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Assessment of different strategies to reduce environmental fungicide contamination in vineyards

Paula Ortega Rioja

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DOCTORAL

THESIS

Assessment of different strategies to reduce environmental fungicide contamination in vineyards

Paula Ortega

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PhD in Agri-Food Technology and Biotechnology program at UPC

Thesis by compendium of publications

Assessment of different strategies to reduce environmental fungicide contamination in vineyards

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Abstract

Vineyard cultivation has a rich history in the European region. To ensure its production, it has been traditionally relied on the use of Plant Protection Products (PPP) for the pest and diseases control. One of the biggest challenges facing the vineyard is *Plasmopara viticola* commonly known as downy mildew. Traditionally, copper-based fungicides have been used to combat this disease. Even though nowadays there are also products from chemical synthesis and biological sources, copper remains the most widely used option. However, it has been observed that their prolonged use can cause environmental contamination, especially in the soil and water bodies. Therefore, the use of PPP in agriculture is currently an issue of major concern due to the risks they pose to both the environment and human health. The prolonged use and sometimes abusive or careless use of them has raised alarm bells in the European Union.

This doctoral research project has been focused on finding alternative solutions to reduce the contamination caused by fungicides in grapevines from different angles, from mitigation to prevention. The PhD dissertation firstly explores the impact of cover crops on reducing pesticides leaching from the surface to groundwater and the role of the vegetated buffer strips in reducing runoff into surface water courses. Secondly, it assesses the potential of vegetation to degrade these products in different soil environment, including their transformation products. Thirdly, this work involves the development and evaluation of a copper microencapsulated product that enhances deposition, and therefore reduces the amount of copper required in crop protection treatments. Finally, in the fourth stage, factors influencing farmers' adoption of biopesticides as alternative product to for copper fungicides are evaluated. This work demonstrates that the three proposed alternatives have been shown as potential strategies to reduce pollution.

The findings presented in this thesis demonstrate promising results. Cover crops and vegetated buffer strips are effective strategies for reducing groundwater and soil contamination resulting from fungicide application, due its capacity to degrade the pollutant compounds. Vegetation accelerates the degradation kinetics of fungicides in soil and their transformation products. The evaluated microencapsulated product, allows to increase the efficiency during the spray application, generating a potential copper reduction. As well, the knowledge of copper legislation has been detected as crucial for introducing biological plant protection products as an alternative to chemical or copper fungicides. All these strategies can be combined to achieve even greater impact at different levels of spray application activities. Further research is needed to achieve the lowest environmental impact and to produce safer food in a more sustainable way.

Keywords: Buffer zones · Copper · Cover crops · Crop protection · Fungicides · Microencapsulation· Sustainability · Vineyard

Resumen

El cultivo de la vid tiene una rica historia en la región europea. Para garantizar su producción, se ha confiado tradicionalmente en el uso de productos fitosanitarios (PPP) para el control de plagas y enfermedades. Uno de los mayores retos a los que se enfrenta el viñedo es *Plasmopara viticola*, comúnmente conocido como mildiu. Tradicionalmente, para combatir esta enfermedad se han utilizado fungicidas a base de cobre. Aunque hoy en día también existen productos de síntesis química y de origen biológico, el cobre sigue siendo la opción más utilizada. Sin embargo, se ha observado que su uso prolongado puede causar contaminación ambiental, especialmente en el suelo y las aguas. Por ello, el uso de productos fitosanitarios en la agricultura es actualmente motivo de gran preocupación debido a los riesgos que plantean tanto para el medio ambiente como para la salud humana. Su uso prolongado y, en ocasiones, abusivo o descuidado, ha hecho saltar las alarmas en la Unión Europea.

Este proyecto de investigación doctoral se ha centrado en la búsqueda de soluciones alternativas para reducir la contaminación causada por fungicidas en la vid desde diferentes ángulos, desde la mitigación hasta la prevención. En primer lugar, la tesis doctoral explora el impacto de los cultivos de cobertura en la reducción de la lixiviación de plaguicidas a las aguas subterráneas y el papel de las franjas de protección vegetadas en la reducción de la escorrentía a los cursos de agua superficiales. En segundo lugar, evalúa el potencial de la vegetación para degradar estos productos en distintos medios edáficos, incluidos sus productos de transformación. En tercer lugar, este trabajo implica el desarrollo y la evaluación de un producto microencapsulado de cobre que mejora la deposición y, por tanto, reduce la cantidad de cobre necesaria en los tratamientos fitosanitarios. Por último, en la cuarta fase, se evalúan los factores que influyen en la adopción por parte de los agricultores de los biopesticidas como producto alternativo a los fungicidas de cobre. Este trabajo demuestra que las tres alternativas propuestas se han mostrado como estrategias potenciales para reducir la contaminación.

Los hallazgos presentados en esta tesis demuestran resultados prometedores. Los cultivos de cobertura y las bandas de vegetación son estrategias eficaces para reducir la contaminación de las aguas subterráneas y del suelo resultante de la aplicación de fungicidas, debido a su capacidad para degradar los compuestos contaminantes. La vegetación acelera la cinética de degradación de los fungicidas en el suelo y de sus productos de transformación. El producto microencapsulado evaluado, permite aumentar la eficacia durante la aplicación en pulverización, generando una reducción potencial de cobre. Asimismo, se ha detectado que el conocimiento de la legislación del cobre es crucial para la introducción de productos fitosanitarios biológicos como alternativa a los fungicidas químicos o de cobre. Todas estas estrategias pueden combinarse para lograr un impacto aún mayor en diferentes niveles de actividades de aplicación de pulverización. Es necesario seguir investigando para lograr el menor impacto ambiental y producir alimentos más seguros de forma más sostenible.

Palabras clave: Cobre · Cultivos de cobertura · Fungicidas · Microencapsulación · Protección de cultivos · Sostenibilidad · Viñedos · Zonas tampón

Resum

El cultiu de vinyes té una rica història a la regió europea. Per assegurar la seva producció, s'ha basat tradicionalment en l'ús de productes fitosanitaris (PPP) per al control de plagues i malalties. Un dels reptes més grans a què s'enfronta la vinya és *Plasmopara viticola* coneguda comunament com a mill de riu. Tradicionalment, s'han utilitzat fungicides basats en coure per combatre aquesta malaltia. Encara que avui en dia també hi ha productes de síntesi química i fonts biològiques, el coure continua sent l'opció més utilitzada. No obstant això, s'ha observat que el seu ús prolongat pot causar contaminació mediambiental, especialment en el sòl i les masses d'aigua. Per tant, l'ús de la PPP en l'agricultura és actualment un tema de gran preocupació a causa dels riscos que plantegen tant per al medi ambient com per a la salut humana. L'ús prolongat i, a vegades, abusiu o imprudent d'aquestes substàncies ha despertat l'alarma a la Unió Europea.

Aquest projecte de recerca doctoral s'ha centrat en trobar solucions alternatives per reduir la contaminació causada pels fungicides en les vinyes des de diferents angles, des de la mitigació fins a la prevenció. En primer lloc, la tesi doctoral explora l'impacte dels cultius de cobertura en la reducció dels pesticides que es lixivien cap a l'aigua subterrània i el paper de les franges tampó vegetatives en la reducció de l'escorrentia cap als cursos d'aigua superficials. En segon lloc, avalua el potencial de la vegetació per degradar aquests productes en diferents entorns del sòl, inclosos els seus productes de transformació. En tercer lloc, aquest treball implica el desenvolupament i l'avaluació d'un producte microencapsulat de coure que millora la deposició i, per tant, redueix la quantitat de coure requerida en els tractaments de protecció de cultius. Finalment, en la quarta fase, s'avaluen els factors que influeixen en l'adopció de biopesticides per part dels agricultors com a producte alternatiu als fungicides de coure. Aquest treball demostra que les tres alternatives proposades s'han mostrat com a estratègies potencials per a reduir la contaminació.

Els resultats presentats en aquesta tesi demostren resultats prometedors. Els cultius de cobertura i les franges tampó vegetatives són estratègies efectives per reduir la contaminació de les aigües subterrànies i del sòl resultants de l'aplicació de fungicida, a causa de la seva capacitat de degradar els compostos contaminants. La vegetació accelera la cinètica de degradació dels fungicides en el sòl i els seus productes de transformació. El producte microencapsulat avaluat, permet augmentar l'eficiència durant l'aplicació d'esprai, generant una possible reducció del coure. Així mateix, s'ha detectat que el coneixement de la legislació sobre el coure és crucial per a la introducció de productes fitosanitaris biològics com a alternativa als fungicides químics o de coure. Totes aquestes estratègies poden combinar-se per a aconseguir un impacte encara major en els diferents nivells de les activitats d'aplicació de polvorització. Es necessita més recerca per a aconseguir el menor impacte mediambiental i per a produir aliments més segurs d'una manera més sostenible.

Paraules clau: Coure · Cobertes vegetals · Fungicides · Microencapsulació · Protecció de cultius · Sostenibilitat · Vinyes · Zones tampó

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List of Acronyms

AIC - Akaike information criterion

BG – Bare Ground

Bio PPPs – Biological Plant Protection Products

BS – Buffer strip

DSS - Decision Support System

EC - European Commission

EFSA - European Food Safety Authority

EUROSTAT - Statistical Office of the European Communities

EU – European Union

F2F – Farm to Fork Strategy for a fair, healthy and environmentally-friendly food system

FAOSTAT- Food and Agriculture Organization Corporate Statistical Database

FG- Focus group

IFV - Institut Français de la Vigne et du Vin

IPM - Integrated Pest Management

Koc - Organic carbon-water partition co-efficient.

Kow - Octanol-water partition coefficient

MC – Monoculture Cover Crop

ORs - odds ratios

PC – Polyculture Cover Crop

PCA - Principal component analysis

PPPs - Plant Protection Products

RMSE - root mean square error

SUD - The Sustainable Use Directive (2009/128/EC)

Sw - Solubility

TPs – Transformation Products

VRA – Variable Rate Application

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CHAPTER I. General introduction

i. Vineyard crop relevanceAcknowledgments

According to FAOSTAT, the global vineyard production in 2020 was estimated to cover a total harvested area of almost 7,000,000 ha (Food and Agriculture Organization of the United Nations, 2020). Nowadays 3.2 million hectares are dedicated to grapevine cultivation in EU, equivalent to about 45 % of the world's wine-growing areas. Notably, this cultivation is mainly concentrated in three Mediterranean counties, Spain, France, and Italy which together account for three-quarters (74.9 %) of the area under vines in the EU and about two-fifths (38.7 %) of vineyard holdings in 2020 (Figure 1.1). The historical cultivation of this crop is translated in the fact that vine trees are relatively old, being over one-third (36.7 %) over 30 years (Statistical Office of the European Communities, 2020). The size of the vineyard fields in EU is also a significant factor to consider. In 2020, there were 2.2 million vineyard holdings dedicated to wine, the vast majority of which were tiny; 83.3 % had less than 1 ha of vineyards (Statistical Office of the European Communities, 2020). Grape cultivation in Europe is manly destinated to wine production. In 2020 the European Union was the world-leading producer of wine, accounting for 45% of global wine-growing areas, 64% of production and 48% of consumption. Wine is the largest EU agri-food sector in terms of exports, 7.6% of agri-food value exported in 2020 (Statistical Office of the European Communities, 2020).

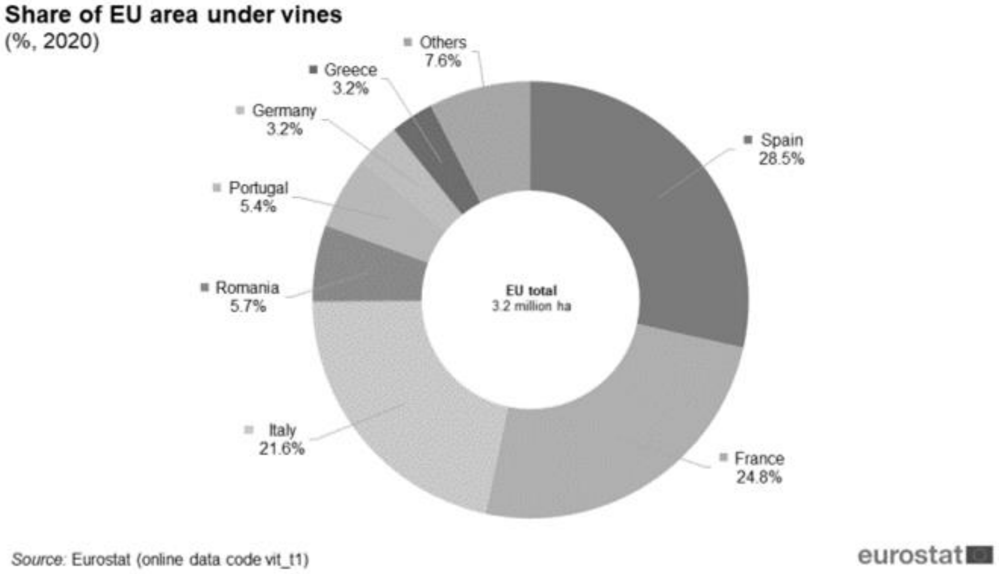

***Figure 1.4:** EU area under vines in 2020 (Statistical Office of the European Communities, 2020)*

Wine is an integral part of the Mediterranean diet and the customs and traditions of the people, playing a significant role in many festivals and celebrations. This aspect attracts both national and international tourism, making the wine sector in Spain essential not only in economic terms but also in terms of its significance for the country's society and culture. Currently, Spain has an area of 121,200 hectares dedicated to the cultivation of vines in organic production, making it the country with the largest surface area used for this purpose. This area produces over 400,000 tonnes of wine grapes (FEV, 2020).

ii. Challenge of crop protection in vineyards

Currently, the four countries with the highest recorded pesticides volumes sold are Germany, Spain, France, and Italy. The use of plant protection products (PPPs) is especially relevant in crops such as vines or fruit trees, where their use is a difficult challenge, both from the point of view of the difficulties of guaranteeing high quality of application, as well as the enormous dependence of these crops on plant protection products. According to EUROSTAT (Statistical Office of the European Communities, 2023), Spain, France and Italy recorded around 50% of the total amount of fungicides in Europe, as the main producers of grapes and fruits. In France, one of the largest European wine producers, the vineyard sector uses more than 20% of the total amount of PPP, in a reduced agricultural area of less than 3% of the total productive surface. This trend has been also detected in other countries like Spain, Italy, Portugal, and Greece, where grapes and fruit production represent most of the production. An important action focused on vineyard has been arranged in France by the implementation of DEPHY network, a huge organization involving more than 250 farmer’s organizations and more than 2,800 farmers, with a common goal to test and evaluate technics and systems developed to reduce the use of PPP. DEPHY group already demonstrated PPP reductions in vineyard up to 33%. Concerning orchards fruits and the potential reduction of PPP, recent research demonstrated that the amount of PPP can be reduced up to 43% just applying best management practices and considering canopy structure as a basis for a proper way of pesticide dose expression. According to the report developed by EIP-AGRI Focus Group (FG) Sustainable Ways to Reduce Pesticides in Pome and Stone Fruit Production, compared to other crops, fruit production uses a significantly higher quantity of pesticides to control pests, diseases, and weeds, and to regulate growth (e.g., apples are treated with various pesticides 20-30 times/year). Economic, social, and environmental benefits will be noted after the proposed objectives to reduce the PPPs, including positive impacts on environmental biodiversity (European Union, EIP-AGRI operational groups, 2022).

The grapevine (*Vitis vinifera*) is sensitive to pests and diseases, which can negatively impact its health. These issues have the potential to impair the functioning of the vines, cause damage or even lead to their demise, resulting in significant economic losses for farmers. Given the grapevine’s vulnerability and its status as a high-value crop, crop protection becomes a critical undertaking. Specifically, the use of fungicides plays a crucial role in safeguarding grapevines. In some cases, farmers may require to apply fungicides as frequency as 12 to 15 times per year to mitigate potential fungal threats (European Parliamentary Research Service, 2021). Spain, being one of the world's largest consumers of phytosanitary products, uses 38% of them in vineyards. This high use has led to problems regarding the presence of pesticide residues in food and water bodies (Alonso González et al., 2021).

iii. Downy mildew control

Vineyard crops face several diseases that can damage vineyard crops, and for instance farmers must take care of, like Botrytis (Elmer & Michailides, 2007) or Powdery mildew (Gadoury et al., 2012), among many others. Even though, *Plasmopara viticola*, commonly named as grape downy mildew, poses one of the greatest risks to viticulture growers. This type of oomycete, came from North American and became one of the most damaging diseases of grapevine (Fontaine et al., 2021). Downy mildew infects new plant tissues such as young green leaves, twig, and fruit tissues, causing severe losses in short periods of time. These infections can destroy 40–90% of plants in the field in optimal humidity and temperature (Toffolatti et al., 2018). There are several mildew types that can affect may kind of plants, but grapevine downy mildew remains the most destructive downy mildew in Europe, being most damaging foliar diseases of grapevine, leading to organoleptic defects and productivity reduction (Darriet et al., 2002; Gessler et al., 2011; Jermini et al., 2010; Stummer et al., 2005). Mildews are similar to fungi in many respects. Still, they do not belong to this group of organisms, but to the Peronosporaceae, a morphologically diverse group of oomycetes, mainly obligate parasites with conidia or sporangiophores of determinate growth (Koledenkova et al., 2022).

P.viticola is an obligate biotrophic endoparasite, that is adapted also to the vineyard crop cycle. During the winter it remains as an inactive oospore in the ground or the remains of pruned branches or leaves. When temperatures and humidity are optimal, usually in spring, the oospore matures and germinates (Gessler et al., 2011). Helped by the meteorological agents, like wind and rain, the sporangium reaches the hosts and releases the zoospores that infects it (Figure 1.2). After the infection is produced, the mycelium grows inside the plant tissue, causing the well-known symptoms of downy mildew, brownish-yellow spots on the upper side of the leave (Orlandini et al., 2008). After this part of the cycle, asexual reproduction takes place, when the mycelium produces sporangiophores that emerges from the infected plant tissue and liberate zoospores that will start the second infection. The whole cycle typically lasts from less than one week to 18 days (Velasquez-Camacho et al., 2023). If the conditions are optimum, this infection cycle is repeated during the growing season (Gilles, 2004). When temperatures become unfavourable, the sexual part of the cycle takes place again, producing the oospores that will remain in the field to start the infection the next season (Thind et al., 2006).

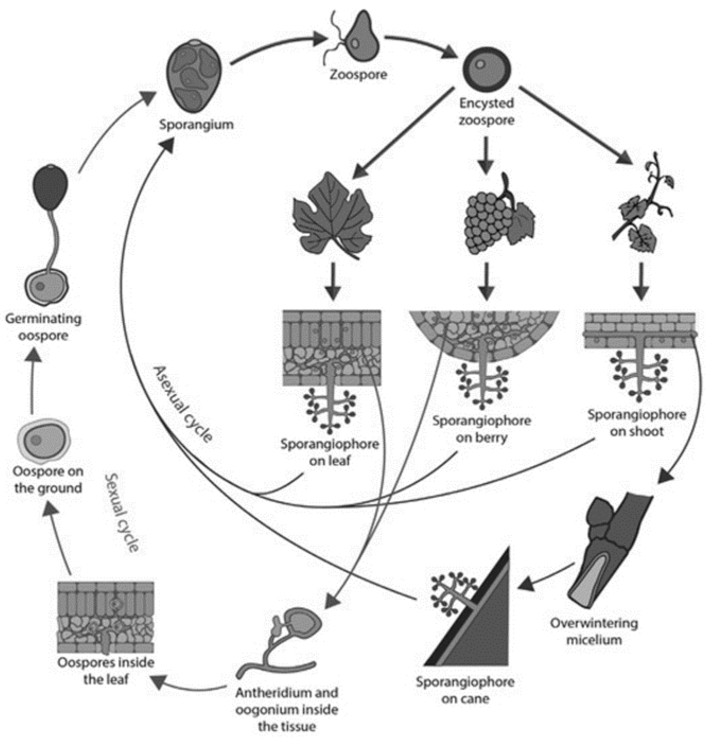

Figure 1.5: *Schematic diagram of the disease cycle of downy mildew caused by Plasmopara viticola (Velasquez-Camacho et al., 2023).*

In 1885, Millardet found the effect that copper sulphate has suppressing many pathogens, including downy mildews. After this discovery, many contact and penetrating single-site fungicides have been developed to control this pathogen and have been traditionally used in vineyard crops. Nowadays, coper fungicides can be presented in different formulations, such as copper oxychloride, copper oxide or copper hydroxide (Millardet, 2018). Cupric fungicides act by contact and preventive action, forming a protective barrier on the leaf surface. The Cu^{2+} ions function as the active ingredient, effectively targeting pathogen spores until their accumulation is lethal to pathogen cells. What makes copper so effective its ability to employ three distinct pathways of action against the disease: blocking of the respiratory process, inhibition of protein synthesis, and reduction of cell membrane activity. This fact also makes the emergence of resistance very unlikely, due the multisite action. These facts, makes copper fungicides almost indispensable in the actual viticulture farming. Anyhow currently there are other products besides copper available on the market, such chemical synthesis products (Gadoury et al., 2012). Regardless, in this case, fungicide mixtures provide better control of the individual chemicals applied alone or rotation of active ingredients needed to avoid resistances, such as these products mostly have just one action pathway (Ghule et al., 2020a; Massi et al., 2021).

While copper fungicides remain popular, there are nowadays other options available on the market. Synthetical compounds like, dimethomorph, metalaxyl or zoxamide are used as active ingredients for fungicides used for downy mildew control (Campbell et al., 2021; Ghule et al., 2020). Those type of chemical formulates have been demonstrate effective against *Plasmopara viticola*, but they mostly have a single way of action against the pathogen. This can result in easier resistance to the components and a shorter lifespan for these products. Even thought, there is also another type of products in the market, biopesticides (Tucker et al., 2022). Those are made from a natural source or living organisms, and therefore tend to be safer for the environment and human health. However, most of them lack a specific target and tend to act by preventing the infection before it occurs so the efficacy of these formulates is often not very high.

iv. Technology and difficulties of crop protection in vineyards

Crop protection strategies are inevitably linked to actual agricultural production systems (Damalas, 2015). At present, pesticide application is the main activity performed by farmers as for maintaining the crop in optimum conditions against pests and diseases. Currently, this makes farming highly dependent on Plant Protection Products (PPPs). The number of treatments performed during a vine campaign, usually relies on the area meteorological conditions, as well as the year, which marks the level of the disease pressure (de Oliveira et al., 2021). The application of PPPs in vineyards, can be performed using different methods of spraying, ranging from manual application using a knapsack sprayer (seen in Figure1.3-A) to specialized machinery tailored to the crop and field conditions. The choice of technology will depend on different factors such as crop characteristics, field attributes, and the economic resources of the farmer or company. The most widely used sprayer are air-assisted sprayers, due the vineyard canopy structure (Y. Gil & Sinfort, 2005; Salcedo et al., 2019). Those spraying technologies uses a fan or turbine to transport droplets to the target area, while also moving vegetation, which allows for better penetration of the product into the canopy. Apart from that, there are multiple options available, ranging from simple conventional sprayers to highly specific ones like Figure1.3-B.

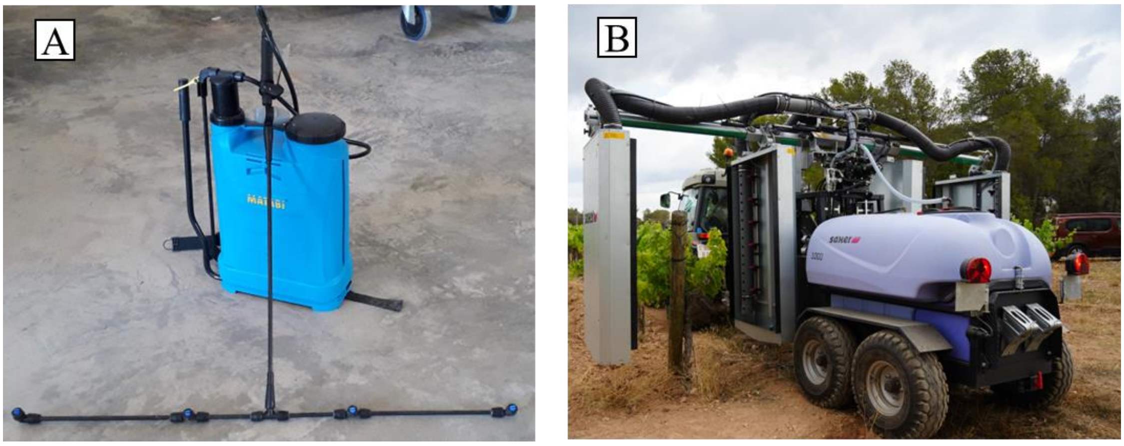

Figure 1.6: *A- Knapsack sprayer / B- multi row sprayer with recovery panels*

Even if we use the most advanced technology on the market, the application process will produce product losses that consequently end up in the environment. Of course, the level of technology of the application method is directly related to the amount of waste produced. Drift is the amount of product that remains suspended in the air after the spraying process, that therefore can be transported and deposited in places out of the target area. It is considered a dangerous source of contamination derivate from the crop protection activities, and for instance a topic of concern for the EU. According to Article 18 of the proposed the Sustainable Use Directive of Pesticides (SUD) by the European Commission, the use of all plant protection products is prohibited in sensitive areas and within 3 meters of these areas. The buffer zone of 3 meters cannot be reduced by using alternative risk-mitigation techniques. The regulation also proposes that buffer zones should be extended to at least 5 meters in the case of sensitive areas and 10 meters for surface waters. Each country has the liberty to decide and is encouraged to establish larger buffer zones if deemed necessary. To address this, the Institut Français de la Vigne et du Vin (IFV) has developed an innovative test bed called EoleDrift. This test bed measures vertical drift and compares sprayers and practices based on the associated drift risks. The EoleDrift test bed is designed to compare the levels of drift generated by various wine spraying techniques to determine acceptable handling time and cost limits. It also measures the amount of drift on a vertical plane generated by different spraying techniques in viticulture (Sage et al., 2021).

