factor for the susceptibility of the grapevine plants. This also becomes apparent when we compare, e.g. the berry weights in the next chapter (4.3.2). Another indication of the result of the powdery mildew infestation is that although a full protection with sulphur should have prevented the infestation, considerable symptoms still occurred, especially on the grapes. It is conceivable that the application technique also played a role. This can be seen in comparison to the neighbouring plot, where no symptoms of powdery mildew could be detected. There, a Fischer sprayer head (300 l, Collombey-Muraz, Switzerland) was used to spray through anti-drift nozzles in every second row on both sides. The higher pressure (16-20 bar) results in a finer spray pattern, and the ventilation ensures that the underside and hidden sides of the leaves were treated. In the hand sprayed treatments, the spray pattern was often too liquid in the grape zone, which most likely led to runoff and thus to less effective protection.

4.3.2 Must oenological parameters

The experimental design of a randomized block experiment made it possible to exclude the factor of local bias within statistical analysis. This was done in section 4.3.2.1 for some selected must oenological parameters by setting the blocking factor as random. In chapter 4.3.2.2, on the other hand, the blocks were included as a fixed factor for the evaluation of possible influences due to site-specific influences. The results of both chapters will be discussed at the end.

4.3.2.1 Must oenological parameters' analysis by treatment

In 2022, the year in which the evaluation was carried out, there were no significant differences between the treatments for any of the must oenological parameters (see Tab. 8). All treatments were repeated four times (n=4), with 100 randomly selected berries being analysed in each repetition.

Table 8: (Incomplete) List of the results of the analysis of the oenological parameters of the must: average berry weight in grams (±SD), sugar content in °Oe (±SD) and pH (±SD) depending on the treatment. No significant effects of the treatments were observed.

Treatment	Berry weight (g)	Sugar content (°Oe)	pH
Control	1.53±0.13	85.75±4.43	3.2±0.06
Novo-1	1.62±0.17	87.00±2.45	3.28±0.07
Novo-2	1.63±0.26	84.75±2.06	3.20±0.08
Novo-3	1.55±0.32	84.50±1.91	3.19±0.05
Reference	1.65±0.20	87.75±2.36	3.26±0.06
Reference/2	1.47±0.23	86.50±5.45	3.23 ±0.04

Treatment with Novochizol™ had no effect on berry weight, sugar content and pH. Other parameters crucial for a proper vinification process, such as total acidity and yeast assimilable nitrogen (YAN) were also not significantly affected (data not shown). All values were average values, with weight and YAN being rather low.

4.3.2.2 Berry weight analysis by location

A further comparison of the distribution of values across the plots showed that the location of the plots had some influence. This effect was significant, e.g. for the berry weight (see Fig. 26).

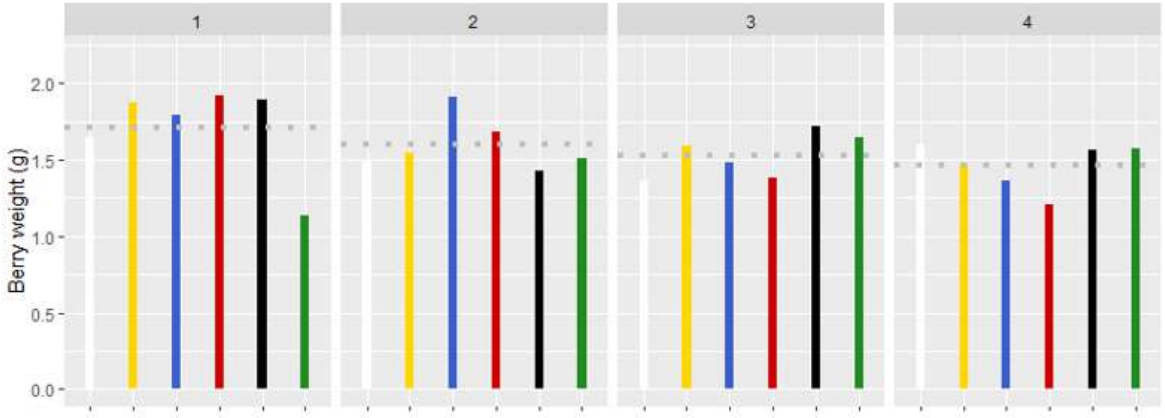


Figure 26: Average berry weight of 100 berries (in grams) by treatment and block before harvest of 2022 season, with the dotted grey line indicating the mean berry weight of 100 berries (in grams) per block.