Drift is especially high risky when we talk about air-assisted sprayers, since the air currents blow the droplet away with more energy, thus causing them to reach further and thus spreading the contamination even further. It is not possible to avoid spray drift completely, but it can be minimized and reduced by adjusting the spraying parameters (Felsot et al., 2011). The type and efficiency of the machinery used not only determines the extent of the treatment, but also the amount of product loss that occurs and affects the environment, according to Gil et al. (2021). However, there are other ways to reduce these losses. Adjusting equipment, improving operator skills, or using anti-drift nozzles as mitigation measures can help to reduce the amount of airborne product loss (Otto et al., 2015).

Moreover, apart from drift, product losses can also be deposited on the soil, referred as losses to the ground, if this is not the target (Grella et al., 2017). Even in other cases the objective can be applying PPPs to the soil (like herbicides) when we talk about *Plasmopara viticola* applications are made on the leaves or grapes, so the target will never be the ground, so that all the product that does not reach this objective, will be considered as pollution. As well as in drift the spraying type and its calibration its crucial to reduce this pollution source. Therefore, it should also be noted that during vine campaigns, several pesticides are applied repetitively, and that is why the machinery efficiency for the treatments is a key factor that can cause a huge impact on the amount of PPPs ending into the environment.

v. Pesticides pollution

The use of pesticides in vineyards has become a pressing concern due to the potential risks they pose for the environment and the human health (Regus et al., 2022). Fungicides have been detected far from their application site, transported by air and water streams which extends their pollutant action far away from where is applied (Sergazina et al., 2021). The negative effect this kind of substances have on the environment have been extendedly reported. Furthermore, it worth mentioning that several fungicides are poorly removed in vineyard soils, and depending on their physicochemical properties, they can be accumulated in the soil (Pérez-Mayán et al., 2020). Fungicides include compounds like dimethomorph or zoxamide, but the most extended compounds against downy mildew are copper-based fungicides. Anyway, chemical compounds are frequently detected in runoff water courses and soil, like Gregoire et al. (2010), who found dimethomorph in 72% of the water samples from vineyard runoff catchments. Furthermore, it has been observed that this type of chemicals can also migrate from the soil and be bioaccumulate in plants (Margenat et al., 2018).

Nevertheless, in the context of vineyards, the use of copper, as previously elucidated, takes on a particularly crucial role, highlighting the exceptional significance of this problem. Copper fungicides are applied as preventive contact foliar product which remains deposited on the leaf surface, not being absorbed by the plant. For this reason, the metal easy reaches the ground though the action of the wind, or after being washed by the rain (Pérez-Rodríguez et al., 2015). Even copper is an essential element to life, which is present in all the environmental media naturally, due to the prolonged use of copper-containing products over time in pest management strategies, it has been accumulated specially in the soil, and the tendency is to be increasing (Droz et al., 2021a). Copper causes soil and water contamination mainly thought the crop protection agricultural activities according to Apori et al, (2018). There is a relation between the traditional vineyard cultivation areas of Europe, the Cu concentration in the topsoil, as it can be seen in Ballabio et al. (2018). Fernández-Calviño et al. (2008) founded an average copper concentrations of 31 mg kg^{-1} in woodland soils, 30 mg kg^{-1} in pasture soils, and eight times higher, 246 mg kg^{-1} (range 157 – 434 mg kg^{-1}), in productive vineyard soils. Since copper is a heavy metal, it cannot be biodegraded or transformed like the organic compounds, which make the accumulate into the soil or circulate to surface or ground water courses (Eisler, 1998). Cu ions into the soil, have different behaviour depending on the soil characteristics, but the most relevant ones are the organic matter contain and the pH (Sauvé et al., 1997). Fernández-Calviño et al., (2008) found in their studies that about fifty per cent of copper content into the soil by was generally bound to soil organic matter, highlighting the importance of this parameter in the soil and the accumulation of copper. Moreover, it tends to boned with the organic matter of the soil getting strongly attached into the ground composition. Although it is also a micronutrient for plants, its uptake is low, and has so far been balanced by the amount that is lost to the soil through the spray application process. Furthermore, these high concentrations can have a bad impact in many levels of the soil biodiversity. Some studies suggested that copper can supress nitrogen fixation rates, at high concentrations (Addo-Fordjour et al., 2013), creating a nutrition deficit in one of the main macronutrients in plants. It has also been observed, that these high copper concentrations of the vineyard soils can affect also to the enzymatical activity of the microorganisms that lives int it (Fernández-Calviño et al., 2010). Furthermore, that extensive contain of copper fungicides also affect to macroinvertebrates, such as eliminating large earth worms of the soil (Norgrove, 2007).

Not all the copper remains fixed in the soil, so some of it is mobilized into surface or underground watercourses. Rivers close to cultivated areas with high copper concentrations into the soil can also have high concentrations of this metal in their water (Xue et al., 2000). Also in river sediments, has been found a relation between the coper concentration and the proportion of close vineyard land ($r = 0.915$, $p < 0.05$), ranging from 18 to 209 mg kg^{-1} (Fernández-Calviño et al., 2008). Copper can also leach from the surface to the groundwater, and may also affect contaminating potentially drinking water (Dousset et al., 2010; Hildebrandt et al., 2008). But the risk of it leaching into the water carries with it an implicit risk of affecting wildlife. It has been shown that copper used as a fungicide in vineyards can cause an ecotoxicity impact (Peña et al., 2018). It can result hazardous to invertebrate, fish, vertebrates, and mammals depending on the concentrations, and may have lethal or sublethal effects (Eisler, R. 1998). Human health can also be affected, Rengaraj et al, (2004) found that elevated levels of copper may cause health damages such as liver and kidney failure or Wilson’s disease and Asthma or other respiratory diseases (Mamane et al., 2015; Raherison et al., 2019).

Recent studies have highlighted transformation products (TPs) as pollutants, in addition to the contamination caused by conventional pesticides (Krier et al., 2022). These types of substances can also be long-persistent in the soil and remain in it even more than the parental compound. According to a study by Chiaia-Hernandez et al., (2017), TPs were found in 47% of the cases where the original pesticide was applied. The study discovered that residues of nearly 80% of all applied pesticides were present, with half of them being TPs that persisted for over a decade.

vi. EU legislation trends on plant protection products use

Over the past two decades, European legislation has been actively promoting a shift towards more sustainable agricultural practices. The European Union is committed to a sustainable use of pesticides and protecting surface waters. This is reflected in initiatives such as the Sustainable Use of Pesticides Directive, which requires the use of Integrated Pest Management (IMP) as per Directive 2009/128/EC, and the protection of surface waters as per Directive 2000/60/EC. Recently, the European Union has announced the Farm to Fork (F2F) strategy, which aims to reduce the use of plant protection products by 50% by 2030. The baseline for this reduction will be calculated using the average sales of 2015, 2016 and 2017, which were the three most recent years for which data were available at the time of the announcement of the F2F Strategy. Nowadays, this tendency is getting even stronger trying to transform the current economic model to a new one, more productive and respectful reducing environmental pollution. It is part of the Green Deal (EC, 2019), which addresses different lines of action, and aims to transform the European agrarian systems into a more sustainable sector, with the lowest possible environmental impact, while increasing the level of food quality. It also pushes farmers to become into organic growing systems, which have a growing tendency. In the case of vineyards, choosing organic management does not suppose a risk of vine grape production, and it improves mitigations on the environmental impacts, according to Borsato et al. (2020) so it an easy step to make. Although, organic vineyard, makes a higher use of copper-based fungicides compared to a conventional growing system (Tamm et al., 2022), since there are no chemical products authorized in this type of farming, and the use of biological plant protection products is not really extended nowadays. Therefore, it makes these farmers more dependent to copper for their crop protection proposes. But of course, copper is no exception, and legislation is increasingly restrictive on its use as a fungicide, due to its accumulation over time and the risk it poses. Its use as a fungicide has been limited to 28 $kg \cdot ha^{-1}$ for a total period of 7 years (commission implementing regulation EU 2018/1981 of 13 December 2018). Alternative ways of protecting vines are emerging but they are still marginal, so copper still being the main fungicide used in vineyard crop protection.

vii. Future challenges for sustainable solutions

There are different research approaches to address vineyard contamination caused by pesticide use. Resistant/tolerant cultivars to downy mildew may provide a solution for reducing fungicide crop protection treatments, however, planting such hybrids in large areas has led to a decrease in the characteristics of the grape and the wine ("Genetics, Genomics, and Breeding of Grapes," 2016). Another way of action is by reducing the number of applications performed during the crop campaigns. Most of the time, fungicides are overused, and an example is that the first fungicide treatment is applied at least six weeks before disease onset in more than 50% of the vineyards according to Chen et al. (2020). In addition, decision support systems (DSS) are tools that can help farmers reduce the amount of pesticides applied (Pertot et al., 2017) and are increasingly being developed on a crop- and area-specific basis (Trilles et al., 2020). Alternatively, another approach to reduce the amount of product is by adjusting the dose rate required. This can be adjusted to the characteristics of the canopy at the time of treatment as well as to the type of sprayer used, thus adjusting the volume to the state of the specific field conditions at the time of application (Gil et al., 2019). In the same line, improving the spraying application technologies can also be a way to reduce the amount of pesticides applied in vineyard or the pollution produced. New machines like tunnel sprayers, with recovery panels can help to reduce the soil ground loses (Pergher & Petris, 2009). variable rate application (VRA), adjusts the volume rate to the crop characteristics with a map (Campos et al., 2019, 2020) or real time (Llorens et al., 2010). Even so, most of the times making a good calibration and adjustment of the sprayer, together with a good maintain and correct inspection, can reduce significantly the plant protection products needed, by increasing the efficiency (Pergher, 2004).

Acting on the products or the formulates is another way to address this problematic. Microencapsulation is a new way to achieve the reduction of pesticides. Nowadays, microcapsule of pesticides can act based on with different stimulus-responsive performance for pH value, temperature, and enzymes (Cen et al., 2022; Li et al., 2023), making them more efficient in terms of crop protection, compared to the conventional products. This approach allows for greater specify in product timing, aligning it with disease or meteorological conditions. For instance, it can result in a reduction of the amount of product used, which is directly linked to environmental pollution. Another alternative type of product is biopesticides, mostly derived from botanicals or biocontrol agents. They offer another way for reducing treatment-related contamination. There are many types of biopesticides, and they are classified based on their extraction sources and the type of molecule/compound used for their preparation (J. Kumar et al., 2021). However, the common thread among them is that they consist of natural or biologically occurring compounds already present in the environment.

Once pollution has already occurred, there are different lines of research to contain, mitigate or remediate the impact of pollution, including strategies such as cover crops or mulching. Research has shown the potential of aerated compost tea to enhance8 the mobility and phytoextraction of copper in vineyard soil (Eon et al., 2023). Also, the use of biochar has been analysed to capture the generated contamination (Varjani et al., 2019). Cover crops have been studied as another approach. These are plants that either grows spontaneously or are intentionally planted between the rows of the main crop. Their introduction into cropping systems has been shown to reduce pesticide transfer in soils (Cassigneul et al., 2015). This effect is likely associated with their other beneficial actions, such as enhancing soil biological activity, increasing organic matter content, and improving soil water dynamics compared to bare soil during the fallow period (Potter et al., 2007; Reeves, 2018). Vegetated strips operate in the same principle and offer an additional means to mitigate pollution, or at the very least, reduce its impact, but at the field margins. These strips serve as a physical barrier to runoff water, including with pesticides from the spray applications, and contribute to reducing soil pesticide content. Additionally, they can help mitigate the off-field flow of nutrients (Prosser et al., 2020). Unfortunately, all existing research is conducted with herbicides, whereas in the context of vineyards, it is more pertinent to study the behaviour of fungicides.

The focus of this work is to propose solutions for preventing and mitigating contamination resulting from fungicides. These solutions can be applied in the short-term, while also exploring long-term options that offer a more sustainable approach to fungicide application practices.

CHAPTER II. Structure and objectives

Considering the ongoing contamination issue arising from fungicide use in vineyards, the main objective of this thesis is to explore different approaches to reduce this pollution. To achieve this goal, three different strategies has been assessed, each addressing the issue from a different starting point (Figure2.1): i) pollution attenuation (chapter III); ii) copper reduction (chapter IV); iii) biopesticides use as an alternative (chapter V).

To address pollution mitigation, the option of cover crops was chosen. Cover crops are easy to implement and have been proven effective in the case of herbicides. The following specific objectives have been outlined:

- To study the use of cover crops and their impact on reducing fungicide leaching and their capacity to degrade PPPs.

- To determine the influence of soil type and cover crop biodiversity, on potential reduction of contamination.

- To evaluate the effectiveness of using vegetated buffer strips in decreasing fungicides runoff and their role in fungicides elimination and the generation of transformation products.

Moreover, copper reduction was addressed by evaluating the use of a new microencapsulated product, given the participation of this group in the COPPEREPLACE project (https://coppereplace.com/):

- To evaluate a new microencapsulated copper product with the aim of reducing copper usage and assessing its environmental impact.

Finally, in the context of exploring the use of biopesticides as a replacement for copper, the main aim is to understand why such products are not widely used, despite already being available on the market:

- To identify the factors influencing farmers' adoption of biopesticides as a substitute for copper-based products.

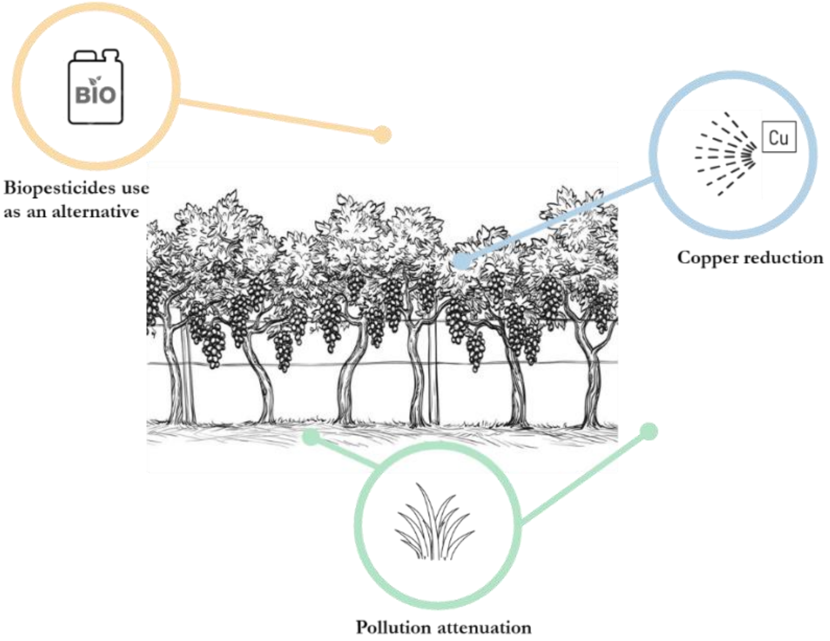

Figure 2.1: *Graphical abstract of the thesis objectives*

CHAPTER III. Evaluation of the use of vegetation to mitigate fungicide pollution in vineyards

Many fungicides have been recorded as hazardous substances for water bodies and their living organisms. From crop protection treatments, these compounds reach mainly the water by leaching or runoff. Attenuation of this type of pollution, using cover crops or vegetated buffer zones has been demonstrated useful in other types of pesticides, such as herbicides. Therefore, in this work, its use has been studied to reduce contamination by copper-based fungicides, as well as other fungicides used to control downy mildew.

3.1 Article 1: Use of cover crops in vineyards to prevent groundwater pollution by copper and organic fungicides. Soil column studies

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Use of cover crops in vineyards to prevent groundwater pollution by copper and organic fungicides. Soil column studies

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HIGHLIGHTS

- Fungicide soil leaching was highly affected by soil texture.
- Crop covering reduced fungicide soil leaching.
- Rhizosphere interaction time is key for reducing fungicide leaching.
- Crop roots enhanced the depletion kinetic rates of almost all tested fungicides.

GRAPHICAL ABSTRACT

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ABSTRACT

Several fungicides, such as copper and organic products (synthetic or natural), are currently being used in vineyards to control downy mildew (*Plasmopara viticola*) resulting in soil, surface water, and groundwater pollution. This study aims to assess the effectiveness of using cover crops as an agricultural practice in vineyards to protect soil and groundwater pollution. For that purpose, we performed different soil column studies to quantify soil leaching of selected fungicides (copper, dimethomorph, oxathiapiprolin, zoxamide, acibenzolar-s-methyl, and laminarin) following a rainfall event after a conventional fungicide vineyard application. Two types of vineyard soils (loam and sandy-loam soil textures) and three ground covers (bare ground, monoculture cover, and polyculture cover) were assessed. These studies were completed with hydroponic assays to check the effectiveness of cover roots in the fungicide degradation. Mass balance results show that whereas 3 fungicides (Cu, zoxamide, and dimethomorph) were leached through sandy soil columns, only copper was leached from loam soil columns. The effect of cover crops was only significant for Cu and zoxamide when fungicides were applied 24 h before the rain event, reducing the fungicide leaching by 30%. Hydroponic studies showed that cover roots enhanced the kinetic rates of almost all tested fungicides by 5-467%, suggesting that they are relevant to improving the degradation of fungicides in the soil column. These results are relevant to drawing up recommendations on the use of cover crops to protect soil and groundwater pollution by fungicides.

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Use of cover crops in vineyards to prevent groundwater pollution by copper and organic fungicides. Soil column studies

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Abstract

Several fungicides, such as copper and organic products (synthetic or natural), are currently being used in vineyards to control downy mildew (*Plasmopara viticola*) resulting in soil, surface, and groundwater pollution. This study aims to assess the effectiveness of using cover crops as an agricultural practice in vineyards to protect soil and groundwater pollution. For that purpose, we performed different soil column studies to quantify soil leaching of selected fungicides (copper, dimethomorph, oxathiapiprolin, zoxamide, acibenzolar-s-methyl, and laminarin) following a rainfall event after a conventional fungicide vineyard application. Two types of vineyard soils (loam and sandy-loam soil textures) and three ground covers (bare ground, monoculture cover, and polyculture cover) were assessed. These studies were completed with hydroponic assays to check the effectiveness of cover roots in the fungicide degradation. Mass balance results show that whereas 3 fungicides (Cu, zoxamide, and dimethomorph) were leached through sandy soil columns, only copper was leached from loam soil columns. The effect of cover crops was only significant for Cu and zoxamide when fungicides were applied 24 hours before the rain event, reducing the fungicide leaching by 30%. Hydroponic studies showed that cover roots enhanced the kinetic rates of almost all tested fungicides by 5-467%, suggesting that they are relevant to improving the degradation of fungicides in the soil column. These results are relevant to drawing up recommendations on the use of cover crops and the application of fungicides to protect soil and groundwater pollution.

Keywords: Copper · Fungicides · Cover crops · Vineyard · Soil leaching · Groundwater pollution

1. Introduction

Downy mildew (*Plasmopara viticola*) is one of the most severe and devastating diseases for grapevines (A. Hadwiger, 2005). Although there are various plant protection products (PPPs) (copper and organic fungicides) that can solve this issue, viticulture has traditionally used copper-based products as a fungicide, which is the most efficient way controlling it, and has become an indispensable product for a large number of grape growers worldwide (Dagostin et al., 2011a). The reliance on this product is even higher for organic farming since there are no synthetical alternatives accepted to be used and copper-based pesticides are indispensable for organic vine cultivation (la Torre et al., 2007). Therefore, the continued use of these kinds of products in viticulture has triggered the accumulation of this metal in several vineyard area soils around Europe and worldwide (Ballabio et al., 2018; MacKie et al., 2012; Rusjan et al., 2007) which may even affect the productivity of food crops (Adrees et al., 2015). Although Cu is fixed by organic and clayey matter content in the soil, Sonoda et al.(2019) observed copper mobility due to organic matter degradation (Pensini et al., 2021), suggesting possible groundwater contamination. The high potential ecotoxicity of copper in freshwater (Peña et al., 2018) and its accumulation in European soils have raised concerns in the European Union, leading to a tightening of the legislation on the use of copper-based products, limiting the use to 28 kg/ha of copper for seven years (Commission Implementing Regulation EU 2018/1981).

Even though other PPP such as synthetic organic fungicides could be considered to replace copper in conventional vineyard production, their use is also not exempt from environmental risks such as soil accumulation and surface or groundwater pollution (Peña et al., 2018) depending on their composition and the site characteristics (soil properties, climate, and site conditions) (Arias-Estévez et al., 2008; Bereswill et al., 2012; Peña et al., 2018). For example, a recent publication study (Zambito Marsala et al., 2020) observed that synthetic organic fungicides (dimethomorph and pyrimethanil) used for grapevine cultivation in northern Italy were present in 80% of monitored water wells, exceeding the Environmental Quality Standards (EQS) limits for groundwater in 30% of them. The toxicity of dimethomorph has been observed in several living organisms, such as fish and invertebrates (Lunn et al., 2007), other soil organisms (Cycoń et al., 2010; Wang et al., 2017), soil and water microflora (Andreazza et al., 2010) and aquatic plants (Dosnon-Olette et al., 2009, 2010; Megateli et al., 2013). Concerning zoxamide, low risk was observed for non-target arthropods, soil microorganisms, and non-target terrestrial plants (EFSA, 2017).

The increase of organic vine cultivation during recent years has increased the application of other kinds of organic fungicides, those named natural or biopesticides, produced from a natural source (plant parts) and with minimum adverse effects on the physiological processes of plants (Dagostin et al., 2011a; Zaker, 2016), as is the case of laminarin, which is considered to have low environmental toxicity by EFSA (2017). However, although some ecological products are considered good candidates for reducing copper dependency in vineyards, there is no treatment as effective as copper for controlling grapevine downy mildew (Dagostin et al., 2011, Kraus et al., 2021). The replacement of copper-based products can be done with the use of plant resistance-inducers (PRIs) or biological control agents (BCAs) (Tziros et al., 2021), which have no environmental hazards.

Henceforth, the use of PPPs such as copper-based and organic fungicides in agriculture can involve a human threat due to the intake of crops cultivated under those conditions (Fantke et al., 2012) and a negative impact on other non-target receptors such as water, other plants, and animals. In addition to promoting the use of organic fungicides in viticulture, the sustainable use of PPPs in agriculture must include an application rate according to the characteristics of the vegetation and adjusted spraying, in order to reduce product losses due to drift (Arias-Estévez et al., 2008; Felsot et al., 2011; E. Gil et al., 2021). Despite this, it is worth knowing that active substances of pesticides are washed away by rainfall, causing the deposition of pesticides on the soil (Pérez-Rodríguez et al., 2017), which means continuing to seek solutions for preventing groundwater contamination from agricultural activity.

Bare soil, commonly used in low rainfall areas, allows the rainfall water to drag with it the PPPs losses remaining on the topsoil without any obstacles and can even be simulated and modelled (Thinh et al., 2019). Moreover, erosion and nutrient loss in bare soil would be more pronounced due to torrential rainfall events that are expected to be more recurrent due to climate change (IPCC, 2014). On the contrary, cover crops in vineyards have been proven to be effective in protecting the soil from erosion and nutrient loss, improving soil fertility, structure, soil microbial functional diversity, and balancing the productive, and vegetative parts of the vine (Novara et al., 2020). The use of cover crops could also be beneficial in reducing the copper content in the soil (Andreazza et al., 2010). Although the phytoextraction effect of covers in viticulture is not sufficient to eliminate the entire volume of copper annually applied as a phytosanitary product against mildew (Andreazza et al., 2010; Mackie et al., 2014), cover crops are suggested to reduce the amounts of pesticide leached and, consequently, the risk of groundwater contamination (Dousset et al., 2010). Despite this, there has been no research on the effect of covers on the soil leaching of the most commonly used organic fungicides in vineyards (dimethomorph, oxathiapiprolin, zoxamide, acibenzolar-s-methyl, and laminarin) (http://optima-h2020.eu/es/16219-2/).

The main objective of this work is to demonstrate the capability of using cover crops in the mitigation and prevention of soil and groundwater pollution caused by the application of fungicides against mildew in vineyards. Also, studying if the biodiversity of this cover crops has an effect on it. Copper, 3 conventional/synthetic organic fungicides (dimethomorph, oxathiapiprolin, and zoxamide, and acibenzolar-s-methyl), and 1 ecological fungicide (laminarin) were selected. The specific objectives of this work were as follows: i) assessment of the soil type on fungicides leaching, ii) quantification of pollution reduction due to the use of cover crops, and iii) analysis of the vegetation effect on fungicides degradation.

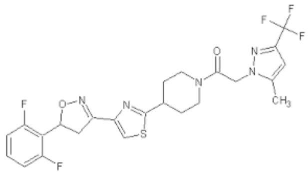
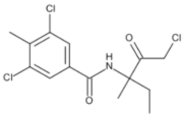
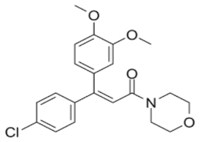
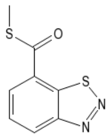
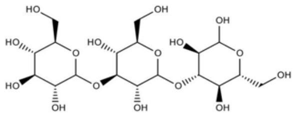
2. Materials and methods

2.1. Fungicide selection and toxicity

Five different PPPs (Table 3.1.1) were selected based on previous trials carried out at OPTIMA project (Optimised Pest Integrated Management to precisely detect and control plant diseases in perennial crops and open-field vegetables, H2020 Grant Agreement N.773718, http://optima-h2020.eu/es/16219-2/) according to fungicide efficacy against downy mildew in vineyards and lower environmental impact (Pugliese et al., 2021).

Table 3.1.1 shows the commercial name, register number, the active ingredient, the molecule structure, the octanol-water partition coefficient (Kow) and the environmental risk for each of the fungicides studied. Codimur 50 (Copper oxychloride), which is a copper-based product used as preventive treatments, is used as a control/reference treatment due to its preferential use in viticulture against mildew. Forum (dimethomorph) and Zorvec Vinabel (oxathiapiprolin and zoxamide) are novel synthetic organic fungicides, with a systemic action, with dual preventive and curative effect whereas Bion MX (Acibenzolar-s-methyl) is a synthetic inducer and activator of plant self-defense mechanisms. Finally, Vacciplant (laminarin) is an ecological organic fungicide product. All experimental studies were performed by using commercial fungicides supplied by the manufacturer's brands (Zorvec Vinabel and Forum) or purchased from Agrogava (Barcelona, Spain) (Codimur 50, BionMX and Vacciplant).

Table 3.1.1*. Formulates, chemical characteristics, soil adsorption coefficient (Kow) and environmental hazard of selected fungicides according to the Spanish Register of Phytosanitary Products (https://www.mapa.gob.es/es/agricultura/temas/sanidad-vegetal/productos-fitosanitarios/fitos.asp).*

Comercial name and register number	**Active ingredient**	**Molecule structure**	**Log (Kow)**	**Environmental hazard[1]**
Codimur 50® (23622)	Copper oxychloride 50% [WP] P/P	Cu^{+2}	-	Acute aquatic 1 Chronic aquatic 1 Very toxic to aquatic organisms, with long lasting effects.
Zorvec™ Vinabel® (ES-01243)	Oxathiapiprolin 4 % (P/V) 40 g/L		5.74 (EPI Suite™).	Acute aquatic 1 Chronic aquatic 1
	Zoxamide 30 % (P/V)		3.76 (PubChem 122087).	Very toxic to aquatic organisms, with long lasting effects.
Forum® (19411)	15.0 % (p/v) Dimethomorph		2.68 (PubChem 5889665).	Acute aquatic 1 Chronic aquatic 3 Very toxic to aquatic organisms, with long lasting effects.
Bion MX® (22598)	Acibenzolar-s-methyl 50% [WG] P/P		3.1 (PubChem 86412).	Chronic aquatic 1 Very toxic to aquatic organisms, with long lasting effects.
VACCIPLANT® (25561)	Laminarin 4,5% [SL] P/V		-7.10 (EPI Suite™)	-

[1]Classification based on Directive 67/548/EEC and Directive 1999/45/CE;

1.2. Experimental set-up

Experiments were conducted in the research greenhouse facilities of the Agropolis Research Centre from Polytechnic University of Catalonia (UPC, Viladecans, Spain) during May 2021. The average temperature inside the greenhouse was 21 ◦C (16 ◦C min and 28 ◦C max) and the relative humidity was 47% (45% min and 52% max). Experimental units consisted of 2.5 L cylindrical amber glass columns (Ø = 15 cm and 20 cm high, surface area of 160 cm2) fitted with a bottom outlet connected to drainage tubing (Ø = 3 cm) through which the leachate was collected (Figure 3.1.1A). Two vineyard soil types from commercial vineyard fields located in the Tarragona region of Catalonia (Spain) were selected. One of them, hereafter quoted as sandy, had a sandy-loam texture (80.7% sand, 11.3% silt, and 8% clay) and 0.29% of total organic carbon content. The other soil, hereafter quoted as loam, had a loam texture (51.2% sand, 30% silt, 18.8% clay), 0.95% of total organic carbon content. Further details on the soil physicochemical characteristics are shown in Table 10.1.1-supplementary material (SM). Nine columns of each soil types were filled with 2.3 kg of air-dried soil sieved to a 2 mm particle size. Moreover, three different soil covers were tested in the two soil types to evaluate the capacity for leaching attenuation of the PPPs: Bare ground (BG) which is the traditional way to maintain the soil on vineyard crops, monoculture cover (MC) with grass plants (Lolium perenne) and a polyculture cover (PC) with a mix of gramine and legume species (Trifolium subterraneum; Trifolium resupinatum; Trifolium michelianum; Biserrula pelecinus, Medicago polymorpha, Medicago truncatula, Hedysarum coronarium; Trifolium cherleri; Trifolium isthmocarpum; Dactylis glomerata; Lolium perenne; Lolium multiflorum; Festuca rubra). Columns were planted with 1 g of seeds for the monoculture cover crop (450 ± 22 seeds, on average for each column) and 1.5 g for the polyculture one (420 ± 19 seeds on average for each column). The aim was to obtain a 100% of vegetation coverage. Columns were watered three times a day 2 min each time with a flow rate of $2L\cdot h^{-1}$. After concluding the experiment, the cover crops were dried (TCF 400 Argo lab, Italy) at 60 ◦C until constant weight and shoots and roots biomass were weighted separately. Monoculture crop had an average of 6.1 g ± 1.0 and 21.5 g ± 2.1 for shoots and roots, respectively, whereas polyculture crop had an average of 8.9 g ± 0.7 and 11.2 g ± 0.7 for shoots and roots, respectively.

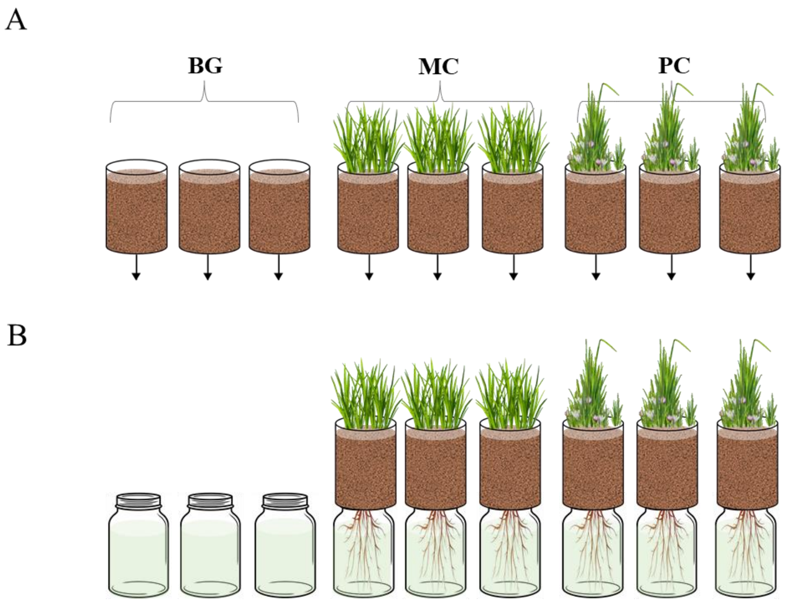


Figure 3.1.1. *A) Experimental design layout for leaching assays; B) Experimental design layout for hydroponic assays. Bare ground (BG), monoculture cover (MC) and polyculture cover (PC).*

1.3 Fungicide leaching assays

Fungicide soil leaching assays were carried out in nine columns for each soil type with three different soil cover strategies, as shown in Figure 3.1.1-A. Monoculture and polyculture covers were developed for one month until the end of the tillering stage, when the fungicide assay started. It is important to note that at this stage, above and below-ground parts of gramineous plants were covering all soil columns (Fig 10.1.1-SM)

During the spray application process carried out for crop protection, there is an inevitable loss of fungicides to the ground. According to Gil et al. (2001), the amount of ground losses due to the spraying application process in a vineyard was calculated as 4.62 $\mu g \cdot cm^{-2}$ for an application of 1kg $Cu \cdot ha^{-1}$ under the worst conditions (a primary crop stage and a conventional sprayer). Therefore, based on those experiments, and taking into account the highest recommended doses of the selected products according to their labels, ground losses for each selected formulation were estimated as indicated in Table 3.1.2 and applied using a manual sprayer to each soil column. After application, a rainfall event of 120 mm was immediately simulated during a period of 2 hours to imitate an average rainfall event from the Tarragona region for the treatment months ([Meteorological Service of Catalonia (SMC)]). A total of 2 L of rain water was supplied to each soil column. The leachate water from each column was collected in consecutive 200 mL samples, and the water leaching time was recorded.

***Table 3.1.2**. Products maximum label dose, expected product losses and expected active ingredient losses of selected fungicides according to Gil et al. (2001).*

Commercial product name	**Label higher dose (Kg·ha^{-1})**	**Expected product losses (µg·cm^{-2})**	**Expected active ingredient losses (µg·cm^{-2})**
CODIMUR 50 (Copper oxychloride)	3	13.86	6.93
ZORVEC VINABEL (Oxathiapiprolin and Zoxamide)	1.37	6.33	0.25 Oxathiapiprolin 1.89 Zoxamide
FORUM (Dimethomorph)	2.5	11.55	1.73
BION MX (Acibenzolar-s-methyl)	0.3	1.38	0.69
VACCIPLANT (Laminarin)	2	9.24	0.41

A second experimental process was repeated one week later, only in the sandy soil columns, increasing the time between fungicide application and rain event simulation to 24 hours, to study the interaction of the pesticides with the rhizosphere. In this case, leachate water samples were collected every 500 mL, as a lower concentration of the fungicides was expected. As in the previous study, the time between water sample collection was recorded.

1.4 Hydroponic assay

At the IDAEA-CSIC laboratory, hydroponic experiments were performed to study the role of plants in the attenuation of selected fungicides. The six sandy soil columns with vegetation, from previous studies were placed in 800 mL water nutritional solution glass containers as shown in Figure 3.1.1B. The drainage system was removed from the bottom of the columns to let the roots come out. Cover cropped soil columns were placed under room conditions with 35 µmol m^{-2}s^{-1} light intensity and a 12 h:12 h light/darkness cycle. The nutrient solution composition was as follows: 516 mg·L^{-1} KNO_3, 820 mg·L^{-1} $Ca(NO_3)_2$, 130 mg·L^{-1} KH_2PO_4 , 33.8 mg·L^{-1} TARSSAN® MIX and 223 mg·L^{-1} $MgSO_4$. The containers were covered to avoid sunlight and UV exposure. Moreover, three more containers with nutritive solution were left without vegetation as control containers. After 15 days, the roots elongated until they were out of the column, and reached the containers. The containers, were then spiked with up to 500 µg·L^{-1} of each PPP, including control containers without roots. At 0, 24, 48, 72, 96, 168 and 240 h, 20 mL water samples were taken. The water solution level was topped up to maintain the same conditions (800 mL) in all columns during the whole process. The refill quantities were annotated to recalculate the real fungicide concentrations.

1.5 Analytical methodologies

Fungicides were measured as follows. Water samples were filtrated with a 0.22 µm hydrophilic PTFE filter (Frisenette, DK), and, 400 ng of diclofenac were added as an instrumental standard. The samples were then injected into a Nexera X2 ultra high-performance liquid chromatograph (UHPLC) equipped with a photodiode array detector (SPD-M30A) (Shimadzu UK Ltd, Milton Keynes, UK). The chromatographic separation was achieved on a core–shell Ascentis® Express RP-Amide column (15 cm × 2.1 mm, 2.7 µm particle size, Supelco, Bellefonte, USA) with a guard column (0.5 cm × 2.1 mm, Supelco, Bellefonte, USA) containing the same packing material. The flow rate and injection volume were 0.35 mL min^{-1} and 25 µL, respectively. More information about the reagents used and the binary gradient elution programme is contained in the supplementary material (SM) section. Linearity ranged from 2.5 to 2000 µg L^{-1}. The used wavelength was specific for each compound (dimethomorph 235 nm, zoxamide 211 nm, acibenzolar-s-methyl 325 nm, oxathiapiprolin 258 nm and laminarin 225 nm).

A Varian SpectrAA 110 (Mulgrave, Victoria, Australia) flame atomic absorption spectrometer, equipped with a copper hollow cathode lamp, and a deuterium lamp for background correction, was used for the determination of copper in water samples. The instrument was operated under the conditions recommended by the manufacturer: lamp current of 4 mA, wavelength of 324.7 nm, slit width of 0.1 nm, burner height of 14 mm, acetylene flow rate of 1.0 L min^{-1} and air flow rate of 10.0 L min^{-1}. Linearity ranged from 0.5 to 5 mg/L.

1.6 Data analysis

The mass balance of the leached PPPs was calculated for each soil column condition, taking into account the amount of fungicide losses and the concentration contained in each active ingredient. The experimental results were statistically analysed using RStudio (RStudio Team, 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). The Shapiro–Wilk test was used to check the normal distribution of the variables and their homoscedasticity. ANOVA test was performed to compare differences between the different hypotheses. HSD Tuckey test was conducted to find the group means that are different from each other. The same procedure was applied to analyse the statistical differences between the soil leaching flowrates and the hydroponic study results.

3. Results and discussion

3.1. Effect of soil type on the water infiltration

Table 3.1.3 shows the water flow rates (mL·s^{-1}) for each soil type and each cover type in the first performed trial. Loam soil shows a statistically lower water leaching rate in relation to sandy soil (0.15 *vs*. 0.25 mL·s^{-1}, p-value<0-05), which means an increment in the time interaction between fungicides and soil, and a higher potential soil's capacity to retain and/or degrade them. The results are in agreement with Ma et al. (2016), who found that water infiltration rate and cumulative infiltration were higher in sandy than in loamy soils. According to Liu et al. (2019), soil moisture is the main factor affecting water conductance, decreasing the water infiltration capacity. This is consistent with the fact that loam soil has a lower infiltration rate, as it retains more moisture. The presence of vegetation had no statistical effect on the water leaching rate for the sandy or loam soil (Table 3.1.3). This is partially in disagreement with current studies that indicate that plant covering increases water soil infiltration (Islam et al., 2021; Gulick et al., 1994). This difference can be explained by the fact that in our study we assessed the water leaching rate, so plant evapotranspiration or moisture retention by roots can reduce water leaching. This is in agreement with the soil retention studies performed by Leung et al (Leung et al., 2015) who observed that rooted soil preserved higher water suction than bare soil by 100%–160% during rainfall. Therefore, future water infiltration studies should also take into consideration water leaching to explore the real impact of water infiltration on groundwater table. Furthermore, root biomass, even it was different between cover crops (21.5 vs. 11.2 g for monoculture and polyculture crop roots respectively), did not show statistical differences.

Table 3.1.3. *Leaching flow rate for the different soils and cover types. Average ± standard deviation (n=3). HSD Tukey (P < 0.05) differences expressed with a letter.*

Soil type	**Cover crop type**	**Q (mL·s^{-1})**	**Tukey**
Sandy soil	BG	0.24 ± 0.015	a
	MC	0.26 ± 0.001	a
	PC	0.26 ± 0.001	a
Loam soil	BG	0.17 ± 0.007	b
	MC	0.16 ± 0.003	b
	PC	0.14 ± 0.012	b

3.2. Effect of soil and cover crops on fungicide leaching

Figure 3.1.2 shows that the most relevant parameter for fungicide leaching was the soil composition. Despite 6 fungicides being applied on the soil surface, only 3 (Cu, zoxamide, and dimethomorph) were detected in the leached water from the sandy soil columns, and only one (copper) was leached through the loam soil columns. Oxathiapiprolin, laminarin, and acibenzolar-S-methyl were not detected in the leaching water from any of the studied soil columns and conditions. The absence of these compounds in the leachates could be due to the low calculated ground losses taking into account the recommended application dose (Table 3.1.2), but also to the high soil sorption coefficient in the case of oxathiapiprolin (log Kow 5.74) and the high biodegradation rates described for laminarin and acibenzolar-S-methyl in aquatic and soil environments (highly biodegradable compounds by prediction BIOWIN models contained in EPI Suite™). Figure 2 indicates that copper shows a linear increasing trend for the three column types (BG, MC, and PC) in both soils, whereas dimethomorph and zoxamide behave differently in bare soil columns than in vegetation covered soil columns. Both accumulated leached synthetic fungicide products continued to maintain the linear trend in cover cropped soil while bare soil columns showed a sigmoid curve for dimethomorph and an exponential curve for zoxamide. This may indicate that at the beginning, the bare soil columns were capable of retaining greater amounts of these fungicides, but later on, the retention capacity of cover cropped columns were greater.

The mass balance assessment shows that fungicides (Cu, zoxamide, and dimethomorph) leached through the cover cropped sandy soil columns by a range of 7 to 64% (Figure 3.1.3). Specifically, dimethomorph was leached from 37 to 64%, whereas these leaching values were 16-21% for zoxamide and 7-15% for copper. These results are in agreement with pesticide leaching values found by Singh et al. (2002) in packed soil columns (silt loam texture) for metolachlor and terbuthylazine (3-38%). The greatest leaching of dimethomorph in comparison to zoxamide is in accordance with the reported sorption coefficient for these compounds (log Kow of 2.68 for dimethomorph and 3.76 for zoxamide, Table 3.1.1). Although our results show a high dependence on soil texture, no significant differences were observed between covered soil columns and bare soil columns in sandy soil. These results are in disagreement with Dousset et al. (2010) who found that lower amounts of pesticides leached through cover cropped soil columns (2.7–24.3% of the initial amount) than bare soil columns (8.0–55.1%). These differences can be explained due to the soil composition. While our sandy soil has a sandy-loam texture, the soil used by Dousset et al. (2010) had a sandy texture, and as it has been seen in Table 3.1.3, the texture of the soil affects the water leaching rates, thus the leaching of the fungicide products. Furthermore, differences in cover species types or water loading rates employed in each study can explain fungicide mass leaching differences.

However, mass balance studies performed on loam soil columns show that fungicide leaching was much lower than that from sandy soil columns, probably due to the longer soil interaction time (Table 3.1.3) as well as the higher organic matter and clay content of the loam soil, except for Cu which showed greater mass leaching in the loam soil columns. It is important to notice that loam soil had a very high concentration of copper (21.3 mg dw Kg^{-1}), whereas it was low in sandy soil (4.8 mg dw Kg^{-1}), so copper leaching was strongly affected by copper soil composition. The greater retention of fungicides by loam soil is in agreement with different studies that have found that organic matter and clay play a relevant role in enhancing the soil retention of pesticides and copper (Copaja, 2021; Uchimiya et al., 2011; Sauvé et al., 1997). Furthermore, our results show that the use of covers on loam soils significantly reduced the amount of copper leached through the columns (from 15% to 9 and 11% for MC and PC columns, respectively) probably due to the longer water soil contact time in these columns (Duplay et al., 2014).

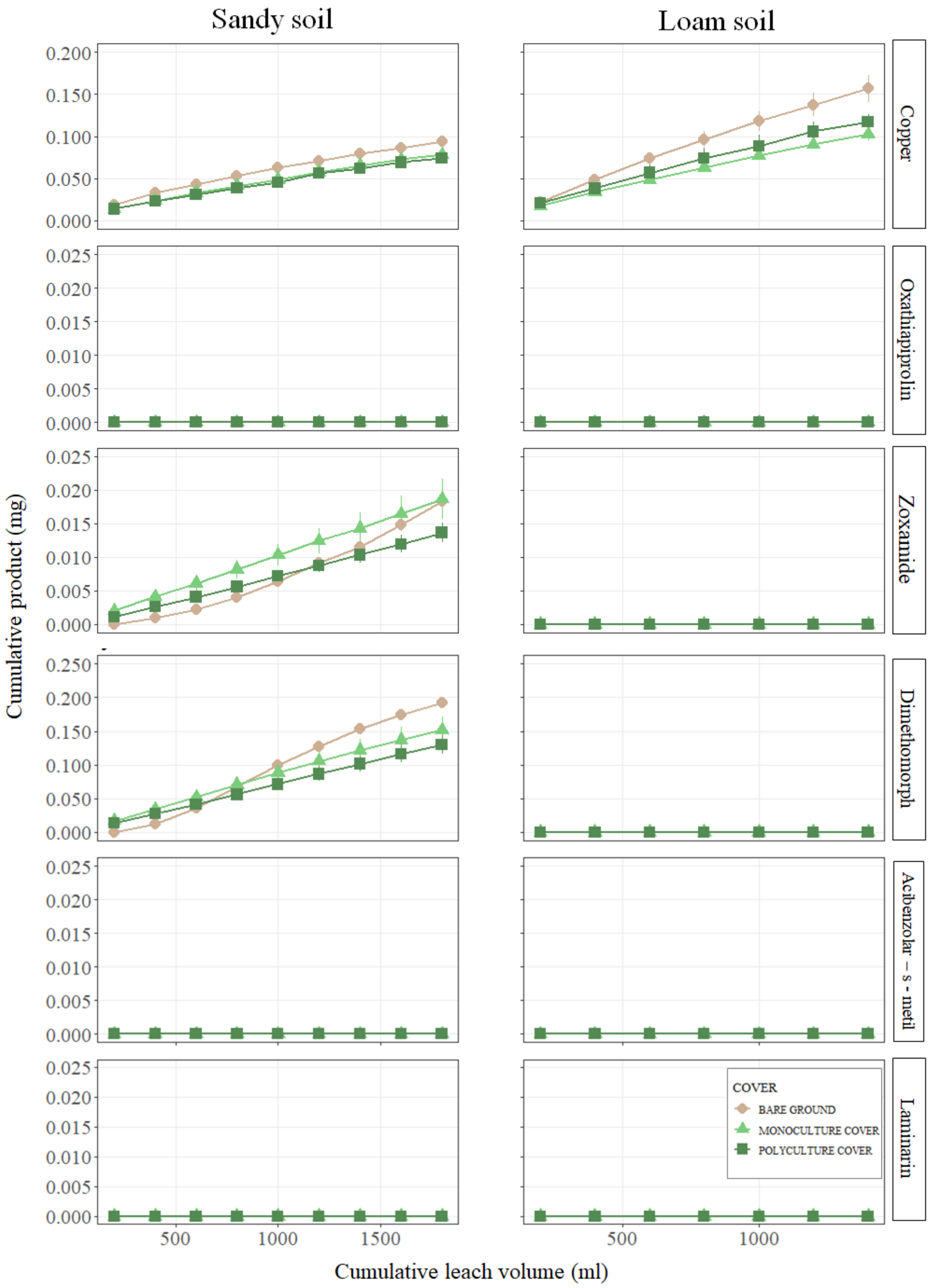


Figure 3.1.2. *Cumulated mass fungicide product in leached water in relation to cumulated volume for the three tested covers (BG, MC, and PC) and two the soil types.*

Table 3.1.4. *Mass balance of leached fungicide products (%). BG: Bare ground; MC: Monoculture cover; PC: Polyculture cover. Average ± standard deviation (n=3). HSD Tukey (P < 0.05) differences expressed with a letter.*

	Sandy soil			**Loam soil**		
Cover crop type	**BG**	**MC**	**PC**	**BG**	**MC**	**PC**
Copper	8±1.0 a	7±0.6 a	7±0.5 a	15±2.6 a	9±1.1 b	11±1.3 b
Oxathiapiprolin	n.d -	n.d -	n.d -	n.d -	n.d -	n.d -
Zoxamide	21±2.3 a	21±5.8 a	16±3.0 a	n.d -	n.d -	n.d -
Dimethomorph	64±4.0 a	46±14.2 a	37±9.5 a	n.d -	n.d -	n.d -
Acibenzolar-s-metil	n.d -	n.d -	n.d -	n.d -	n.d -	n.d -
Laminarin	n.d -	n.d -	n.d -	n.d -	n.d -	n.d -

3.3. Effect of fungicide application before rain event on PPP leaching

Since no differences on fungicide leaching were found when a rain event was simulated immediately after fungicide application in sandy soil columns (Table 3.1.4), we tried to observe if the same behaviour occurred with a 24h elapsed between application and rainfall, as this is conventionally done as a cultural practice in a real vineyard, letting a timelapse between the application and the rainfall. Figure 3.1.3 shows that the leaching of fungicides in bare soil columns is greater after 24 h of application than when the rain event was produced immediately after the application (0 h). This is mainly due to the accumulation of fungicides in the soil, as the second study was performed only one week after the first one. Even so, the fungicide mass leaching in the covered soil columns were lower than in the first study. In fact, our findings show that applying fungicides 24 before rain event resulted in a significant reduction in the amount of leached fungicides (copper and zoxamide) between covered soil columns (<10% leaching) versus bare soil columns (30% leaching). Despite this, no statistical differences were observed for dimethomorph, most likely due to the compound's very high overall leached amount (>40%).