Block 1 was the block with the highest average berry weight of 1.71g. Block 4 comes last with 1.46 g, what was significantly different (ANOVA, p-value 0.03066). The order of ranking correlates with the infestation of downy mildew (see chapter 4.3.1). Interestingly, YAN did not show the same correlation, although there was also a cluster formation depending on the location in the plot independently of the block.

4.3.2.3 Interpretation and Discussion: Must oenological parameters and berry weight analysis

The effects of Novochizol™ applications on grapevines and its influence on must oenological parameters remain uncertain and require further research. While the eliciting effect of Novochizol™ has been questioned in terms of its potential beneficial effects on must oenological parameters, the literature on chitosan offers further insights. Studies such as Gutiérrez-Gamboa et al. (2019) show that the application of chitosan reduces the concentrations of various amino acids in must, leading to a decrease in total amino acid content. In addition, the application of chitosan increased the alcohol content in wines from certain grape varieties.

Analyse of the berries showed no reduced nitrogen levels (YAN), and no higher sugar content (°Oe), which would increase the wine alcohol content, due to the treatment. These effects would have been undesirable for wine quality. The trial showed site-dependent YAN values and berry weights, possibly influenced by the absence of fertiliser, and competing vegetation. Interestingly, weak, and low-yielding vines showed less susceptibility to disease. The grapevine plants are more vigorous where the lower part of the plot is flatter and can store both water and nutrients. This is reflected in increased berry weights. The increased availability of water and nutrients increases the susceptibility of the vines. What is discussed in theory (Marcianò et al. 2023) also becomes obvious in the experiment with downy mildew.

4.4 Wine

4.4.1 Fermentation

The winemaking process and wine quality were not affected using Novochizol™. This was tested on a sample containing Novochizol™ and a control sample (see Fig. 27).

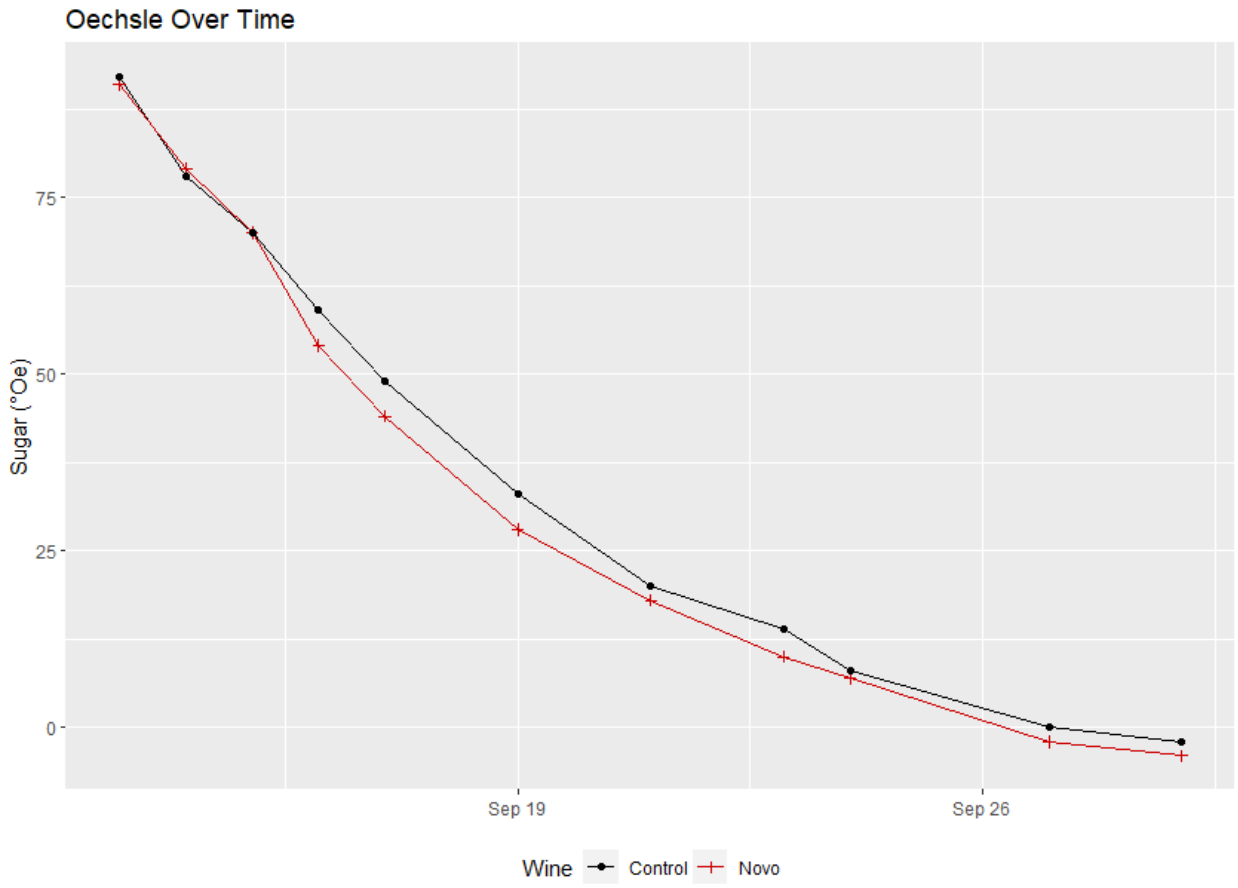


Figure 27:'Fermentation curves', with development of sugar (°Oe) over time for the two wine samples in 2022 season.

At harvest day, on the 13th of September in 2022, the plot was divided in two: the first sample contained the grapes from all plots without Novochizol™ (black) and the second sample contained the grapes from all plots that had been treated with Novochizol™ (red). The control sample measured 92°Oe on the day of harvest, while the sample with Novochizol™ measured 91°Oe. The decisive period of fermentation was documented and is shown in Fig. 27 ('Oechsle over time'). The resulting curves were very similar, and no fermentation stagnation occurred. Fermentation was completed at 29th of September 2022, for both samples. Resulting in 16 days of fermentation. Additionally, the temperature was recorded during fermentation (data not shown) where 23°C was not exceeded.

4.4.2 Wine sampling

The analysis of the wine showed that both samples have completed malolactic fermentation after fermentation. Both samples had difficulties with clarification after fermentation. The rest of the parameters tested, including the results of the tasting, are shown in Table 9:

Table 9: Selected - i.e. the most important - results of the wine sampling of the two wine samples, whereby the residues with (*) were measured with classical methods and the rest with fourier transform infrared spectroscopy analysis. The results of the tasting are in the original French language in order not to falsify them.

	Novo	Control
Alcool [%vol.]	12.1	12.1
Total acidity [g l^{-1}]*	5.02	4.92
pH*	3.59	3.60
Residual sugars by enzymatic method [g l^{-1}]*	1.1	2.2
Glucose [g l^{-1}]	0.2	0.2
Fructose [g l^{-1}]	1.7	2.8
Acetic acid ("volatile acidity") [g l^{-1}]	0.6	0.6
Tasting	Beau rubis, turbidité Nez discret, marqué Bon équilibre en bouche	Beau rubis, turbidité Bon nez, cerises Joli fruits, fraicheur, bons tannins, belle longueur, léger CO_2

The above results show that the resulting wines had the same profile, with no value being significantly different. The slightly higher residual sugar content is negligible compared to 212 g l^{-1} and 215 g l^{-1} as the initial sugar content of the Novochizol™ sample and the control sample, respectively. The same applierd to the fructose content. Fermentation was incomplete in either wine. The volatile acid content - mostly acetic acid - was at a low level in both cases (threshold value according to the 'International Organisation of Vine and Wine' is 1.2 g l^{-1} (OIV 2023)). The tasting revealed slight differences in expressivity, with the Novochizol™-containing sample being more discreet on the nose. No false notes were detected during the tasting.