Furthermore, results show that although there are no statistical differences between covered cropped strategies (MC vs PC) for the leaching of zoxamide, monoculture covered columns resulted in a greater reduction of copper leaching than those observed in polyculture covered columns. This is in agreement with the fact that ray grass *(Lolium perenne)* is a metal-accumulating plant (Healy et al., 2016; Johnson & Singhal, 2015), but also due to the fact that MC columns had greater root biomass than PC columns (22 vs. 11 g in dry weight).

Overall, our study demonstrates that the presence of cover crops significantly reduces the quantity of fungicides leached through the soil. This can be explained by the positive effect of the plant rhizosphere in biodegradation as well as the plant uptake of synthetic and ecological organic fungicides (Tarla et al., 2020). Therefore, since covers do not act as a physical resistance against product leaching, it seems that the attenuation of potential groundwater contamination occurs when the fungicide-rhizosphere interaction time is as long as possible prior to a rainfall event to allow phytoremediation to take place.

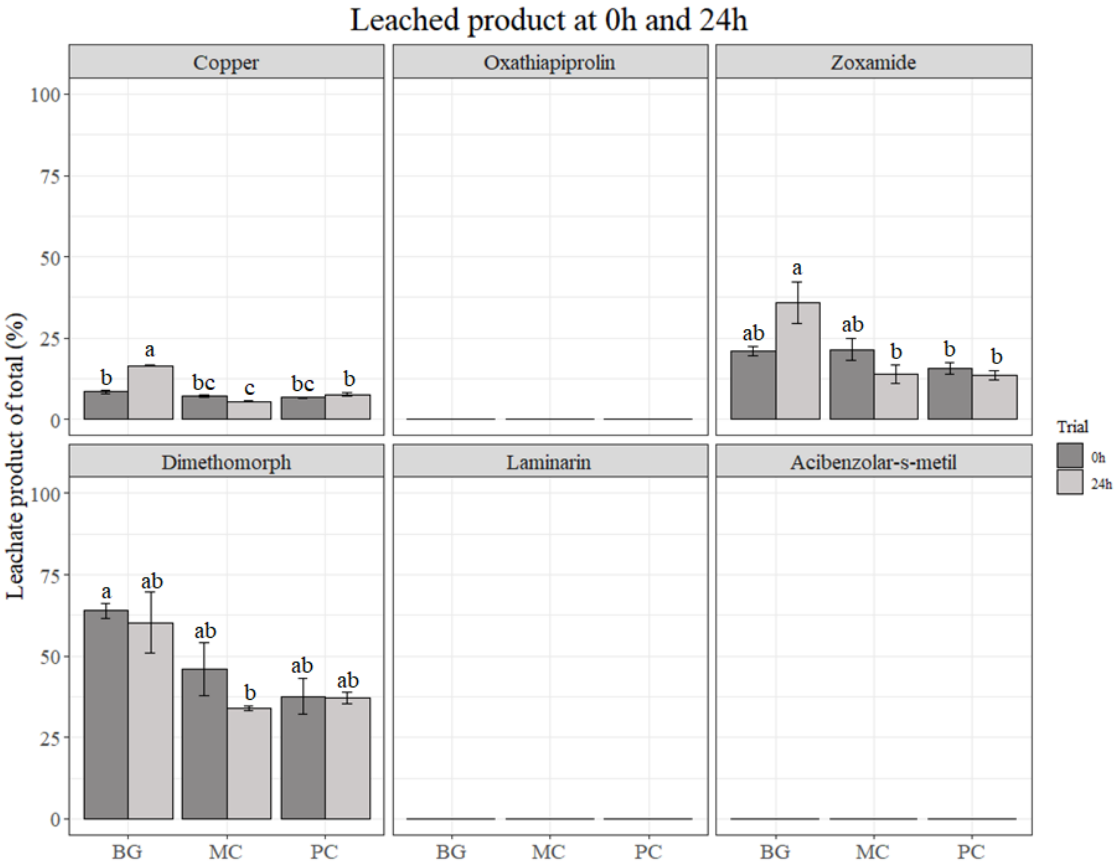


Figure 3.1.3. *Mass balance of the leached fungicide products through the sandy soil columns (BG, MC and PC) at 0 and 24 h after their application.*

3.4. Hydroponic degradation studies

Different hydroponic studies were performed to assess the direct impact of vegetation on the attenuation of fungicides without taking into consideration soil interaction. The concentration decay of fungicides in hydroponic containers followed a first-order kinetic with concentration decay rates ranging from 0.06 to 0.63 d^{-1} and half-lives from 1 to 21 days (Table 3.1.5). These kinetic rates are in the range of those found in other hydroponic studies that explored the impact of rooted crops on the attenuation of pesticides (Lv et al., 2016; Ni et al., 2018). Fungicides can be classified as highly degradable (>0.5: Acibenzolar-s-methyl), moderately degradable (between 0.5 and 0.2 d^{-1} for at least rooted containers, oxathiapiprolin, and laminarin) and poorly degradable (<0.2 d^{-1}: dimethomorph and zoxamide). Acibenzolar-s-methyl was the fungicide with the highest kinetic decay rate (>0.5 d^{-1}) in any of the studied hydroponic conditions, indicating that this is a highly degradable compound as it was suggested in previous soil leaching studies where it was not detected (sections 2.2 and 2.3). Similarly, oxathiapiprolin and laminarin showed kinetic rates of between 0.2 and 0.5 d^{-1} for rooted hydroponic containers, indicating moderate biodegradability. In this regard, for laminarin, the presence of roots enhanced the concentration decay from 0.06 d^{-1} in the control containers to 0.22 and 0.34 d^{-1} for the rooted MC and PC containers, respectively. This positive effect of roots may be explained by the increase in biodegradation due to the root exudates or the plant uptake of this highly polar compound (Kow of -7.1). Finally, dimethomorph and zoxamide were only poorly removed. These findings are consistent with results from leaching studies where these two fungicides and copper were the only ones that leached through soil columns. Overall, hydroponic studies show that dimethomorph, zoxamide, and laminarin are highly affected by the presence of rooted crops (kinetic rates increased from 51 to 467%, p-value<0.05), indicating that covering has a highly positive impact on enhancing concentration decay, either by biodegradation, sorption, and/or plant uptake. These results are in agreement with previous hydroponic studies carried out by Lv et al. (2017), who found that the concentration decay of fungicides such as tebuconazole and imazalil is enhanced by the use of highly rooted plants like *Phragmites australis*. Furthermore, our studies show that using a mixture of cover spices (PC) enhances the reduction of dimethomorph under hydroponic conditions. This last finding suggests that plant biodiversity may aid in enhancing soil bioremediation (Moosavi et al., 2013).

Focusing on the removal of fungicides after 10 incubation days, it ranged from 36 to 98%, so that at least 36% of all tested fungicides degrade after that time. Similarly, the findings for kinetic rates in removal results show that vegetation has a significant positive effect on all tested fungicides, with the exception of oxathiapiprolin. The reduction in concentration of zoxamide, dimethomorph and acibenzolar-s-methyl due to the presence of vegetation increased by 27-51%, 108-148% and 42-50%, respectively. For laminarin, elimination is gathered with the root action, practically reaching total elimination after 10 days (>90%), but with a low degradation in the control containers (65%).

Overall, hydroponic results indicate that cover crops roots are capable of enhancing the removal of almost all studied fungicides, whereas increasing plant biodiversity can play a relevant role in improving the attenuation of dimethomorph.

Table 3.1.5. *First order kinetic and elimination. Average ± standard deviation. HSD Tukey ($P < 0.05$) differences expressed with a letter.*

Active ingredient	**Cover type**	**K (day^{-1})**	**Tukey**	**Vegetation effect (%)**	**T ½ (day)**	**Elimination (%)**	**Tukey**	**Vegetation effect (%)**
Oxathiapiprolin	C	0.22±0.04	a	-	3.31	88±1	a	-
	MC	0.22±0.01	a	ne	3.19	86±1	a	ne
	PC	0.22±0.03	a	ne	3.27	88±3	a	ne
Zoxamide	C	0.08±0.03	a	-	8.97	58±10	a	-
	MC	0.12±0.01	ab	51	5.58	74±2	ab	27
	PC	0.18±0.03	b	117	3.96	87±4	b	51
Dimethomorph	C	0.05±0.01	a	-	14.24	36±4	a	-
	MC	0.15±0.04	b	202	4.98	75±8	b	108
	PC	0.24±0.04	c	381	3.01	89±4	b	148
Acibenzolar-s-metil	C	0.63±0.05	a	-	1.08	72±1	a	-
	MC	0.53±0.05	b	ne	1.53	74±4	a	42
	PC	0.53±0.05	b	ne	1.54	89±2	b	50
Laminarin	C	0.06±0.04	a	-	25.67	65±5	a	-
	MC	0.22±0.01	b	260	3.23	92±1	b	3
	PC	0.34±0.09	b	467	2.13	98±1	b	24

3.5. Agricultural implications

The results of this study show that cover crops can be used to mitigate soil and groundwater pollution. Furthermore, fungicide application practices are very relevant to enhancing the covering effectiveness. Therefore, our results suggest that fungicide spraying should be performed at least 24 hours before rain forecasting. This is in line with the European strategy to reduce the use of pesticides and soil pollution. According to Chapagain et al. (2020), covers minimize soil disturbance and erosion, improve soil structure and water-stable aggregates, and support pollinators and beneficial insects, among other things. However, Sharma et al. (2018) noticed that over crops can be problematic in some points, including the method of killing, host for pathogens, regeneration, and not immediate benefits of using them. There is also a concern about water competition concerns between the main crop and the cover crop. But according to Delpuech et al. (2018), even in the Mediterranean region, where most vineyards are located, the implementation of a cover crop strategy is feasible.

4. Conclusions

The results from this study indicate that cover crops can be a suitable strategy to protect groundwater pollution from fungicide application in vineyards. Despite 6 fungicides being applied on the ground, only 3 (Cu, zoxamide, and dimethomorph) and 1 (Cu) were leached through sandy and loam soil columns respectively due to soil interactions. The use of cover crops was not relevant on fungicide leaching when a rain event occurred immediately after fungicide application, but it reduced the quantity of leached fungicides (copper and zoxamide) when fungicides are applied 24 h before the rain event. The hydroponic studies indicate that vegetation plays and important role for almost all studied fungicides, increasing kinetic rates by 51-467% and suggest that increasing plant biodiversity may improve the attenuation of certain fungicides such as dimethomorph. Further studies, therefore, are needed to find out the impact of covering and biodiversity on the attenuation of fungicides under real agricultural conditions as well as on how these new fungicides are transformed and leached down into the soil.

3.2 Article 2: Attenuation and soil biodegradation of fungicides by using vegetated buffer strips in vineyards during a simulated rainfall–runoff event

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RESEARCH ARTICLE

Attenuation and soil biodegradation of fungicides by using vegetated buffer strips in vineyards during a simulated rainfall–runoff event

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Abstract

Rainfall–runoff events occurring in vineyard fields can result in pesticide ground losses and the subsequent pollution of surface water bodies, derivate from the crop protection spray applications. In this study, the capacity of vegetated buffer strips (BS) to prevent surface water pollution due to the application of five fungicide products typically used in vineyards (copper, dimethomorph, oxathiapiprolin, zoxamide, acibenzolar-s-methyl, and laminarin) following a simulated run-off event has been assessed, and compared to that from a bare ground soil (BG). Two strips (5 m in length, each), one with vegetation and the other without were built up, and two different experiments were performed, a runoff event and a soil fungicide degradation kinetic evaluation. The runoff results show that fungicide mass retention in the strips ranged from 73 to 98% and that the presence of vegetation in BS increased the fungicide mass retention in the strips by almost 10% (on average) in comparison to the unvegetated strip. Moreover, soil degradation studies highlighted that the presence of vegetation reduces significantly the half-time life of almost all the studied fungicides by 55%, on average. Eight fungicide transformation products (TPs) were identified following a runoff event in the soil strips, but the abundance of these TPs was up to 78% lower in vegetated strips. These results highlight the effectiveness of using vegetated buffer zones in vineyards to protect aquatic ecosystem pollution.

Keywords Copper · Fungicides · Vineyard · Runoff · Buffer zones · Buffer strip · TPs

Introduction

The use of pesticides for crop protection in vineyards is an essential part of today's agricultural production system. As a result, the runoff of pesticides following a rainfall event has been identified as one of the most important sources of pesticide pollution into surface water bodies (Reichenberger et al., 2007, Freitas et al., 2008; Frey et al., 2009; Wohlfahrt et al., 2010). However, despite fungicides being the main pesticides used in viticulture, only limited studies have been conducted to investigate their behaviour and fate following a run-off event (Lefrancq et al., 2014). Lefrancq et al. (2017) reported the occurrence of fungicides such as dimethomorph in the runoff water of a vineyard plot with concentrations up to 13 µg L^{-1} among others, posing a risk for aquatic organisms. Moreover, in vineyards, the traditional use of fungicides against downy mildew (*Plasmopara viticola*), such as copper-based products, has resulted in a negative effect on soil organisms (Ballabio et al., 2018).

In view of this, the European Commission (EC) has launched the European Green Deal programme (EC, 2019), with the aim of changing the current agricultural production model to one that is more environmentally friendly. This programme includes several focal points for action, including the "farm-to-fork" strategy (EC, 2020). This initiative aims to turn the European agricultural system into a more sustainable sector which reduces the environmental impact and increases at the same time the food quality level. To this end, a number of targets have been set, including a 50% reduction in the overall use of chemical pesticides, and in this way contribute to reducing the environmental pollution. Furthermore, the Sustainable Use Directive of Pesticides (2009/128/EC) establishes a framework for European agriculture to

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Attenuation and soil biodegradation of fungicides by using vegetated buffer strips in vineyards during a simulated rainfall–runoff event

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Abstract

Rainfall–runoff events occurring in vineyard fields can result in pesticide ground losses and the subsequent pollution of sur-face water bodies, derivate from the crop protection spray applications. In this study, the capacity of vegetated buffer strips (BS) to prevent surface water pollution due to the application of five fungicide products typically used in vineyards (copper, dimethomorph, oxathiapiprolin, zoxamide, acibenzolar-s-methyl, and laminarin) following a simulated run-off event has been assessed, and compared to that from a bare ground soil (BG). Two strips (5 m in length, each), one with vegetation and the other without were built up, and two different experiments were performed, a runoff event and a soil fungicide degradation kinetic evaluation. The runoff results show that fungicide mass retention in the strips ranged from 73 to 98% and that the presence of vegetation in BS increased the fungicide mass retention in the strips by almost 10% (on average) in comparison to the unvegetated strip. Moreover, soil degradation studies highlighted that the presence of vegetation reduces significantly the half-time life of almost all the studied fungicides by 55%, on average. Eight fungicide transformation products (TPs) were identified following a runoff event in the soil strips, but the abundance of these TPs was up to 78% lower in vegetated strips. These results highlight the effectiveness of using vegetated buffer zones in vineyards to protect aquatic ecosystem pollution.

Keywords: Copper · Fungicides · Vineyard · Runoff · Buffer zones · Buffer strip · TPs

1. *Introduction*

The use of pesticides for crop protection in vineyards is an agronomic practice that has been carried out all over the years, and which has become an essential part of today's agricultural production system. Nevertheless, runoff of pesticides following a rainfall event has been identified as one of the most important sources of pesticides pollution into surface water bodies (Reichenberger et al., 2007, Freitas et al., 2008; Frey et al., 2009; Wohlfahrt et al., 2010). However, although fungicides are the main pesticides used in viticulture, only a few studies have focused on their behaviour and fate following a run-off event (Lefrancq et al., 2014). Lefrancq et al. (2017) reported the occurrence of fungicides such as dimethomorph in the runoff water of a vineyard plot with concentrations up to 13 $\mu g\ L^{-1}$ among others, posing a risk for aquatic organisms. Moreover, in vineyards, the traditional use of fungicides against downy mildew (*Plasmopara viticola*), such as copper-based products, has resulted in a negative effect on soils (Ballabio et al., 2018), especially in nearby water courses.

In view of this, the European Commission (EC) has launched the European Green Deal programme (EC, 2019), with the aim of changing the current agricultural production model to one that is more environmentally friendly. This programme includes several focal points for action, including the "farm-to-fork" strategy (EC, 2020). This initiative aims to turn the European agricultural system into a more sustainable sector which reduces the environmental impact and increases at the same time the food quality level. To this end, a number of targets have been set, including a 50% reduction in the overall use of chemical pesticides, and in this way contribute to reducing the environmental pollution. Furthermore, the Sustainable Use Directive of Pesticides (2009/128/EC) establishes a framework for European agriculture to achieve more sustainable use of pesticides and reduce the risks to human health and the environment that derivates from the use of these products. In the case of copper, it has become a major environmental and toxicological problem in vineyards and EU legislation has been adopted to limit its use (Commission Implementing Regulation EU 2018/1981).

In this frame, the EU established the use of buffer zones to protect non-target organisms and safeguard water bodies from pesticide spray drift, drain flow and runoff (Directive 2009/128/EC), and also training and awareness-raising projects for farmers have also been carried out (http://www.topps-life.org/). Even that, the use of vegetation in those zones is not mandatory. Buffer strips are linear bands of permanent vegetation adjacent to an aquatic ecosystem intended to minimize the pollution from diffuse sources by trapping the pollutants (Barling & Moore, 1994; Mancuso et al., 2021) and have therefore been found to be an effective way of reducing the transfer of pesticides, caused by surface runoff from fields to watercourses (Aguiar et al., 2015; C. Chen et al., 2019; Lacas et al., 2005; Otto et al., 2008). For example, at the EU level, the FOCUS working group on landscape mitigation factors recommends assuming 50%, 75% and

90% runoff reduction for 5 m, 10 m and 15–20-m wide buffer strips (FOCUS, 2007), respectively (Ohliger & Schulz, 2010). Nevertheless, most of the pesticide runoff studies focus on herbicides, even in vineyard fields (Jurado et al., 2012) and results from the use of buffer strips for reducing fungicide run-off in vineyards seem to be contradictory. For instance, Bereswill et al. (2012) observed that there is no substantial difference between buffer strip width and the reduction of fungicides such as copper or dimethomorph. Furthermore, the application of organic fungicides on agricultural soils can also lead to the generation of transformation products (TPs) (Menger et al., 2021), which may have the same or greater toxicological effects on watershed ecosystems than parental fungicides (Iwafune, 2018; Meffe et al., 2021). Currently, none of the studies performed until now has addressed the soil degradation of fungicides in buffer strips nor assessed the impact of vegetation on that. Nevertheless, a previous study carried out by Ortega et al (2021) demonstrated the capacity of using cover crops to reduce groundwater pollution by fungicides, but how the use of buffer strips can help to reduce fungicide surface run-off and the fate of these compounds in soil buffer strips is still missing.

This work, therefore, aims to reveal the capability of using buffer strips for the reduction of organic and inorganic fungicide pollution following a simulated rainfall-runoff event in vineyards and to assess, for the first time, the behaviour and fate of fungicides retained in the buffer strips by monitoring their TPs.

2. *Materials and methods*

2.1 Experimental set-up

2.1.1 Buffer strip set-up

Trials were conducted in the greenhouse facilities of the Agropolis Research Centre from Polytechnic University of Catalonia (UPC, Viladecans, Spain) in May 2022. Two filter strips of 5.00 x 0.30 x 0.15 m (length x width x height) were built to simulate semi-field conditions buffer zones, according to Franco et al. (2016). They consisted of a 1% slope and an exit to collect samples at the end. The buffer strips were filled with 80 kg of vineyard soil (sized < 2 mm) from a commercial vineyard field located in the Tarragona region of Catalonia (Spain). The soil had a loam texture (51.2% sand, 30% silt, 18.8% clay), 0.95% of total organic carbon content, pH of 8.8 and a C/N relation of 7.7 (further information is detailed in the supplementary material (SM) section).

Two types of ground management were tested, one in each channel: Bare ground (BG) and Buffer Strip (BS), planted with a cover crop mix for a total soil coverage (*Trifolium subterraneum; Trifolium resupinatum; Trifolium michelianum; Biserrula pelecinus, Medicago polymorpha, Medicago truncatula, Hedysarum coronarium; Trifolium cherleri; Trifolium isthmocarpum;*

Dactylis glomerata; Lolium perenne; Lolium multiflorum; Festuca rubra). The total biomass was measured after the experiments removing the cover and drying it at 60º using a stove (TCF 400 Argo lab, Italy) until constant weight. The total weight of the vegetation at the BS was 406 g of aerial part and 292 g of roots.

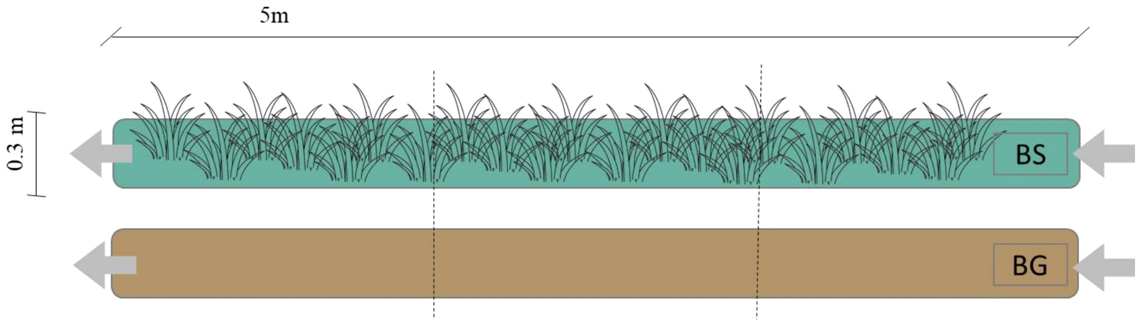


Figure 3.2.1: *Buffer zones strips setup scheme. Arrows show the liquid flow.*

2.1.2 Fungicide selection

With the aim to follow the same research line, the five fungicides used in Ortega et al. (2022) were based on previous trials carried out at OPTIMA project (Optimised Pest Integrated Management to precisely detect and control plant diseases in perennial crops and open-field vegetables, H2020 Grant Agreement N.773718, http://optima-h2020.eu/es/16219-2/) and according to Pugliese et al (2021).

In fungicide application processes, there is a part that is inevitably lost to the soil. According to Gil et al. (2001), during this spraying process, for a conventional copper product (1 kg/ ha) in vineyards and under worst-case conditions (an early crop stage and a conventional sprayer), the produced spray ground losses are estimated in 4.62 µg·cm^{-2}. Table 3.2.1 shows the estimated soil losses, and the maximum dosage of the selected products according to their labels, the total soil losses were estimated for each selected product. These losses were calculated for a theoretical square hectare (Figure10.2.1-SM), assuming that all losses are carried over as run-off, from the field area to the constructed buffer zone (considering the same width, 0.3 m).