The slight CO_2 detected during the tasting could simply render the wine more expressive. However, as there were no false notes, both wines can be described as clean. This also signify the content of volatile acidity. Volatile acidity mostly comes from secondary fermentations, which impact the overall quality of wines. These fermentations are conducted by microorganisms such as acetic acid bacteria leading to acetic fermentation. The presence of acetic acid and certain yeasts may negatively impact the wine. Interestingly, chitosan is also being discussed as a means of reducing the risk of acetic acid bacteria occurring during winemaking (Castro Marín et al. 2021). Based on the results, nothing can be said about such a positive effect of Novochizol™ on wine quality. In addition, favourable conditions during the season did not allow contamination of the grapes by unwanted micro-organisms.

In terms of alcohol, acidity, and pH, both wines are identical. The slight difference and, above all, the fact that the sugar was fermented incompletely is normal. Sugar levels around 2 g l^{-1} are hardly noticeable and are not uncommon.

5 Overall discussion / Synthesis

The combination of Novochizol™ with simple copper salts as an agent to prevent downy mildew infections has proven to be valuable. The in vitro results indicated that the efficacy is probably dose-dependent, depending mainly on the dose of copper metal used in the formulations. The anti-oomycotic efficacy of Novochizol™ only comes into play at high doses, where, however, it proves to be very effective. The behaviour in terms of doses and concentration in relation to efficacy was more ambiguous: then both in literature (Asgari-Targhi et al. 2018; Suarez-Fernandez et al. 2020) and in the plant pathogen bioassay, a non-linear relationship is described. It should also be mentioned that the invariant results in the in planta trials can probably be attributed to non-uniform (initial) chitosan samples, the inoculation process itself and the complex interplay of direct and indirect effects of copper and Novochizol™ respectively.
Nevertheless, depending on the different copper formulations used, very high efficiencies were achieved in the plant pathogen bioassays - especially with copper sulphate [$CuSO_4$] and copper oxychloride [$(ClCu_2H_3O_3)_2$]. Halving or in some cases even quartering the amount of reference copper doses (Kocide® Opti) did not significantly reduce the effectiveness against downy mildew infestation. The added concentration of Novochizol™ proved to be crucial. At low concentrations of Novochizol™ (e.g. 0.0188%), the most interesting phenomenon of the work presented itself: an effect against grapevine downy mildew that was almost completely comparable to the full effect of Kocide® Opti. The bimodal way in which Novochizol™ acted on its own and in combination with copper should be thus investigated further. The renewed increase in efficacy of the copper-Novochizol™ compounds at low Novochizol™ concentrations, although the individual elements of the compounds lose efficacy linearly as they are diluted, is a strong indication of more effective delivery. An additional effect of Novochizol™ can be assumed, whereby this mechanism is known and discussed in connection with copper-chitosan formulation (Hashim et al. 2019; Lemke et al. 2022; Saharan et al. 2015). For the exact mechanism, Lemke et al. (2022) propose: chitosan interacts with the fungal cell wall, disrupting enzymes and nucleic acids and makes therefore copper ions get into the cell easier. About the occurrence of such a wave-like pattern – literature doesn't deliver any hints. So, it can again only be speculated about the reasons for such a behaviour. The answer must be found in the different mode of action of the compound of the copper-Novochizol™ formulations:

1. Direct anti-oomycotic effect of Novochizol™
2. Direct anti-oomycotic effect of copper
3. Indirect anti-oomycotic effect through plant elicitation of Novochizol™
4. Mechanism of copper delivery by Novochizol™

Depending on the concentration of Novochizol™ added, the mode of action of the individual substances - copper and Novochizol™ - comes into play differently.

While copper sulphate [$CuSO_4$] in combination with 0.3% Novochizol™ caused phototoxicity, such a phenomenon was not observed with copper oxychloride [$(ClCu_2H_3O_3)_2$], with having almost the same efficacy. This is another reason why this study sees the greatest potential in the combination of Novochizol™ with copper oxychloride [$(ClCu_2H_3O_3)_2$]. The lack of improved formulations and the lack of systematic tests regarding pre-treatment and rain-fastness does not really allow any statements to be made that copper could also be saved in this respect. The question of extended treatment intervals due to the longer-lasting effect of the Cu^{2+} ions delivered by Novochizol™ can therefore not be answered.

As far as the field trial is concerned, no clear conclusion can be drawn, as the disease level was too low in both the 2022 and 2023 seasons. Although the efficacy of the compounds could not be tested, it can still be said that the handling of the compounds is possible, and no phytotoxic reactions were observed with copper oxychloride [$(ClCu_2H_3O_3)_2$] in combination with Novochizol™. With regard to the (not significant) infestation of grapevine downy mildew in terms of treatments, however, it was shown in 2023 that the microclimate can ultimately determine susceptibility. The most productive vine plants were the most infected ones. Winemaking was not affected due to the use of Novochizol™. It can thus be ruled out that the use of Novochizol™ affects the quality and sensory properties.

The utilization of the tested copper-Novochizol™ compounds should not only be effective but also economically viable. Notably, the compound with the lowest Novochizol™ concentration (0.0188%) is likely to be cost-effective, as suggested by Novochizol SA (Loroch 2023, personal communication). Compared to Airone, a widely used commercial copper product, the addition of Novochizol™ showed: by halving the copper metal used in Airone, copper oxychloride [$(ClCu_2H_3O_3)_2$] in combination with 0.0188% Novochzol™ achieves almost twice the effectiveness of Airone. What was shown in the plant pathogens bioassays would mean an application of 0.96 kg ha^{-1} y^{-1} of Novochizol™ under field conditions. Compared to other additives (e.g. CropCover CC-1000, a starch-based adhesive from Andermatt Biocontrol) with 3-4 ls ha^{-1} per treatment and a price of CHF 291.30 for 20 l, this would be a comparable use. Thus, if a 3% Novochizol™ solution would be used, this would mean an application volume of 4 l ha^{-1} per treatment.

The question posed at the beginning, whether the addition of Novochizol™ to simple copper salts used against *P. viticola* in organic viticulture enables a significant reduction in copper concentration, can therefore be answered in the affirmative regarding the (semi-)controlled environment. It is certainly worth further testing the effect in the field after improving the formulation.

6 Conclusions

Given that it is unlikely to replace copper in the short term, copper reduction seems reasonable. It should also be mentioned that the use of copper has been somewhat relativised in terms of its environmental risk. And further, copper is a cheap, very effective measure without the risk of causing resistance - which is probably one of the most difficult challenges for conventional agriculture in the future. And it has been shown that farmers have already taken own ways to reduce their copper use in different region in Europe: be it by weather-dependent warning systems for a more precise application time, the use of lower copper doses or by delaying the first treatment. Sticking with copper as a plant protection product would make even more sense if the spraying intervals could be extended and (organic) farming could thus be more economically and socially sustainable. This due to the contact nature of copper-containing PPPs - and therefore their ability to be washed away by rain. Controlled and more effective delivery of [Cu^{2+}] and agrochemicals in general is key.

Chitosan has proven effective in many areas, as it is very valuable in delivering copper ions with its ability to complex with active substances. Given the low solubility of bulk chitosan, it has been therefore tested an improved chitosan formulation - Novochizol™ - to circumvent this issue and combined it with copper compounds. The results indicate that this combination is a possible way to reduce copper. Mixed with low concentrations of Novochizol™, copper may exert its effect differently: being bound and thus protected in the Novochizol™ sphere, it can be released more precisely. Not to be forgotten is the stand-alone anti-oomycotic effect of Novichizol™ against *P. viticola* - the causing agent of grapevine downy mildew. Investigations into the structure of the molecules, the binding kinetics and, above all, the bulk chitosan (DDA and MW) that Novochizol™ is made of could provide further insights. Despite, it will be very difficult to draw conclusions about efficacy entirely from theory. Plant pathogen's bioassay must ultimately define the efficacy. This is also because chitosan, and therefore probably also Novochizol™, is considered being a plant elicitor and the plant defence only comes into play in this interaction between plant and pathogen. Even if direct comparisons with the literature concerning Novochizol™ are difficult, the work that has already been done on copper-chitosan compounds leads to the conclusion that possible copper reductions can be considered. The use of copper PPP against many other important plant pathogens, such as olives and potatoes, should also be mentioned.