Table 3.2.1. *Fungicides used in the study. Sw: solubility. Log Kow: Octanol-water partition coefficient.*

Commercial name	**Active ingredient**	**Expected ground losses for the calculated area (mg)**	**Sw ($mg L^{-1}$) / Log Kow**	**Use**
Codimur 50®	Copper oxychloride 50% [WP] P/P	416	-	Control/reference
Zorvec™ Vinabel®	Oxathiapiprolin 4 % (P/V) 40 g/L Zoxamide 30 % (P/V)	190	0.175 / 5.74 (Pesticide Properties DataBase /EPI Suite™) 0.681 / 3.76 (PubChem 122087).	novel synthetic organic fungicides with systemic action
Forum®	15.0 % (p/v) Dimethomorph	347	49.2 / 2.68 (PubChem 5889665).	novel synthetic organic fungicides with systemic action.
Bion MX®	Acibenzolar-s-methyl 50% [WG] P/P	42	7.7 / 3.10 (PubChem 86412)	synthetic inducer and activator of plant self-defence mechanisms
Vacciplant®	Laminarin 4,5% [SL] P/V	277	301.5/ -7.10 (EFSA website /EPI Suite™)	ecological organic activator of plant self-defence mechanisms product

2.1.3 Effect of buffer strips on the fungicide runoff

Experimental runoff design was based on Franco et al. (2016). 2 L of fungicide-enriched water (according to the expected ground losses of Table 3.2.1) were injected on the surface of each of the buffer strips by a peristaltic pump with a hydraulic loading rate (HLRs) of 1 cm h^{-1}. This simulates the first runoff portion, in which the water washes the products from the field, emulating the worst-case scenario in which pesticide retention in the field is not considered. Continuously, an additional 40 L of water, unenriched, was injected into each system to emulate the remaining runoff water in the field, for a total of 14 m^3 ha^{-1}, according to an average value of the field factors of area and runoff quantity (Ramos et al., 2006). Leached waster was collected at the outputs of the channels every 1L with a total collected volume of 30L for the BG strip and 15L for the BS one. The collection time of each sample was recorded

2.1.4 Effect of buffer strips on fungicide biodegradation

A soil kinetic study was performed to evaluate the degradability of the compounds in the vegetated (BS) and unvegetated (BG) buffer strips after one month of the first assay performance, and taking blank samples to ensure there was no contamination. The same amount of fungicides as in the previous experiment (Table 3.2.1), were applied manually on the soil surface of each buffer strip diluted in 2 L of water in order to distribute it uniformly. Kinetic degradation was assessed by taking 3 composite soil samples from different points and depths within each sampling section (Figure 3.2.1). Sampling among each channel was performed at 0, 24, 72, 144, and 240 h. The buffer strips were irrigated by drip irrigation to keep the cover crops alive, but avoid leaching, with 3.2 L spread over two different times of the day.

2.2 Analytical methodologies

For organic fungicides were followed two different procedures. Water samples were filtrated with a 0.22 µm hydrophilic PTFE filter (Frisenette, DK). Soil samples were processed as follows: 2 mL of Ethyl acetate was added to 500 mg of 24h lyophilized soil, of each sample (Telstar, Madrid, Spain). The mix was ultrasonicated and centrifugated for 10 and 15 min respectively. The liquid fraction was extracted and the process was conducted once more. The liquid fractions were mixed and evaporated with nitrogen. 500 µL of ultrapure water was added before the sample reached dryness. The samples were then injected into ultra-high-performance liquid chromatograph (UHPLC) under the same conditions than Ortega et al. (2021). Specific wavelength was used for each compound (dimethomorph 235 nm, zoxamide 211 nm, acibenzolar-s-methyl 325 nm, oxathiapiprolin 258 nm and laminarin 225 nm) and the linearity ranged from 2.5 to 2000 µg L^{-1} (LOD and LOQ of the method for the tested are specified in the Table 10.2.2-SM).

Soil sample extracts from the fungicide biodegradation experiments were analysed to find possible fungicide transformation products (TPs). To do so, one extract for each kinetic timing (0, 24, 72, 144, and 240 h) and buffer strip was analysed. The samples were run on a UPLC-QToF Impact II (Bruker Daltonics, Bremen, Germany) with a chromatographic separation matching the conditions of the instrument application TargetScreener HR. For this, we used a C18 column (Bruker Intensity Solo; L=100mm, ID=2.1 mm and particle size 1.8µm; with a precolumn) operated at 40°C. Mobile phases consisted of water/methanol (99:1) and methanol, both with 5mM of ammonium formate and 0.01% of formic acid. Electrospray Ionization was performed in positive mode. Once the data was obtained, MetaboScape® software (Bruker) with the inbuilt BioTransformer 3.0 (Djoumbou-Feunang et al., 2019) was used for the identification of TPs by predicting different biotic and abiotic reactions of each fungicide and comparing the resulting molecular formulas with the obtained ones. The exact mass of the predicted formula was compared to the m/z obtained masses to find matches. Among the obtained matches we only considered TPs whose masses were present as $M+H^+$, $M+Na^+$ and $M+ K^+$ and had an m/z tolerance of <3 mDa and a mSigma score < 50 with the proposed molecular formula.

On the other side, DTPA (diethylenetriaminepentaacetic acid) extraction method was used to evaluate the copper in the soil according to Lindsay et al. (1978). All copper determination was quantified with a Varian SpectrAA 110 (Mulgrave, Victoria, Australia) flame atomic absorption spectrometer, equipped with a copper hollow cathode lamp, and a deuterium lamp for background correction. The instrument was operated under the conditions recommended by the manufacturer: lamp current of 4 mA, wavelength of 324.7 nm, slit width of 0.1 nm, burner height of 14 mm, acetylene flow rate of 1.0 L min^{-1} and airflow rate of 10.0 L min^{-1} (linearity ranged from 0.5 to 5 mg L^{-1}).

2.3 Data analysis

The experimental results were statistically analysed using RStudio (RStudio Team, 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL (http://www.rstudio.com/). Kruskal-Wallis test was performed analyse statistical differences between the half-life times of the tested fungicide products in soil with and without vegetation.

3. *Results and discussion*

3.1 Runoff reduction and pollution mitigation in the buffer zones

Figure 3.2.2 shows the cumulative amount of pesticides in the final runoff water, for each of the tested fungicides in the two experimental cases, bare ground (BG) and vegetated buffer strips (BS). All fungicides were detected in the final run-off water, except laminarin, due to the high solubility of the compound (Table 3.2.1). The BS implies a considerable reduction of all the detected compounds in the eluted water. Furthermore, there is a clear difference between the time at which runoff water starts to elute at the end of the two strips (Fig 3.2.2), being greater in the vegetated strip. According to Yu et al. (2016), that can be explained due to the fact that plant roots of BS significantly modify soil hydraulic conductivity and reduce the runoff from a rainfall event. Similarly, BS resulted in a lower recovery of fungicides (1-4%) than BG (1-26%), which means greater fungicide retention due to the presence of vegetation. The low recovery of fungicides (Figure 3) in both systems can be explained by their interaction with soil (Barchańska et al., 2020), whereas plants and their rhizosphere system have been shown to be beneficial for pesticide degradation (Eevers et al., 2017). Regarding the total amount of pesticides leached at the end of the run-off study (Fig 3), dimethomorph is the compound with the highest elution rate (with 26% in the BG and 4% in the BS) which can be explained due to its high solubility and low sorption coefficient (log Kow of 2.68). A correlation has been found with the absorbance of pesticides by roots and log Kow, which in turn correlated negatively with the translocation of these substances into the plant (Wang et al., 2017). Oxathiapiprolin compound has been leached by 14% in the BG case, but it is reduced to almost zero with the use of the cover crop (0.1 %), this is also consistent with the high reported value of the sorption coefficient and the low solubility of the compound (Table 3.2.1). Acibenzolar-s-methyl and Zoxamide are also reduced, and thus their pollution potential, with the presence of the vegetation form an 8% to 2% and 5 to 0.8% respectively. Although copper has the higher application dose, and therefore the higher ground losses, the runoff simulated experiment retained 98% and only 2% of it at the outlet, due to the insolubility of this compound, whereas planted buffer strip leached less than 1%. This is in agreement with the high sorption capacity of Cu in the soil as it has been described by (Babcsányi et al., 2016), where Cu export by runoff from the catchment in vineyard fields, accounted for 1% of the applied Cu mass. These findings are also in agreement with previous studies conducted by Ortega et al. (2021) who found that the presence of vegetation reduces fungicide groundwater pollution (covered crop soil columns had <10% fungicide leaching and bare soil ones had a 30% fungicide leaching). In all cases, the presence of vegetation played a very important role, reducing the runoff of organic and inorganic fungicides, and indicating a greater interaction of these compounds with the vegetation. Nevertheless, in addition to sorption, other attenuation mechanisms such as biodegradation or plant uptake cannot be ruled out.

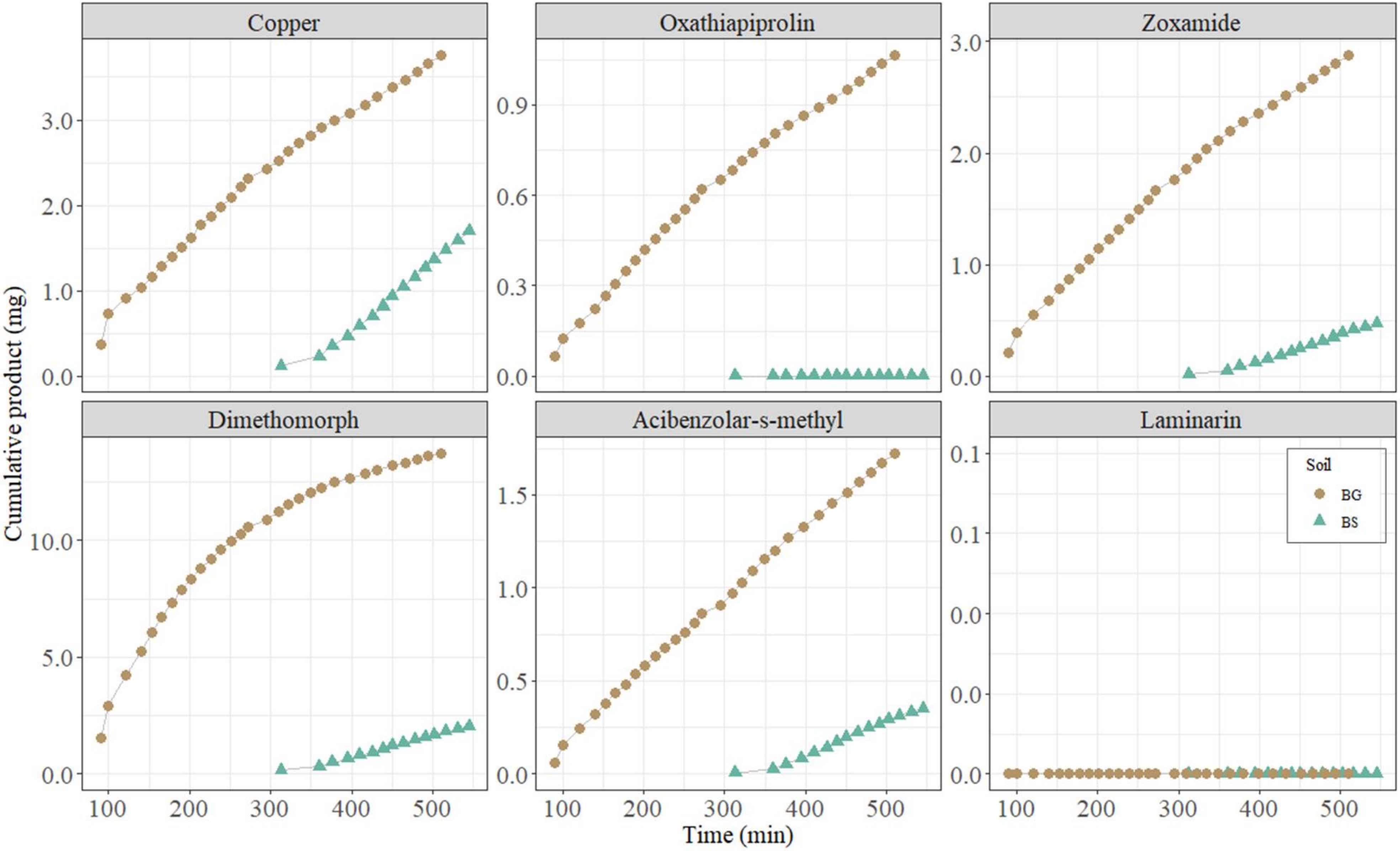


Figure 3.2.2: Cumulated amount of pesticides eluted by runoff for the two tested strips, bare ground (BG) and vegetated (BS).

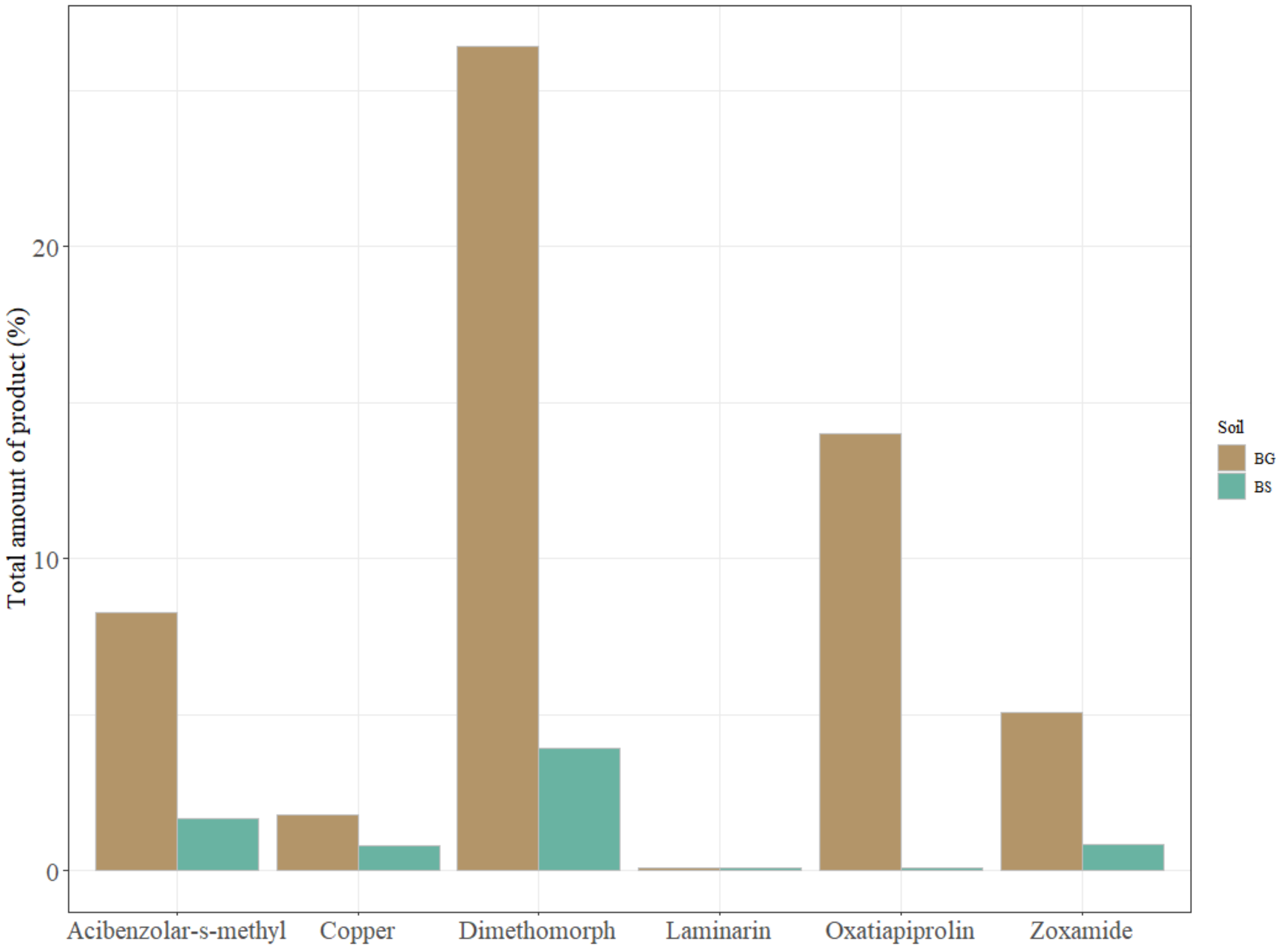


Figure 3.2.3: *Percentage of total amount of fungicides recovered after 7h 30' of the runoff experiment. Bare ground (BG) and vegetated (BS).*

3.2 Fungicide soil degradation and transformation

To check out the impact of soil biodegradation in the attenuation of fungicides in the buffer strip a first-order kinetic study was performed. Table 3.2.2 shows that the kinetic rates for the degradation of fungicides in soil ranged from 0.05 to 0.096 d^{-1} and from 0.05 to 1.54 d^{-1} in the vegetated and unvegetated strips respectively. The variability between the kinetic parameters is quite high due to the high soil heterogeneity (despite the use of soil composite samples), especially in compounds with low mobility like copper. Dimethomorph, oxathiapiprolin and copper had lower kinetic rates, indicating that among studied fungicides these are the most recalcitrant ones. This is in agreement with previous studies indicating that copper is a very stable compound in soil (Rehman et al., 2019), and that dimethomorph had a kinetic reduction rate of 0.034 d^{-1} in vineyard topsoil (Masbou, et al., 2022). The presence of vegetation in the BS enhanced the soil degradation kinetic rates for all the tested compounds, except for laminarin which could not be detected in any of the sampled soils. Similarly, half-lives (t1/2) for fungicide's active ingredients were notoriously reduced by the action of vegetation. Statistically significant differences ($\alpha < 0.05$) were observed for the oxathiapiprolin, zoxamide and dimethomorph compounds, from a half-life of 13.3, 3.7 and 15.2 days for the BG to 3.8, 1.2 and 2.6 days for the BS respectively. Acibenzolar-s-methyl had much lower values of half-live and the effect of the covered crop was not as noticeable as in the previous cases. As copper cannot be degraded, it can be assumed that its concentration decay in the BS was accounted to the plant uptake as a micronutrient (Yruela, 2009), thus reducing the amount of metal in the soil. To a greater or lesser extent, the presence of vegetation in BS reduced fungicides half-lives in soil. Our results are in agreement with previous studies that indicate that the presence of plants decreases the amount of pesticides in the soil due to phytoremediation processes such as phytoextraction, phytodegradation, or phytostimulation, among other occurring processes (Pascal-Lorber & Laurent, 2011; Tarla et al., 2020). For instance, Chen et al. (2018) observed that dimethomorph had similar values of 1.7 to 3.8 days with potato crops and 11.5–18.5 days in bare soil, which agrees with the results of this study. Comparing these results (Table 3.2.2) with previous ones where the impact of vegetation was assessed for the same compounds under hydroponic conditions (Ortega et al., 2022), the kinetic rates in soil are greater for all compounds, except for oxathiapiproline, which remains the same. This suggests that soil enhanced the development of bacteria in the rhizosphere as well as the biodegradation of fungicides.

Table 3.2.2. *First order kinetics parameters for the removal of fungicides in soil. Kruskal-Wallis (P<0.05) (n=3).*

Product	Soil	k (d^{-1})	R^2	t ½ (d)	
Copper	BG	0.05±0.05	0.59	24±15	
	BS	0.05±0.03	0.84	15.9±8.3	
Oxathiapiprolin	BG	0.08±0.06	0.95	13±8.9	*
	BS	0.21±0.10	0.75	3.8±1.5	
Zoxamide	BG	0.22±0.12	0.84	3.7±1.7	*
	BS	0.58±0.16	0.79	1.2±0.29	
Dimethomorph	BG	0.05±0.01	0.66	15±5.3	*
	BS	0.33±0.16	0.64	2.6±1.6	
Acibenzolar-s-methyl	BG	0.96±0.01	0.52	0.7±0.1	
	BS	1.54±0.95	0.78	0.5±0.3	
Laminarin	BG	-	-	-	-
	BS	-	-	-	-

* Values are statistically different at a p-value of 0.05

3.3. Identification of transformation products (TPs) and their behaviour on BG and BS soils

The fungicide concentration decay over time observed in the soil was linked to the identification of several TPs, which as indicated in the introduction can have a similar or greater toxicological impact. Their molecular formulas, retention times and matching literature structures are shown on Table 10.2.3 - SM. For dimethomorph, we identified two TPs that were the result of an oxidation and a demethylation respectively (dimethomorph TP1 and TP2, $C_{21}H_{22}ClNO_5$ and $C_{20}H_{20}ClNO_4$, Figure 3.2.4a). From these, the estimated molecular formula of TP2 matched with that of two common dimethomorph soil metabolites (Z67 and Z69) (Lewis et al., 2016, David Lunn, n.d.). Oxathiapiprolin generated two other TPs with the same molecular formula. These were two different oxidation products (Oxathiapiprolin TP1 and TP2, $C_{24}H_{22}F_5N_5O_3S$, Figure 3.2.4b). Such molecular formula also matched with that of two common soil metabolites of oxathiapiprolin (IN-RDT31 and IN-RDG40) (EFSA et al., 2022). For zoxamide, were found four TPs. Two of the TPs underwent oxidative dechlorination (Zoxamide TP1 and TP2, $C_{14}H_{17}Cl_2NO_3$, Figure 3.2.4c). Their molecular formula corresponds to that of an already identified metabolite of zoxamide (RH-150721) (EFSA et al., 2017). The other two TPs underwent oxidative dechlorination and hydrogenation (Zoxamide TP3 and TP4, $C_{14}H_{19}Cl_2NO_3$, Figure 3.2.4c). No TPs were identified for acibenzolar-s-methyl. Laminarin or its TPs could not be analysed.

Figure 4 shows the formation of all TPs on the soil extracts from the fungicide soil biodegradation experiments. In general, BG soil resulted in higher relative amounts of the identified TPs than the BS soil, suggesting that vegetation enhanced degradation of both parent fungicides and their TPs (Escher and Fenner, 2011). In the case of dimethomorph, the concentrations of its TPs on the BG soil rose or remained stable until day 6 and then they decreased until day 10. In contrast, in the BS soil, dimethomorph TPs concentrations were maintained very low over all the experiment (Figure 3.2.4a). Almost the same behaviour was observed for oxathiapiprolin. While the two TPs were formed within 1d of exposure and then their concentrations decreased until day 10, the same TPs remained very low on the BS soil over the whole experimental period (Figure 3.2.4b). These patterns seem to indicate that plants and their rhizosphere on BS were able to uptake or to metabolize the TPs while they were being formed and accumulated in the soil without vegetation. All TPs behaved as the TPs of dimethomorph and oxathiapiprolim, meaning that they were being formed between days 1 and 6 and then degraded until day 10, except zoxamide TP1, TP2 and TP4. These TPs, unlike the other fungicide were being formed in BS soil over time; reaching concentrations somewhat higher than on BG at day 10 (Figure 3.2.4c). This may indicate that for these TPs there was a plant-uptake and biodegradation equilibrium which was modified over time (Meffe et al., 2021).

All the identified TPs were maintaining the core structure of the parent fungicide. Therefore, these TPs could still maintain some of their biological activity. In this sense, BS soil showed to contain lower amounts of TPs than BG soil, especially when looking at span-times up to 6 days. As a result, the use of BS is not only beneficial to degrade fungicides but also to minimize the amounts of potentially toxic TPs.

Overall, the study indicates that the presence of vegetation enhances soil biodegradation of fungicides and their TPs. Although plant uptake cannot be ruled out, it has been reported to be low (Margenat et al., 2018), suggesting that vegetation can be used for animal feeding or other proposes in a safe way.