A further area of investigation in terms of combining copper with Novochizol™ must clearly be the formulation process: the fact that the copper formulations with Novochizol™ were prepared using a simple method could also explain some of the shortcomings.

The path also seems to be paved from a practical point of view. Because it is obvious that only the possibility of producing stable, well-defined and at the same time affordable copper-Novochizol™ particles on a large scale would enable its use. What remains unconsidered is the question of how cost-intensive and time-consuming it is to approve legally a plant protection product.

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Annex 1: EPPO-Guidelines

- PP 031/1 Plasmopara viticola (see Fig. 28 for evaluation schematics)
- PP 152/4 Design and Analyses of Efficacy Trials
- PP 004/4 Uncinula necator Powdery mildew
- PP 181/4 Conduct and Reporting of Efficacy Trials-GEP
- PP 135/3 Phytotoxicity assessment
- PP 1/223 (2) Introduction to the efficacy evaluation of plant protection products
- PP 1/225 (3) Minimum effective dose

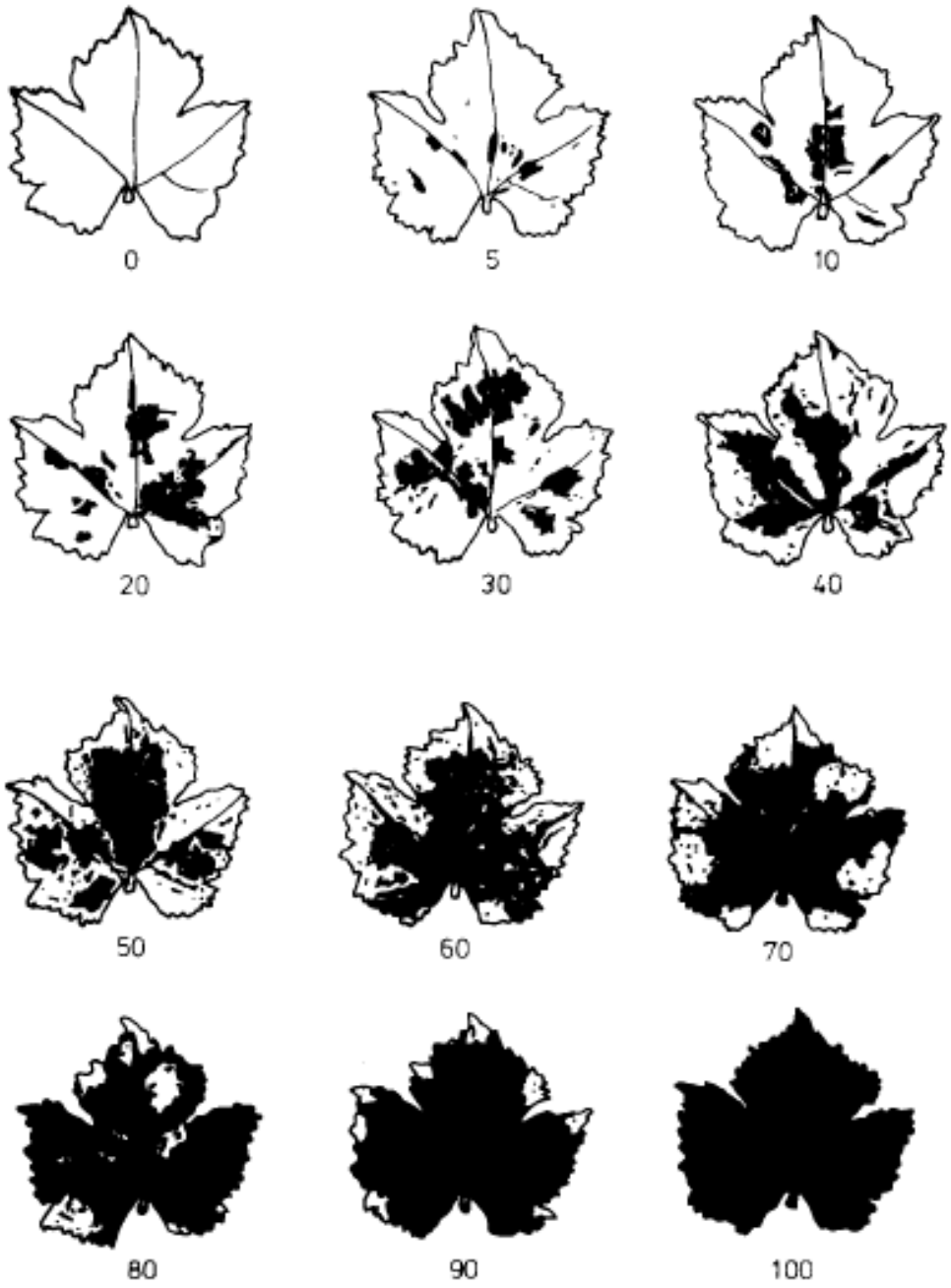

Figure 28: ***Plasmopara viticola*: percentage of lower leaf surface affected** (EPPO 2000)

Annex 2: List of Digital Annex

Folder	Description	Nr.	Year	Designation of the data
1_In vitro			2022	Zoosporen_Hemmtest_invitro.xlx
2_Plant pathogen assay		Exp. 1	2022	1_Kopie von Pv-22-05_Novochizol_1.xlx