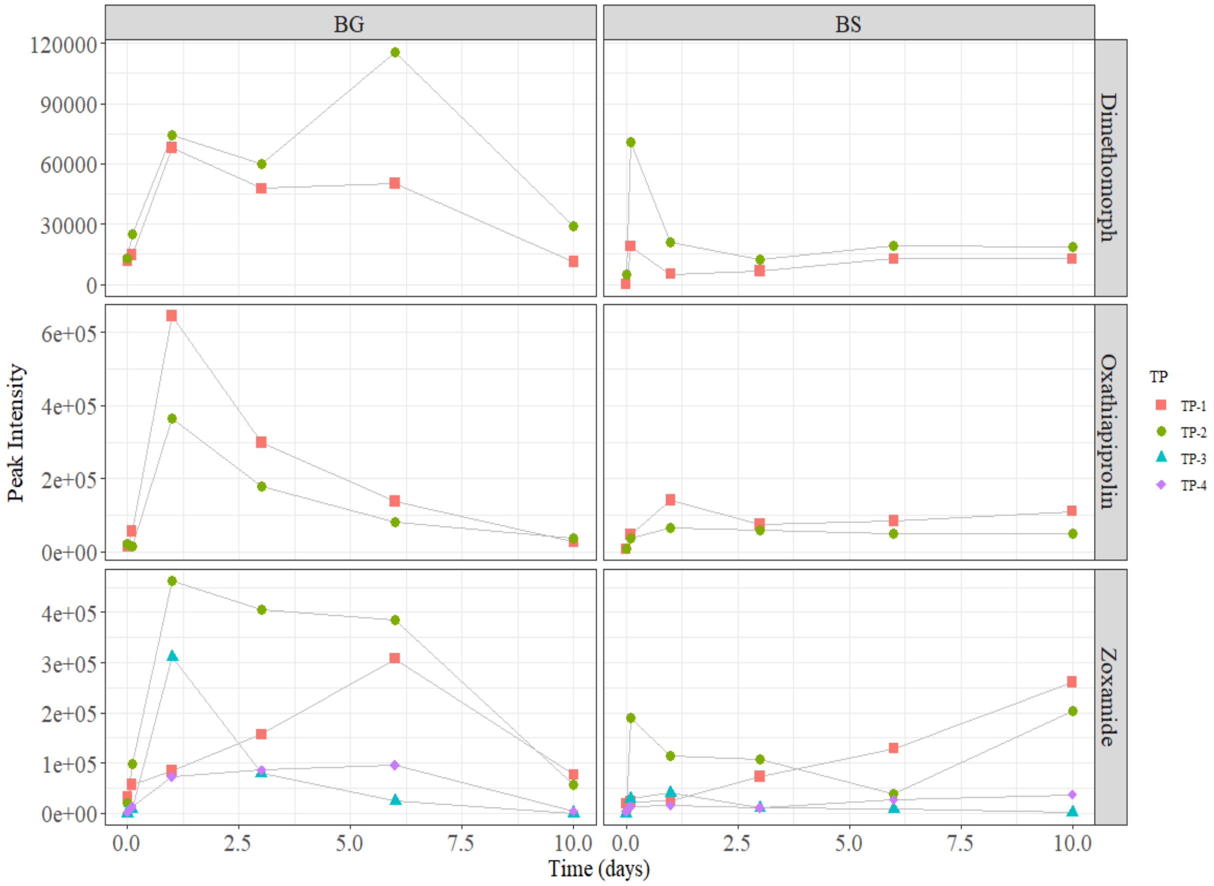


Figure 3.2.4: *Transformation products (TPs) detected into the soil for the different tested compounds in the bare ground (BG) and buffer strip (BS).*

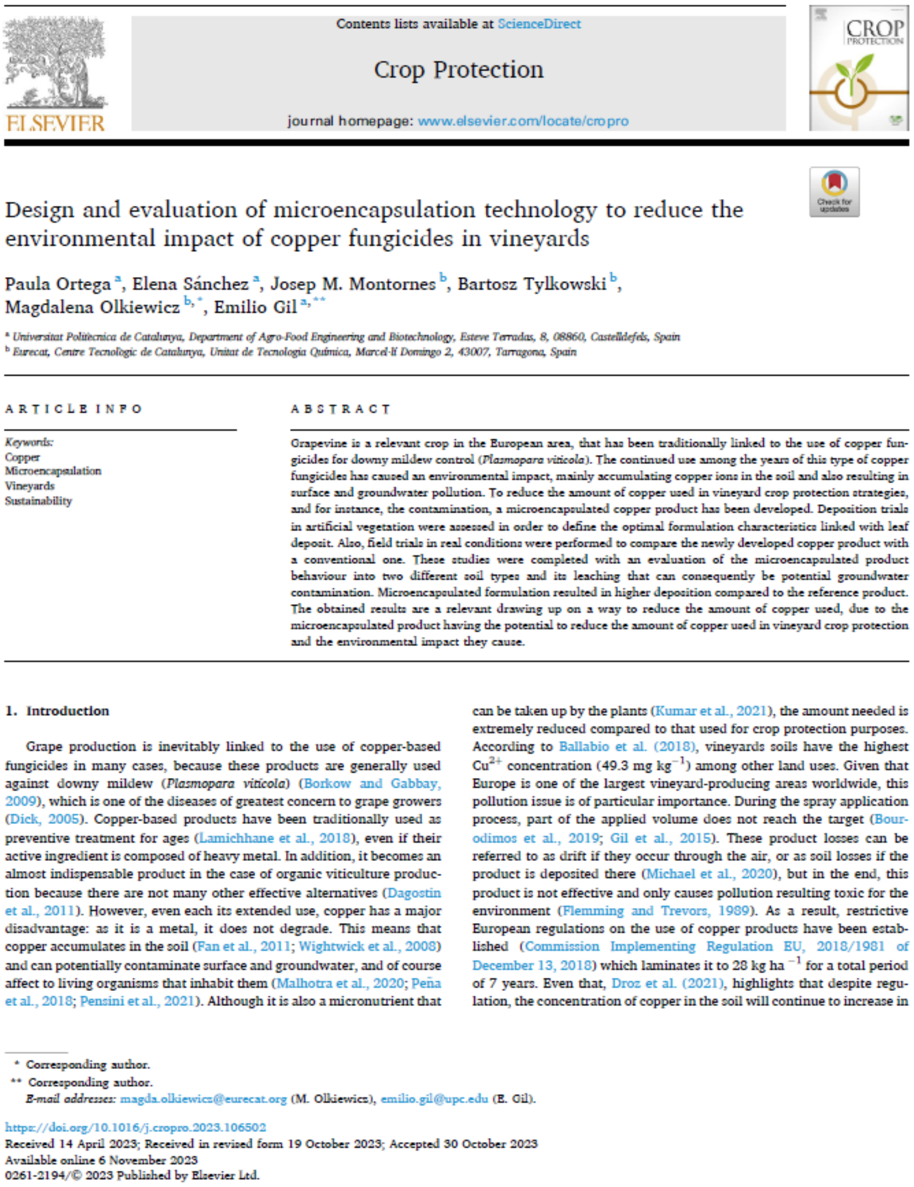
4. Conclusions

This study shows the multiple benefits of implanting vegetated buffer zones in vineyards, since not only reduce the amount of fungicides that can potentially contaminate surface water through runoff, but they also degrade faster the soil retained fungicides and their TPs. Even though laminarin was not detected at the final run-off of the buffer zone, the amount of copper and the tested organic fungicides was reduced, at least by half, due to the action of the vegetation in the buffer strip (retention and biodegradation enhancement). The presence of vegetation had also a significant influence on the soil degradation of the tested compounds, accelerating their kinetic removal rates (from 0.36 to 0.66 d^{-1}, on average). Fungicide TPs of the analysed compounds, were also mitigated by the vegetated buffer strip in comparison to the bare ground one. In view of these results, we recommend the implementation of vegetation at the buffer zones in vineyards, especially when vineyards are close to sensitive aquatic ecosystems or protected water bodies.

CHAPTER IV. Reduction of copper use through microencapsulation technology

It stands to reason that reducing the contamination caused by copper-based fungicides passes through reducing the amount of copper applied in plant protection treatments. Microencapsulation is a new technique that, depending on the compounds used, can help to reduce the amount of pulverized product. In the framework of the project COPPEREPLACE (http://coppereplace.com/), EURECAT proposes the creation of a novel microencapsulated product. Its development was carried out in collaboration with the Agricultural Mechanisation Unit (UPC), which carried out the deposition tests for the choice of the best formulation and its validation. This chapter describes the research carried out to test this new product and its evaluation in terms of deposition and leaching. European Patent Application EP21382965.8.

Article 3: Design and evaluation of microencapsulation technology to reduce the environmental impact of copper fungicides



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Design and evaluation of microencapsulation technology to reduce the environmental impact of copper fungicides in vineyards

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ABSTRACT

Grapevine is a relevant crop in the European area, that has been traditionally linked to the use of copper fungicides for downy mildew control (*Plasmopara viticola*). The continued use among the years of this type of copper fungicides has caused an environmental impact, mainly accumulating copper ions in the soil and also resulting in surface and groundwater pollution. To reduce the amount of copper used in vineyard crop protection strategies, and for instance, the contamination, a microencapsulated copper product has been developed. Deposition trials in artificial vegetation were assessed in order to define the optimal formulation characteristics linked with leaf deposit. Also, field trials in real conditions were performed to compare the newly developed copper product with a conventional one. These studies were completed with an evaluation of the microencapsulated product behaviour into two different soil types and its leaching that can consequently be potential groundwater contamination. Microencapsulated formulation resulted in higher deposition compared to the reference product. The obtained results are a relevant drawing up on a way to reduce the amount of copper used, due to the microencapsulated product having the potential to reduce the amount of copper used in vineyard crop protection and the environmental impact they cause.

1. Introduction

Grape production is inevitably linked to the use of copper-based fungicides in many cases, because these products are generally used against downy mildew (*Plasmopara viticola*) (Borkow and Gabbay, 2009), which is one of the diseases of greatest concern to grape growers (Dick, 2005). Copper-based products have been traditionally used as preventive treatment for ages (Lamichhane et al., 2018), even if their active ingredient is composed of heavy metal. In addition, it becomes an almost indispensable product in the case of organic viticulture production because there are not many other effective alternatives (Dagostin et al., 2011). However, even each its extended use, copper has a major disadvantage: as it is a metal, it does not degrade. This means that copper accumulates in the soil (Fan et al., 2011; Wightwick et al., 2008) and can potentially contaminate surface and groundwater, and of course affect to living organisms that inhabit them (Malhotra et al., 2020; Peña et al., 2018; Pensini et al., 2021). Although it is also a micronutrient that can be taken up by the plants (Kumar et al., 2021), the amount needed is extremely reduced compared to that used for crop protection purposes. According to Ballabio et al. (2018), vineyards soils have the highest Cu^{2+} concentration (49.3 mg kg^{-1}) among other land uses. Given that Europe is one of the largest vineyard-producing areas worldwide, this pollution issue is of particular importance. During the spray application process, part of the applied volume does not reach the target (Bourodimos et al., 2019; Gil et al., 2015). These product losses can be referred to as drift if they occur through the air, or as soil losses if the product is deposited there (Michael et al., 2020), but in the end, this product is not effective and only causes pollution resulting toxic for the environment (Flemming and Trevors, 1989). As a result, restrictive European regulations on the use of copper products have been established (Commission Implementing Regulation EU, 2018/1981 of December 13, 2018) which laminates it to 28 kg ha $^{-1}$ for a total period of 7 years. Even that, Droz et al. (2021), highlights that despite regulation, the concentration of copper in the soil will continue to increase in

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Design and evaluation of microencapsulation technology to reduce the environmental impact of copper fungicides

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Abstract

Grapevine is a traditional crop in the European area, that has been majority linked to the use of copper fungicides for downy mildew control (*Plasmopara viticola*). The continued use among the years of this type of pesticide has caused an environmental impact, mainly accumulating copper ions in the soil and also resulting in surface and groundwater pollution. To reduce the amount of copper used in vineyard crop protection strategies, a microencapsulated copper product has been developed. Deposition trials in artificial vegetation were assessed in order to select the formulation with higher deposition. Once the product was defined, tests in natural vineyard crops were performed in order to compare the newly developed copper product (in two different concentrations) with a conventional one. These studies were completed with an evaluation of the microencapsulated product behaviour into two different soil types and its leaching that can consequently be potential groundwater contamination. Microencapsulated formulation result in higher deposition compared to the reference product and formulation containing 1.5 g · L^{-1} of NA7507 and 2.5 g · L^{-1} of cationic polymer was selected. Microencapsulated product deposition in vineyard leaves was 150% higher than the conventional one, and no statistical differences were found between this last one and the new product deposition at half of the concentration. Soil assays in loam soil showed that conventional copper gets more boned into the ground over time, while the microencapsulated one increases the leaching slowly with time and has approximal 50% half-life time less into the soil. The same tendency, in a more pronounced way, is observed in the sandy soil. The obtained results are a relevant drawing up on a way to reduce the amount of copper used, due to the microencapsulated product has the potential to reduce the amount of copper used in vineyard crop protection and the environmental impact they cause.

Keywords: Copper · Microencapsulation · Vineyards · Sustainability

1. Introduction

Grape production is inevitably linked to the use of copper-based fungicides in many cases, because these products are generally used against downy mildew (*Plasmopara vitícola*)(Borkow & Gabbay, 2009), which is one of the diseases of greatest concern to grape growers (Dick, 2005). Copper-based products have been traditionally used as preventive treatment for ages (Lamichhane et al., 2018), even if their active ingredient is composed of heavy metal. In addition, it becomes an almost indispensable product in the case of organic viticulture production because there are not many other effective alternatives (Dagostin et al., 2011a). However, even each its extended use, copper has a major disadvantage: as it is a metal, it does not degrade. This means that copper accumulates in the soil (Fan et al., 2011; Wightwick et al., 2008) and can potentially contaminate surface and groundwater, and of course affect to living organisms that inhabit them (Malhotra et al., 2020; Peña et al., 2018; Pensini et al., 2021). Although it is also a micronutrient that can be taken up by the plants (V. Kumar et al., 2021), the amount needed is extremely reduced compared to that used for crop protection purposes. According to Ballabio et al. (2018), vineyards soils have the highest Cu^{2+} concentration (49.3 $mg \cdot kg^{-1}$) among other land uses. Given that Europe is one of the largest vineyard-producing areas worldwide, this pollution issue is of particular importance. During the spray application process, part of the applied volume does not reach the target (Bourodimos et al., 2019; E. Gil et al., 2015). These product losses can be referred to as drift if they occur through the air, or as soil losses if the product is deposited there (Michael et al., 2020), but in the end, this product is not effective and only causes pollution resulting toxic for the environment (Flemming & Trevors, 1989). As a result, restrictive European regulations on the use of copper products have been established (Commission Implementing Regulation (EU) 2018/1981 of 13 December 2018) which laminates it to 28 $kg \cdot ha^{-1}$ for a total period of 7 years. Even that, Droz et al. (2021), highlights that despite regulation, the concentration of copper in the soil will continue to increase in the vineyard EU soils, and therefore more sustainable methods are needed. Hence, there is a veritable challenge to reduce the copper use, either by using new technologies, or searching alternative products. The general trends then, are to reduce the use of pesticide, as can be reflected in the EU trends lines like the Green Deal (EC, 2020)13, and move to a more sustainable production friendly with the environment, also in line with the global tendency, like the Sustainable Development Goals (SDG) described by the United Nations. Ergo, there is a need to improve the use of copper, especially when the application of copper has always had easy of washing (Pérez-Rodríguez et al., 2015). Encapsulation technology consists in creating small particles with active materials surrounded by a shell. The purpose of encapsulation is to enable a faster and more effective use of materials for extremely targeted delivery of active ingredients to specific places. Recently, Weitbrecht et al. (Weitbrecht et al., 2021), reported microencapsulation of copper using spray congealing technique. During this process first, matrix material of the particles, a hydrogenated rapeseed oil was melted in a mixing tank at 80 °C. After that, benzoic acid, emulsifier (sorbitan fatty acid ester ethoxylate), copper sulphate and copper hydroxide were added, and the mixture were homogenized by means of ultraturrax. Then, the homogenized dispersion was pumped into a heated two-substance nozzle and solidified by means of a cooled spray tower. The authors were able to produce the capsules with 10% (w/w) copper content. In this work we prepared the microcapsules by complex coacervation method at room temperature. Designed capsule are formed by biodegradable polymers (alginate and chitosan), copper cations and natural polyphenols with additional fungicide properties (Gillmeister et al., 2019; Simonetti et al., 2020).

The aim of this research is to create a new copper formulation, based on microencapsulated copper, capable of reducing the amount of copper used in vineyard fields and, in turn, that can be used in organic farming. In addition, this study also evaluates the deposition efficiency and the environmental impact of the product on the soil in order to assess the overall behaviour of the new formulation when applied in the field.

2. *Materials and methods*

2.1 Development of formulation based on microencapsulated copper

2.1.1 Materials

Two types of sodium alginate, NA4012 and NA7580, with different viscosity 400 and 800 mPas respectively (1%, 20°C, 20 rpm), were purchased from C.E. Roeper GmbH, Germany. Chitosan from shrimp shells and acetic acid (glacial) were purchased from Sigma-Aldrich, Spain. Copper sulfate pentahydrate, CuSO4 5H2O, was obtained from Alfa Aesar, Spain. Wetting agent, BREAK-THRU® S 301, was provided by Evonik Nutrition & Care GmbH, Spain. Polyphenolic extract of grape seeds, Vitaflavan OC-DRT-16-02900, was provided by Purextract®, France.

2.1.2 Preparation of formulation

Two-steps procedure for alginate/copper/polyphenols/chitosan (ALG/Cu/PP/CH) microcapsules preparation were designed based on well-known complex coacervation method for capsule preparation (Trojanowska et al., 2017). The first step is based on ionic gelation method and the second step on polyelectrolyte complexation. The first step involves the preparation of alginate (anionic polymer) microcapsules with a core loaded with polyphenols and copper cations present in the capsule shell matrix (ALG/Cu/PP). Figure 4.1 shows a scale-lab set-up for microcapsules preparation (Figure 4.1a), and a scheme of a SU1A-SS nozzle (Spraying System Spain S.L.) used for alginate/polyphenols micro-droplets formation (Figure 4.1b) held in the 3D printed module (2) and controlled by an automated spray deposition system (ASDS) (3). During this first step 150 mL of alginate/polyphenols aqueous solution (1) containing 1 wt% of sodium alginate and 0.01 wt% of polyphenols were sprayed by a nozzle (2) into a 250 mL of $CuSO_4$ aqueous solution containing 4 g $Cu^{2+}\cdot L^{-1}$ (4) agitated by a magnetic stirrer.

During the experiment, the SU1A-SS nozzle (2) was connected to compressed air. ASDS (3) has been constructed to automate the spraying of solution 1 into uniform micro-droplets. The system was designed on the base of a commercial AVR microcontroller which allows the preparation of high-quality ALG/PP micro-droplets. ASDS permits to control the duration of the pauses between sprays and prevents the formation of undesired alginate film on the surface of Cu^{2+} solution (4).

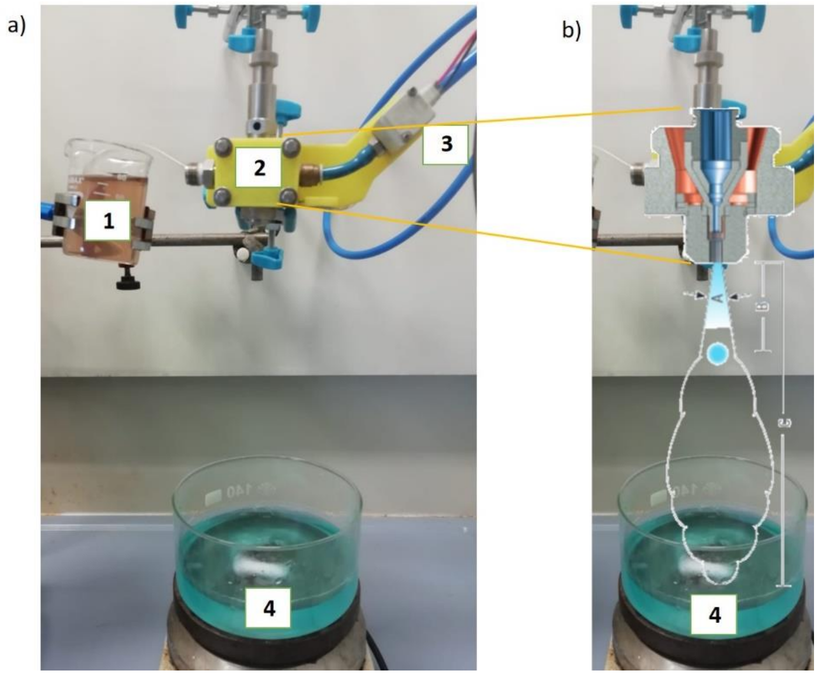


Figure 4.1. *Scale-lab set-up for microcapsules preparation (a), and a scheme of SU1A-SS nozzle used for alginate/polyphenols micro-droplets formation (b)*

The second step consists in coating ALG/Cu/PP microcapsules by chitosan (anionic polymer). The aim of this second step is to reduce the porosity, improve stability and encapsulation efficiency, and delay the release behaviour (Vinceković et al., 2016). By dispersing (ALG/Cu/PP) microcapsules (400 mL solution) into a chitosan acidic solution (600 mL, 0.208 or 0.416 wt%), chitosan rapidly binds onto their surface by electrostatic interactions between protonated amino groups of chitosan and ionized carboxylic acid groups of alginate. The electrostatic interaction between chitosan and alginate tightens and stabilizes the surface of the microcapsules (Jurić et al., 2021). Additionally, a positive charge of chitosan microcapsules loaded with copper cations facilitates better bioadhesion on leaves (Vinceković et al., 2016), which could enhance the deposition of copper on plant surfaces and thus improve its persistence on the leaf during a rainy period. Moreover, the rest of chitosan present in the solution also forms complexes with the rest of non-encapsulated copper (Mekahlia & Bouzid, 2009), protecting the cations against negative influence of the environmental conditions (*i.e.*, rainfall) and therefore leading to their prolonged presence on the leaves.

Thus, the developed formulation contains copper cations which are present in both, microcapsules and solution. Recently, European Patent Application EP21382965.8 has been filed for this new formulation, based on microencapsulated copper, to deliver copper in agricultural application.

In this study four formulation types, presented in Table 4.1 with their tank concentrations, were prepared in order to investigate the influence of anionic polymer (alginate) type and cationic polymer (chitosan) concentration on the copper deposition on vineyard crop, and being able to select the optimum one. Four formulations named: F1, F2, F3, and F4, were developed with copper microcapsules formed by a copper-anionic polymer network and suspended in copper-cationic solution. Each of these formulations contain in total 1.0 g·L-1 of Cu2+ cations and 1.5 g·L-1 of sodium alginate (NA4012 – in case of formulation F1 and F3; NA7580 – in case of formulation F2 and F4). Furthermore, formulations F1 and F2 contain 1.25 g·L-1 of chitosan, while F3 and F4 hold 2.5 g·L-1 of this polymer. Additionally, separately Ccommercially available aqueous solution containing 1 g·L-1 of Cu2+ was prepared using Ossirame 50wp (MANICA COBRE S.L.) following instructions provided by the manufacturer Manica Cobre, Spain., the Ossirame 50wp product which was used also as a reference.

Table 4.1 *– Tested copper product formulations*

Formulation	**Copper Cu^{2+}content [g·L^{-1}]**	**Anionic polymer [g·L^{-1}]**	**Cationic polymer [g·L^{-1}]**
Control	1.0	-	-
F1	1.0	1.5 (NA4012)	1.25
F2	1.0	1.5 (NA7507)	1.25
F3	1.0	1.5 (NA4012)	2.5
F4	1.0	1.5 (NA7507)	2.5

2.1.3 Microcapsules characterization

Microcapsules of prepared formulation were observed by means of optical microscope (Axiovert 40C) for transmitted light brightfield and phase contrast with condenser 0.4, inclusive object traverser M, while the micrographs were captured by DeltaPix Invenio 3S digital camera connected to the microscope. The optical micrographs were analysed by means of Image-J® software in order quantify the microcapsules diameter and provide the characteristic microcapsule size distribution.

Surface morphology and elemental composition of microcapsules were characterized by Scanning Electron Microscope with Focused Ion Beam (FESEM-FIB) equipped with Energy Dispersive X-ray spectroscopy (EDX). For this purpose, drop of washed solutions containing microcapsules were deposited on microscope aluminium supports and dried at room temperature. Prior to capsules observation by FESEM, they were metallized with a gold coating layer. The EDX studies were carried out on non-coated, washed and dried microcapsules samples (40 °C, 24 h) using a system managed by

Inca Oxford. The spectral data were acquired at the working distance 10 mm with an acceleration voltage (AV) of 5kV.

The copper content in the microcapsules were also estimated by thermogravimetric analysis (TGA) carried out by means of Mettler TGA/SDTA 851e thermobalance. The results are also given as DTGA (differential thermogravimetry curve) which is generated as the first derivative of the weight with respect to temperature. Washed and dried microcapsules samples (40 °C, 24 h) with an approximate mass of 7 mg were degraded between 30 and 600 °C at a heating rate of 10 $°C·min^{-1}$ in N_2 with a flow rate of 50 $cm^3·min^{-1}$.

2.2 Experimental set up

2.2.1 Semi-field formulate selection assay

Developed formulas (Table 4.1) were tested in a preliminary selection experiment on artificial vegetation using filter paper as collector (Figure 4.2B). The artificial canopy was designed with 1.2 m long and 0.6 m high, and the leaf area index (LAI) was set at 1.0, standard for vineyards (López–Lozano et al., 2009). The aim of this experiment was to choose the formulation with the highest deposition for a subsequent field test on real vegetation. The deposition of each formula was tested overall target canopy as a target zone. In each trial, 12 filter papers (FANOIA quantitative filter paper, cod. HK1238/30-80, 24 cm^2 area) were placed on the upper side of the artificial plastic leaves (Figure 4.2 A) distributed in three different heights (0.5 m, 0.8 m and 1.1 m) to sample the entire target surface. Collectors had a separation distance between them, in the same row, of approximately 20 cm.

A Matabi Evolution 15 L knapsack sprayer (Goizper-Matabi, Antuzuola, Spain) with a spray boom attached, was used to perform spray application of the products at 3.0 bar spray pressure (Figure 4.2A). Four ISO XR 80/110 025 flat fan nozzles (TeeJet Technologies, Glendale Heights, USA) where used at the boom (50 cm nozzle spacing), and spraying at 50 cm distance from the target to achieve an optimum spray distribution. The tests were conducted with an applied volume of 200 $l·ha^{-1}$ or all the tested formulates at 1 $m·s^{-1}$ of forward speed, as an average condition of a plant protection treatment in this crop.