		Exp. 2	2022	2_Kopie von Pv-22-08_Novochizol-2.xlx
		Exp. 3	2022	3_Kopie von Pv-22-09_Novochizol-3-cleared.xlx
		Exp. 4	2022	4_Kopie von Pv-22-10_Novochizol-4-cleared.xlx
		Exp. 5	2022	5_Kopie von Pv-22-11_Novochizol-5-cleared.xlx
		Exp. 6	2023	6_Kopie von Pv-23-01_Novochizol_Runde1_Nachbonitur.xlx
		Exp. 7	2023	7_Kopie von Pv-23-03_Novochizol_Runde2_Nachbonitur.xlx
		Exp. 8	2023	8_Kopie von Pv-05-23_Novochizol_Runde3.xlx
		Exp. 9	2023	9_Kopie von Pv-07-23_Novochizol_Runde4.xlx
		Exp. 10	2023	10_Kopie von Pv-10-23_Novochizol_Runde5
	Evaluation of inoculation order	Exp. 10	2023	Run_10_order.csv
3_Field assay	Plant protection journals		2022	22_Pflanzenschutzjournal.png
			2023	23_Pflanzenschutzjournal.png
			2022/2023	22_23_Dosierungen.xlx
			2022	22_Analyses baies.xlx
			2023	23_Bonitur.xlx
4_Vinification	Vinification journals	Tank 3	2023	Novo.png
		Tank 20	2023	Control.png
			2022	22_Analyses oenologiques.pdf
5_R	Chapter			0_all_try.R
	4.2.1.1 Disease pressure and related SD			1_mean_sd_all.R
	4.2.1.2 Correlation between inoculation and severity in Exp. 10			2_check_run10.R
	4.2.3 Efficacies depending on copper origin			3_copper sources.R
	4.2.2 Efficacy of Novochizol™ alone			4_Novo solo.R
	4.2.4 Efficacies depending on Novochizol™ concentration			5_CuCl_CuOH.R
	4.2.5 Additive effect of Novochizol™ combined with copper			6_synergies.R
	4.3.1 Disease levels			7_bonitur.R
	4.3.2 Must oenological parameters			8_analyses berries.R