Figure 4.2. *A: Spray application performance. B: Close view of the artificial collectors at the artificial vegetation.*

2.2.2 Field evaluation assay

Experimental fields on real vineyard crop (*Vitis vinifera L.*) cv. Cabernet Sauvignon, located at the UPC research facilities of Agrópolis Research Campus (Viladecans, Spain) (41°17′18.44″N/2°2′43.39″W), were performed to evaluate the deposition of the new microencapsulated copper product in real conditions. The vineyard had a surface of 1,500 m^2 (30 m × 50 m) with a planting scheme of 3.0 m × 1.2 m. and trellised following a double Royat, with two wires in each row. The experiment was performed in a primary crop stage (BBCH 55).

Three hypotheses were tested: Cupprotec 50% (C) at 1 g · L^{-1}; microcapsules selected product, Formulate 4 at 1 g · L^{-1} (M); half concentration of the microcapsule's product at formulation 0.5 g · L^{-1} (M2). Therefore, the field was divided in three equal parts of one for each test, and two entire row lines were assigned for each tested hypothesis, while letting one row to each side between the plots as a buffer zone to avoid cross-contamination (Figure 4.3A). A commercial multi-row orchard sprayer (Hardi Iris-2, Ilemo-Hardi, S.A.U., Lleida, Spain) with a 1,500 L trailed tank was used to perform the application (Figure 4.3 B). The sprayer was equipped with four lateral booms, each having eight nozzles, and able to spray two rows of vine lines simultaneously. The hydro-pneumatic sprayer was provided with a centrifugal fan offering an average air flow rate of 7500 m^3·h^{-1} (E. Gil et al., 2015).

The sprayer was adjusted according to the development stage of the crop at 80 L·ha^{-1}, using two ISO 01 nozzles for each side boom of the sprayer, at 4.9 bar and at a forward speed of 5 km/h. The application volume rate was determined based on the application of the DOSAVIÑA® (E. Gil et al., 2019) to adjust it to the canopy characteristics measurements. Thirty composed samples of 4-5 leaves collectors were picked up at different random points of the sprayed area for each treatment. Before each application, 10 more composed leaf samples were collected as blanks, to ensure there was no cross contamination among the treatments.

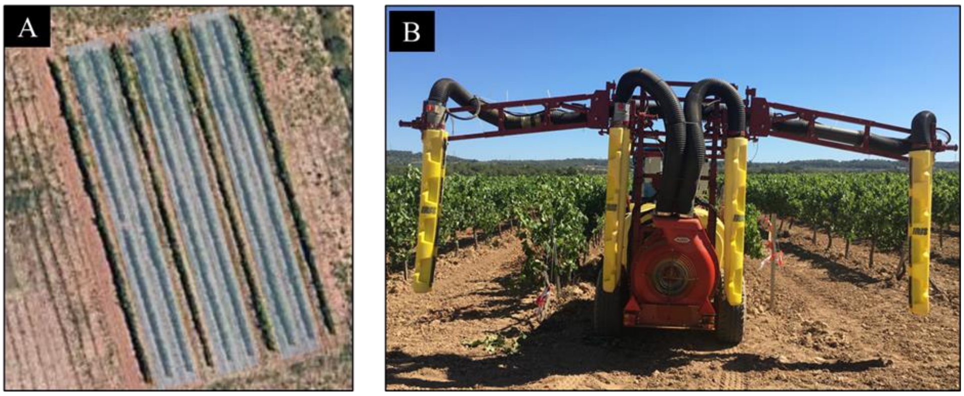


Figure 4.3. *A: Field plot scheme. B: Hardi Iris-2 sprayer used in the field experiment.*

In addition, two petri dishes without a lid were positioned vertically at the same height as the vegetation to evaluate the structure of the microcapsules after the spraying process.

2.2.3 Soil leaching assay

A laboratory assay was performed in order to evaluate the environmental soil assessment of the microencapsulated copper product formulation developed, compared to the control copper product. Two vineyard soil types from commercial vineyard fields located in the Tarragona region of Catalonia (Spain) were selected, for this reason also they already have high copper contain (Table 4.2). For this testing, holes were punctured in the lower part of 50 mL falcon tubes and filled with soil. 36 falcon tubes were prepared with each type of soil, allocating 18 to each product tested (control and microcapsules at 1 mg · mL of copper concentration) and dividing these into triplicates for evaluation at different times (0, 24, 48, 72, 120 and 240 h). Using a pipette, 1 mL of the corresponding product was applied on the surface on each falcon. Evaluation of the soil retention was made by applying 30 mL of distillate water by the top part of the tub and colleting the leached water at the bottom outlet (Figure 4.4) at each established sampling time. The copper content of the two resulting samples, soil and leached water, was analysed.

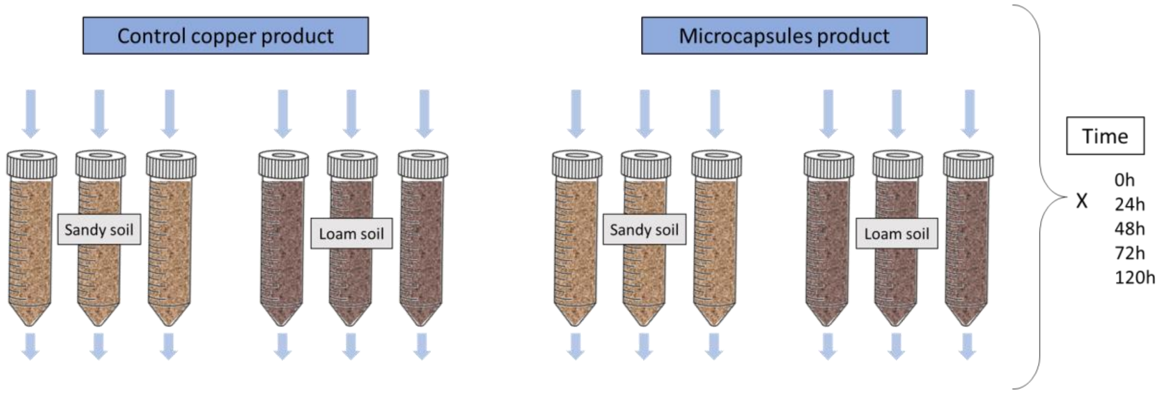


Figure 4.4. *Experimental design of the copper soil assessment evaluation.*

Table 4.2. *Laboratory analysis of the two soil types used in the experiment.*

Soil characteristics	**Loam soil**	**Sandy soil**
pH	8.8	8.9
Electrical conductance (dS·m^{-1})	0.15	0.1
C content (% DMB)	0.95	< 0.29
Organic matter (%DMB)	1.6	< 0.5
Calcium carbonate equivalent (%DMB)	33	< 3
Cu (mg·kg^{-1} DMB)	4.8	21.3

**DMB, dry matter basis*

2.2.3 Analytical methodology for copper determination

Artificial and vegetal collectors used to determinate the copper deposition were analysed as follows: copper content of the samples was measured by washing the collectors with 25 mL of a solution of HNO_3 0.05 N during 5 min. Copper content was measured on the solution extracted, containing the copper cations, according to Salyani et al., 1988 (Salyani & Whitney, 1988). Copper deposition was calculated related to the area of the samples. Artificial collectors had a fixed area of 24 cm^2, whereas the vegetative collector's area was estimated by the weight-surface ratio according to Git el al. (2021). The copper content of the tank mixtures was also measured to ensure that the concentrations of the different applications were the same. On the other side, soil samples were analysed using the DTPA (diethylenetriaminepentaacetic acid) extraction method (Lindsay & Norvell, 1978).

All copper determinations were quantified with a Varian SpectrAA 110 (Mulgrave, Victoria, Australia) flame atomic absorption spectrometer, equipped with a copper hollow cathode lamp, and a deuterium lamp for background correction. The instrument was operated under the conditions recommended by the manufacturer: lamp current of 4 mA, wavelength of 324.7 nm, slit width of 0.1 nm, burner height of 14 mm, acetylene flow rate of 1.0 L·min^{-1}, and air flow rate of 10.0 L·min^{-1} (linearity ranged from 0.5 to 5 g·L^{-1}).

2.2.4 Data analysis

The experimental results were statistically analysed using RStudio (RStudio Team, 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/). An ANOVA test and Tukey HSD post hoc were performed to study the deposition differences by the different formulations, on the artificial and vegetal collectors.

3 *Results and discussion*

3.1 Microcapsule characterisation

Figure 4.5. *Optical microscope micrograph of wet microcapsules maturated in $CuSO_4$ aqueous solutions.*

Representative optical microscope micrograph of prepared microcapsules is shown in Figure 4.5. The microcapsules prepared appear separated, well-formed and globe shaped. By means of Image-J ® software optical micrographs were analysed to provide the characteristic distribution of the microcapsule diameters (see Figure 4.6). From the distributions could be observe that the diameter of the biodegradable microcapsules is between 4 and 64 (±2) µm, and 80% of the capsule diameters is in the range 4–19 µm. Particle size analysis of microcapsule provides additional information on the applicability of the formulation in the field sprayers which might be blocked if particle size exceeds 100 µm.

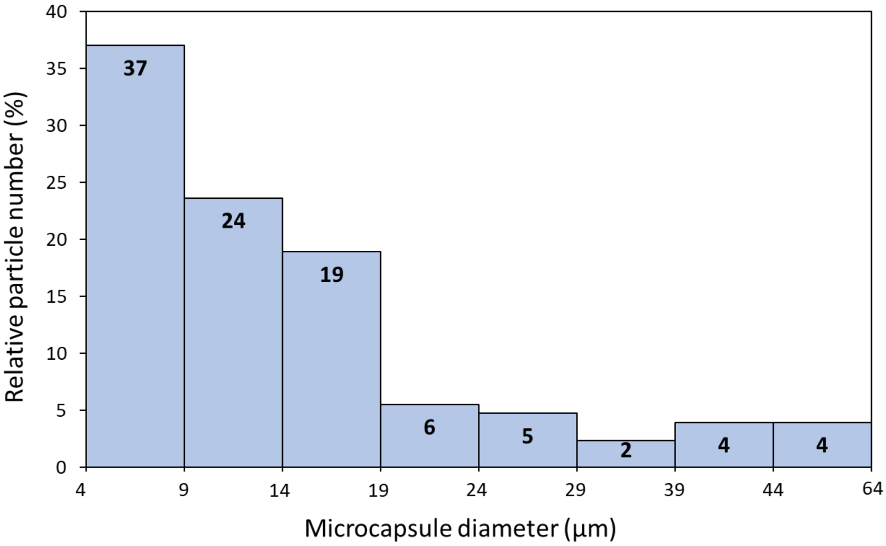


Figure 4.6. *Microcapsules size distribution, measured on 125 microcapsules, on the example of Formulation 4.*

Figure 4.7 shows FESEM image of microcapsules obtained on the example of Formulation 4. All obtained microcapsules exhibit regular (spherical) morphologies, but they are deflated. This evidence can be related to the entrapped water evaporation in the high-vacuum conditions (10×10^{-2} mbar) employed in sample preparation inside of the equipment. Furthermore, the flattened shape of the capsules could indicate their core/shell structure. Besides, the ESEM investigations of their surfaces put into evidence of their rough dense morphologies. In order to evaluate the efficiency of copper encapsulation, EDX investigation were carried out. This method allows to characterize the differences in the elemental composition of investigated samples. The result of this semi-quantitative micro-analysis shows that the microcapsules contain approximately 28.2 ± 3.4% of copper cations in their structures.

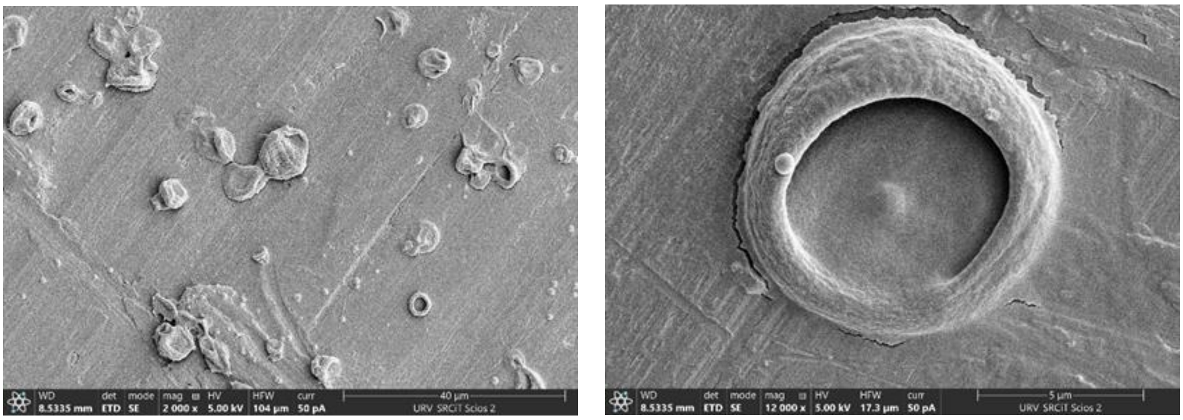


Figure 4.7. *FESEM images of prepared microcapsules in Formulation 4.*

The copper content in the microcapsules was also estimated by dynamic thermogravimetry (TGA and DTGA). As can be observed in Figure 4.8, the TGA and DTGA curves show the loses of around 67% of sample weight during the thermal degradation process until 600°C. The remaining 33% of char is attributed to the copper component of the microcapsules. As shown in Figure 4.7, the first stage of the decomposition process occurred between 48 and 150 °C which is due to dehydration or loss of water (5.8%). The second peak (responsible for major weight loss) occurs between 150 to 400 °C which is attributed to the degradation of organic biomass part in ALG/Cu/PP/CH capsules where bond breaking, conformation changes, and escape of volatiles occurred as the temperature increased. These weight losses constitute of 55.2% indicating the majority component of the capsules are organic compounds (*i.e.*, alginate, chitosan, and polyphenols). The last wave of weight loss at 400 to 600°C (6.2%), is a usual thermal processes characteristic of char formation, degradation of robust inorganic substances, tar cracking and secondary decomposition reactions. For the alginate biomass, the char is mostly composed of metal carbonates or metal oxides because of the interaction with CO_2. Therefore, for a copper alginate matrix, the char would consist of $CuCO_3$ and CuO, and decomposition of CuO to CuO_2. (Camacho et al., 2019).

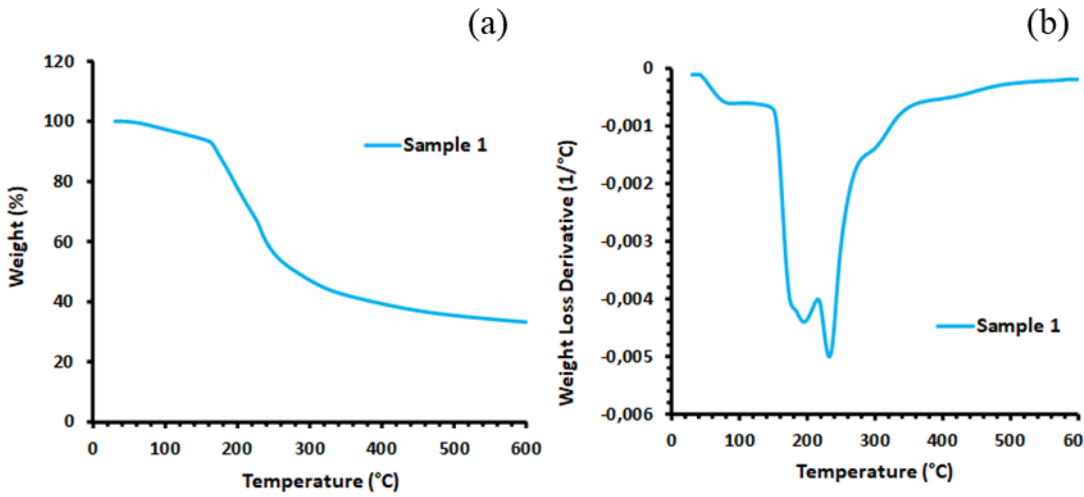


Figure 4.8. *TGA (a) and DTGA (b) curves of the microcapsules using* N_2 *atmosphere.*

3.2 Formulate selection

After performing the deposition trials in artificial vegetation, it was found a higher deposition for all the microcapsule formulations compared to the conventional product (Figure 4.9). It clearly indicates that microencapsulation technique improves the copper deposition on filter paper. It also goes in agreement with Rojas-Lema et al. (2020) which found similar results. Novel copper products deposition into the artificial vineyard, ranged from 38% (Formulation 3) to 85% (Formulation 1) higher than that of conventional copper (Figure 4.9). Nevertheless, three of them had statistically differences amount of copper deposition: Formulation 1, Formulation 2 and Formulation 4. The anionic polymer type (NA14012; NA7507) or the amount of cationic polymer does not seem to have a clear effect on the copper deposition by itself, since there are not differences between the formulates. In this case, the median has been used as the selection criterion as it is more robust and is a parameter that is less affected by the deviation of the data, which can be observed since the median and average are not close values. Formulation 4, therefore, has the highest median value ($0.062 \pm 0.016\ \mu g\cdot cm^{-2}$) in comparation to the other developed formulations (Figure 4.9), and 63% more control than the reference copper-based fungicide tested. This result is supported by the fact that Formulation 4 is one that contains the highest concentration of cationic polymers (Table 4.2). Higher concentration of chitosan (cationic polymer) in general form more stable capsules and higher amount complex polymer-metals, but also increase the adhesion properties of the formulation to the plant surface resulting in better copper deposition as compared to the other formulation tested. However, it is well known that tank mixtures, and polymer concentrations can affect to droplets formation and for instance the leaves deposition (Bouse et al., 1990). This could be the factor that has increased deposition, as well as its deviation at the same time, due to variations in droplet size due to the physic-chemical characteristics of the new formulation.

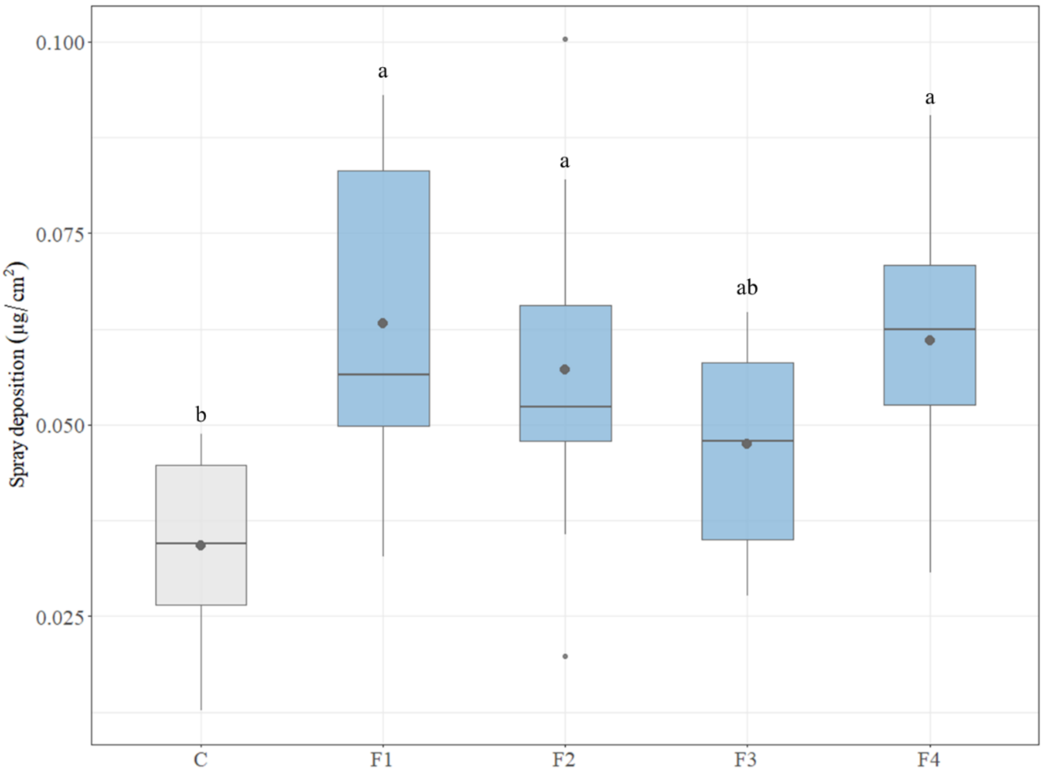


***Figure 4.9**. Boxplot of spray deposition collected (µg·cm-2) for the different formulations tested. HSD Tukey (P < 0.05) differences expressed with a central letter on the boxplot. C: control; F1: Formulation 1; F2: Formulation 2; F3: Formulation 3; F4: Formulation 4. Mean value displayed as a point.*

3.3 Microcapsules efficiency: deposition evaluation

After the spraying process, microcapsule's structure did not suffer any damage, as it is observed in a droplet of the sprayed product (Figure 4.10) observed under the microscope after the spraying performance. This is appropriate as the aim is to preserve the structure of the microcapsules once deposited in the vegetation, in order to a slowly released. Each sprayed droplet contains copper cations in the capsule network and complexed in the formulation medium which are going to act as the active ingredient on the leaves for downy mildew control. Additionally, as can be observed in Figure 4.10, there is no microcapsule agglomeration after spraying, the microcapsules are well dispersed in a whole droplet of the sprayed product. Lack of capsules agglomeration is also crucial for the best effect against mildew since microcapsules must form a homogenous layer of particles on wine leaves after spraying.

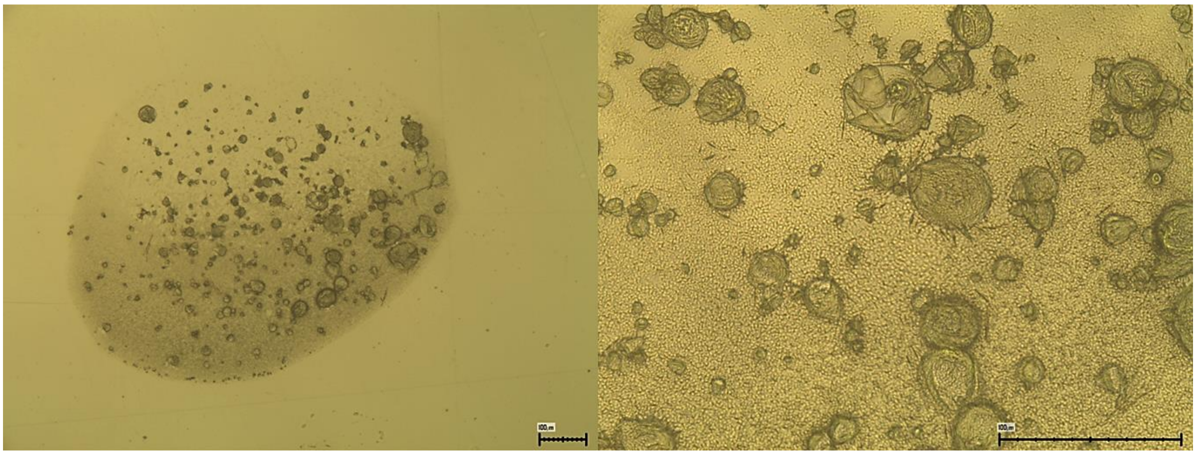


***Figure 4.10**. View of the microcapsules structure with a microscope after performing the spray application. Left- deposit droplet containing the microcapsules. Right- close view of the microcapsules.*

Figure 4.11 shows the deposition results on the real vegetation vineyard leaves. The developed microencapsulated product (M) had a significant higher deposition compared to the conventional copper fungicide tested, confirming the preliminary tests. The novel formulation deposited a copper concentration of 2.072 ± 0.9 $\mu g \cdot cm^{-2}$ on the leaves, while conventional copper deposited 0.802 ± 0.3 $\mu g \cdot cm^{-2}$, which is approximately 2.5 times less than the microencapsulated product. Treatment M2, which is the product formulated with 0.5 $g \cdot L^{-1}$, shows no statistical difference with the control (C), which would reduce the amount of metal sprayed by 50% if used on the farms, and therefore help improve biodiversity by indirectly reducing the amount of losses and therefore the ecotoxicological impact (Karimi et al., 2020). Despite these good results of the novel formula, it can also be observed that the developed product has a larger deposition deviation (Figure 4.11), where the densities of the data are presented in a more elongated form. Moreover, conventional product has a data distribution with marked pics, with the main concentration of the data on the average line, meaning it is more homogeneous, and therefore that is more suitable for crop protection proposes. The high heterogeneity of the microencapsulated product could be due to the agitation process in the machine. Even the spraying conditions where the same, it has been observed that fluid velocity affects to the agitation process (Javier García-Ramos et al., 2018), and that could happen due the new product characteristics.

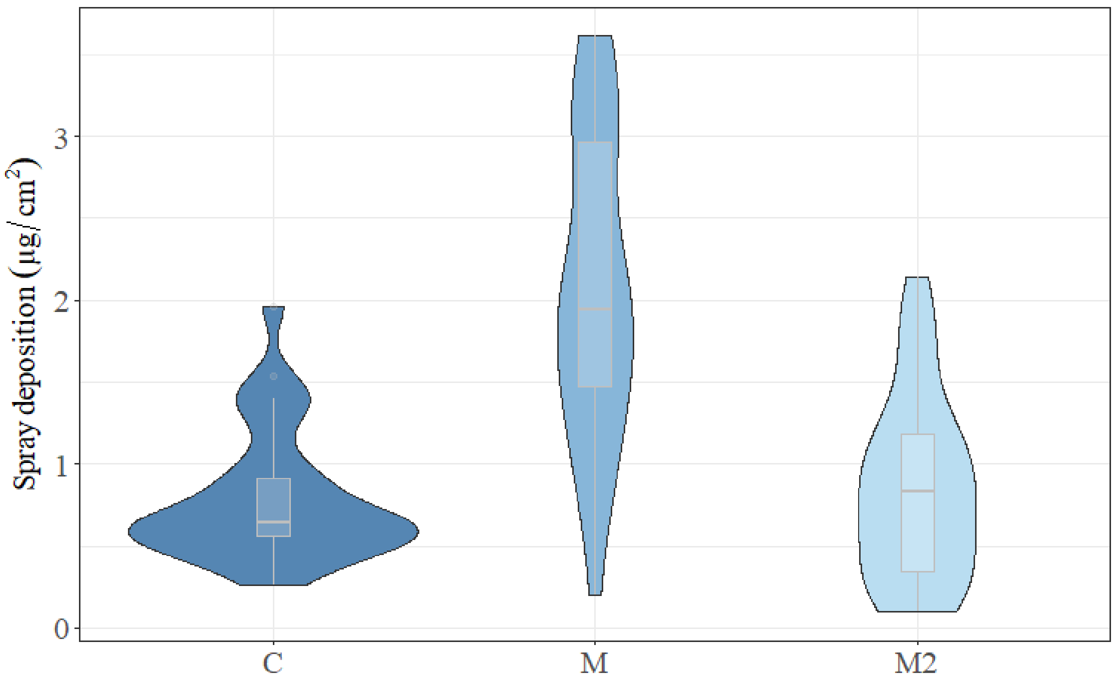


Figure 4.11. *Violin plot of spray deposition collected (µg·cm^{-2}) for the different treatments tested in the field. HSD Tukey (P < 0.05) differences expressed with a central letter.*

3.4 Environmental soil evaluation

There is a clear soil dynamic difference of the copper products, depending on the soil typology (Figure 4.12 and Table 4.5), which goes in concordance with other studies that found different mobility of metal cations depending on the soil characteristics (Smolders et al., 2009) . In the loam soil, the copper leached by the conventional product test decreases related to the time, which indicates this copper becomes fixed to the ground over time. This can be explained because high percent of the copper is bound by the soil organic matter (McLaughlin et al., 2000; Smolders et al., 2009). On the other hand, the microcapsule test increases the leaching of copper over time, suggesting that they degrade and leach copper over a longer period of time rather than as a point source of contamination. This goes in the same line in the kinetics calculated in Table 4.3, where it is observed a big difference between the half-life time of the two tested products, being 1747 days for the coper applied with the conventional product and 852 days for the one applied with the new developed product. Therefore, the results show that for loam soil, these microcapsules would have slow flushing of the soil, thus preventing copper from accumulating in the soil, but without a quick leaching that they could be a point source of contamination.

Moreover, this behaviour is notoriously accentuated by the sandy soil. The leaching of the conventional copper product goes up to 0.22 ± 0.12 and 0.23 ± 0.11 in the 0 and 24h respectively and decreases as soil contact time increases (Figure 4.12). However, the leached amount of copper by the microcapsules is significative minor, but it still can be seen the slow degradation effect observed in the loam soil. In this case, half-life times of the two copper products are completely opposites (Table 4.3). While the microencapsulated product kinetic decreases even faster than in the loam soil, 0.0010 d^{-1}, the conventional copper contain in this soil type increases over time (Table 4.3). That can be explained due the soil parameters as soil carbon contain (Droz et al., 2021b), organic matter (Manceau & Matynia, 2010) and pH which is also a determinant factor in the soil copper bounding (Sauvé et al., 1997) (Table 4.2). In this case, it has not been distinguished between the leached copper originating from the soil (even the high concentration) or from the applied copper products, in order to, be as much reliable to the real field behaviour.

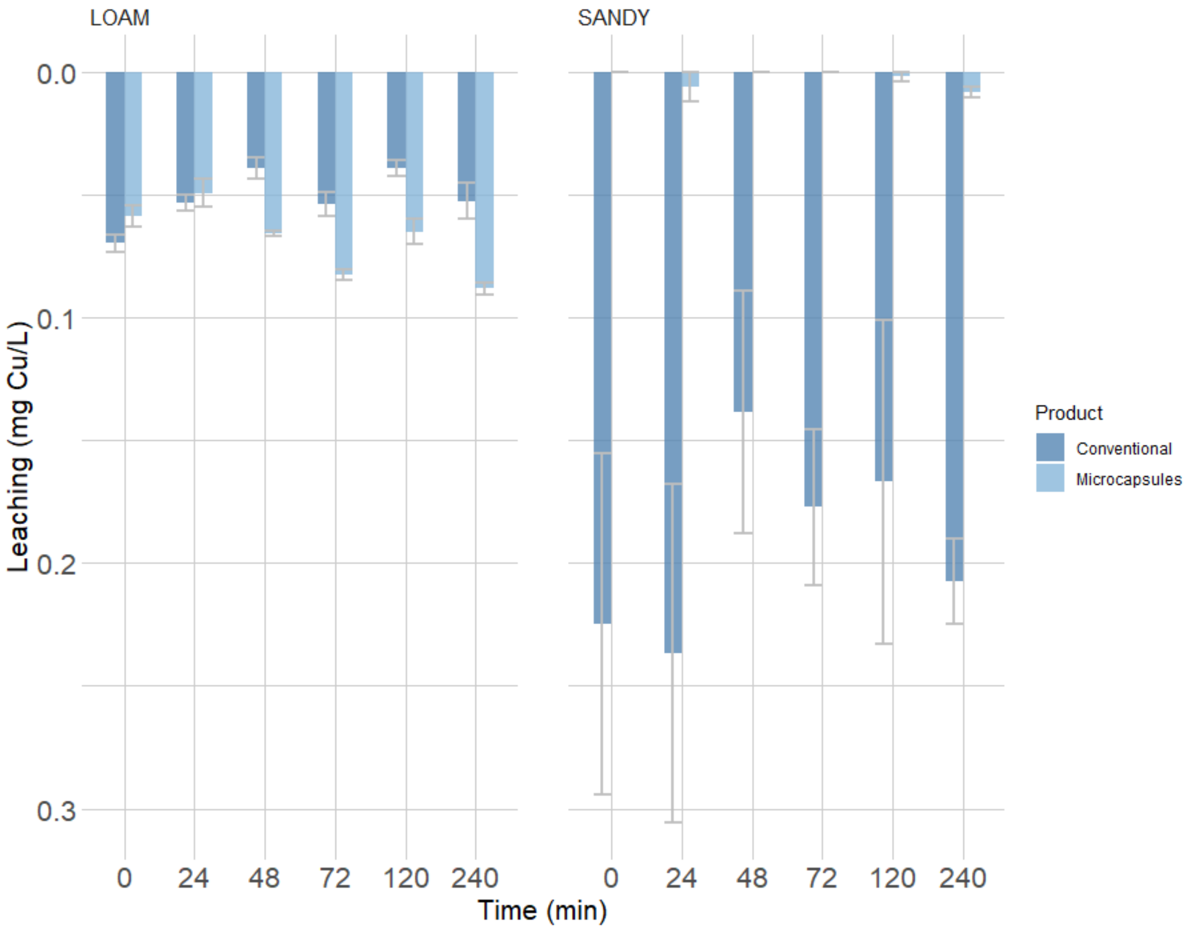


***Figure 4.12**. Bar plot of the leached copper (mg Cu·L $^{-1}$) for the two tested soils and products. Bars means ± SE of the data.*

Table 4.3. *First order kinetic and half-life time of the soil copper contain.*

Soil	Product	K (d^{-1})	R^2	T ½ (d)
Loam	C	0.0004	0.70	1747
	M	0.0008	0.98	852
Sandy	C	-0.0010	0.90	-640
	M	0.0010	0.96	663

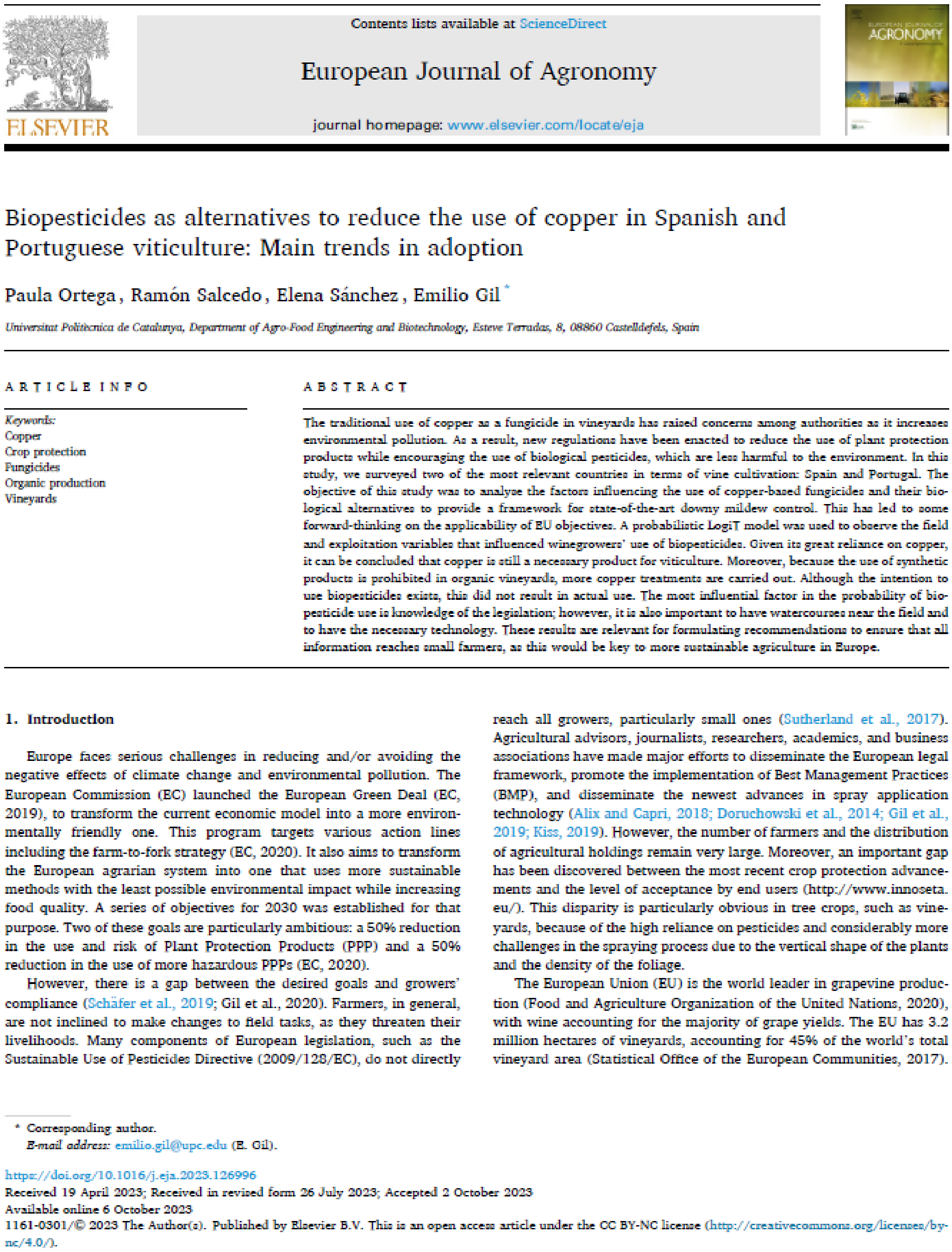
4. Conclusions

All the formulations of the microencapsulated product improved the deposition on the artificial collectors in comparison to the control product. Also, the selected product obtained significantly better results in real vineyards than the reference product. Microencapsulated test with half of the deposition obtained no statistical differences from the conventional one, which indicates it can offer a potential 50% reduction of the metal used as a fungicide. The new copper product has a behaviour that may have less impact on the soil and water, but further results in real soil conditions are needed. Not only does it not bind strongly to the soil over time as the conventional product does, but it also leaches slowly, mitigating the occasional contamination that could occur. It can therefore be concluded that the product developed has been tested with promising results. Further research in biological efficiency is needed for the hole evaluation of the new microencapsulated product.

CHAPTER V. Study on the adoption of alternative products by winegrowers

Copper-based products can be replaced by other types of organic fungicides, the so-called natural or biopesticides, which in general have a lower risk for the environment. The increase in organic vine cultivation in recent years has increased the application of these products, but they are not yet widely used in the fields, although they are an option to reduce pollution. This chapter, therefore, examines the variables that affect the adoption of this type of formulation.

Article 4: Biopesticides as alternative to reduce the use of copper in Spanish and Portuguese viticulture: Main trends in adoption



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Biopesticides as alternatives to reduce the use of copper in Spanish and Portuguese viticulture: Main trends in adoption

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ABSTRACT

The traditional use of copper as a fungicide in vineyards has raised concerns among authorities as it increases environmental pollution. As a result, new regulations have been enacted to reduce the use of plant protection products while encouraging the use of biological pesticides, which are less harmful to the environment. In this study, we surveyed two of the most relevant countries in terms of vine cultivation: Spain and Portugal. The objective of this study was to analyse the factors influencing the use of copper-based fungicides and their biological alternatives to provide a framework for state-of-the-art downy mildew control. This has led to some forward-thinking on the applicability of EU objectives. A probabilistic LogiT model was used to observe the field and exploitation variables that influenced winegrowers' use of biopesticides. Given its great reliance on copper, it can be concluded that copper is still a necessary product for viticulture. Moreover, because the use of synthetic products is prohibited in organic vineyards, more copper treatments are carried out. Although the intention to use biopesticides exists, this did not result in actual use. The most influential factor in the probability of biopesticide use is knowledge of the legislation; however, it is also important to have watercourses near the field and to have the necessary technology. These results are relevant for formulating recommendations to ensure that all information reaches small farmers, as this would be key to more sustainable agriculture in Europe.

1. Introduction

Europe faces serious challenges in reducing and/or avoiding the negative effects of climate change and environmental pollution. The European Commission (EC) launched the European Green Deal (EC, 2019), to transform the current economic model into a more environmentally friendly one. This program targets various action lines including the farm-to-fork strategy (EC, 2020). It also aims to transform the European agrarian system into one that uses more sustainable methods with the least possible environmental impact while increasing food quality. A series of objectives for 2030 was established for that purpose. Two of these goals are particularly ambitious: a 50% reduction in the use and risk of Plant Protection Products (PPP) and a 50% reduction in the use of more hazardous PPPs (EC, 2020).

However, there is a gap between the desired goals and growers' compliance (Schäfer et al., 2019; Gil et al., 2020). Farmers, in general, are not inclined to make changes to field tasks, as they threaten their livelihoods. Many components of European legislation, such as the Sustainable Use of Pesticides Directive (2009/128/EC), do not directly reach all growers, particularly small ones (Sutherland et al., 2017). Agricultural advisors, journalists, researchers, academics, and business associations have made major efforts to disseminate the European legal framework, promote the implementation of Best Management Practices (BMP), and disseminate the newest advances in spray application technology (Alix and Capri, 2018; Doruchowski et al., 2014; Gil et al., 2019; Kiss, 2019). However, the number of farmers and the distribution of agricultural holdings remain very large. Moreover, an important gap has been discovered between the most recent crop protection advancements and the level of acceptance by end users (http://www.innoseta.eu/). This disparity is particularly obvious in tree crops, such as vineyards, because of the high reliance on pesticides and considerably more challenges in the spraying process due to the vertical shape of the plants and the density of the foliage.

The European Union (EU) is the world leader in grapevine production (Food and Agriculture Organization of the United Nations, 2020), with wine accounting for the majority of grape yields. The EU has 3.2 million hectares of vineyards, accounting for 45% of the world's total vineyard area (Statistical Office of the European Communities, 2017).

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Biopesticides as alternative to reduce the use of copper in Spanish and Portuguese viticulture: Main trends in adoption

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Abstract

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Keywords: Copper · Crop protection · Fungicides · Organic production · Vineyards

1. *Introduction*

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Downy mildew, caused by *Plasmopara viticola* is one of the most damaging diseases affecting grapevines (Fontaine et al., 2021). Given its proven effectiveness, copper is the active ingredient in the vast majority of fungicides used to combat this disease(Gessler et al., 2011). Organic viticulture, which also has a substantial economic impact in Spain and Portugal(Willer et al., 2008), is even more important because organic production options are limited. Copper can become toxic at high accumulation levels, harming the biodiversity living in soils and watercourses (Fagnano et al., 2020; Lamichhane et al., 2018). Additionally, copper accumulation in European vineyard soils (Ballabio et al., 2018) prompted the European Union to tighten regulations on the use of copper as a plant protection product (COMMISSION IMPLEMENTING REGULATION (EU) 2018/1981 on 13 December 2018), limiting its use by growers, even when there is no substitute. To maintain the productivity of the sector it is necessary to research new alternatives. One such option is the use of biopesticides such as *Yucca schidigera* and *Salvia officinalis* extracts or *Trichoderma harzianum*, which have already shown promising results for the control of *P.viticola* under greenhouse and field conditions (Dagostin et al., 2011b).

Biopesticides are products or chemicals originating from living organisms, such as microbes and nematodes, as well as plant-derived products, secondary metabolites from microorganisms, insect pheromones, and genes used to induce natural resistance in crops (Copping et al., 2000). Biopesticides generally have fewer hazards in their management than synthetic pesticides, are more considerate of non-target organisms in the ecosystem and have minimal persistence in the food chain (Seiber et al., 2018). Given these considerations, partial substitution of agrochemical products with biopesticides may be a viable option for meeting the Farm to Fork Strategy's 2030 objectives. However, biopesticides are limited in comparison to chemical pesticides because of high manufacturing costs, poor storage stability, and efficacy problems, among other issues (Samada et al., 2020). They still require research and development, but these products generally have a lower environmental impact than chemical pesticides, making them viable alternatives to conventional pesticides (J. Kumar et al., 2021).

However, several questions arise while developing new strategies against pesticides and pests. For example, what is the winegrower's understanding of pesticides and their use? Do they possess the resources necessary for adequate product management? Are farmers prepared to use biopesticides as substitutes for Cu? Are the European Union's targets for reducing pesticide use feasible? However, no research has been conducted to determine the reasons for the farmers' adoption or non-adoption of biopesticides as substitutes for Cu in vineyards. A specific survey was designed and distributed among vineyard growers in Spain and Portugal to expand knowledge and experience about the use of biopesticides as a viable alternative to copper. The survey also intended to assess the degree of knowledge and implementation of ongoing European legislation to reduce the use of copper fungicides. This study aims to identify the factors that influence the adoption of biopesticides. Therefore, the main objective is to assess the factors influencing the use of copper-based fungicides and the adoption of biological alternatives to control downy mildew and relate them to current regulations and the objectives proposed by the EU. The following specific objectives have been established to achieve this: i) to analyse the use of copper-based products by Spanish and Portuguese winegrowers, ii) to assess the use of biopesticides and the factors that influence their adoption, and iii) to evaluate compliance with EU objectives regarding the use of plant protection products in this case.

2. *Materials and methods*

2.1 Data collection, questionnaire, and sample size

A total of 98 farmers (75 from Spain and 23 from Portugal) were surveyed to ascertain the extent of copper and biopesticide use in Portugal and Spain. Data were collected through an online questionnaire. Between September and December 2021, farmers who were approached through the databases of consultants, wineries, and public institutions involved in viticulture research were given access to the online questionnaire. The questionnaire included 30 questions divided into four categories: 1) farm data, 2) crop characteristics, 3) copper products against downy mildew, and 4) biological products against downy mildew. The details of the full survey are listed in Table 5.1. The survey was voluntary, and participants were told the information would only be used for research. Neither the identity nor the affiliation of those consulted were disclosed in accordance with Regulation (EU) 2016/679.

Table 5.1. *Full questionnaire distributed among the farmers*

Farm	**Question type**	**Answer options**
Country	Category	Portugal / Spain
Total area of vineyard fields (ha)	Numeric	-
Average productivity of the last three years (kg/ha)	Numeric	-
Degree of adoption of new technologies in the field (and in the winery if any)	Rank scale	(0-All activities carried out manually/ 10- All activities carried out with machinery)
You consider that the agronomic practices carried out on his farm are rather	Rank scale	(0-Traditional - Conventional / 10-Alternative - Innovative)
Is the farm certified organic?	Category	Yes / No / In process
If yes, from which year?	Numeric	-
Crop characteristics		
Indicate the vineyard variety (if more than one, indicate the three most common)	Category	Open answer
Vineyard training system (if several, indicate the most common)	Category	Trellis / Gobelet / Pergola / Other

Downy mildew pressure	Rank scale	(0-No pressure from this disease/ 10-Very high disease pressure)
Powdery mildew pressure	Rank scale	(0-No pressure from this disease/ 10-Very high disease pressure)
Do you consider water infiltration into the soil a problem?	Rank scale	(0-Yes, the soil is clayey and water stagnates/ 10-No, the soil is sandy and water infiltrates easily)
Do you consider the slope of the ground a problem?	Rank scale	(0-No, the terrain is flat/ 10-Yes, there are areas with steep slopes)
Are there any geographical features close to the farm?	Category	No / Water courses / Forest / Urban nuclei / Other
Mildew applications - copper products		
Do you use copper-based fungicides?	Category	Yes / No
What type of copper product do you use?	Category	Oxychloride / Hydroxide / Oxide / Sulphate / Other
Copper concentration of the product [%] (if more than one, choose the one with the highest amount used)	Numeric	-
How many treatments with copper products do you perform per year (average of the last three years)?	Numeric	-
Are you aware of the current regulation (EU) 2018/1981 on copper containing products?	Category	Yes / No
Have any application parameters changed because of the new restrictions (4 kg/ha*year of copper)?	Category	Yes / No
Mildew applications - biological products		
Do you know about organic products as an alternative to copper products?	Category	Yes / No
Do you use this type of product?	Category	Yes / No
If yes		
What kind of active ingredients are in the biological products you use?	Category	Oils / Bacteria / Seaweeds / Fungi / Other
Do you think this type of product works correctly?	Category	Yes / No

Do you consider that the information on the label is enough for a correct application?	Category	Yes / No
What information would you add to the label (even if you think the existing information is sufficient)?	Category	-
What parameters do you vary for the application of biological products?	Category	No one / Pressure / Speed / Nozzles / Air assistance / Other
If no		
Why don't you use organic products?	Category	Doubt about the effectiveness / Price / Difficulty of application / Other
Do you think you would use them if you saw that their use was widespread among professionals in the sector?	Category	Yes / No / Maybe

2.2 Data analysis

The RStudio software (RStudio Team 2020; Boston, MA, USA; URL; http://www.rstudio.com/) was used to perform the statistical analyses. A flowchart of the methodology is shown in Figure 5.1. The Kruskal–Wallis Test (non-parametric approach) was used to assess differences in the number of Cu treatments for organic and non-organic farmers, with a level of significance of $p < 0.05$.

A binary logistic regression model (logit model) was used to analyse the factors that affected the decision to adopt biopesticides. The logit model analyses an event’s probability of success in the response variable as a linear function of the independent variables. To build the model, all categorical variables were transformed into dummy variables (1 and 0). Each level of the categorical variables was recombined into a single dummy variable. Virtually all the available variables were included as factors in the following equations (Eq. 1 and 2):

Equation 1.

$$log(odds) = logit(p) = ln\left(\frac{p}{1-p}\right) = \frac{probability\ of\ Yes}{probability\ of\ No}$$

Equation 2.

$$logit(p) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_k X_k$$

where (p) is the probability of using biopesticides, $(X_1, X_2, \dots, X_k)$ represents the (k) independent variables, like field area or the presence of water courses near the farm, and $(\beta_0, \beta_1, \beta_2, \dots, \beta_k)$ the coefficients to be estimated through the regression for each one of those variables. This logistic regression is interpreted posteriorly by calculating the odds ratios (ORs) for each variable (Orin =e^{β_i}), which represent the modification that occurs in the response variable for every one-unit change in the independent variable (Pojman et al., 2019). It quantifies the increase or decrease in the probability of adopting a biopesticide when the independent variable increases by one unit. The Akaike information criterion (AIC) was used to assess the different possible models, determine the best fit for the data and estimate how much information was lost overall. Once the model was selected, the Wald index was used to assess each variable's statistical significance at the 95% and 90% confidence levels, after which the Hosmer– Lemeshow test was used to determine the goodness of fit of the model.

Principal component analysis (PCA) was performed to investigate the relationships between numerical variables. The variables were rescaled from 0 to 10 to homogenise their order of magnitude.

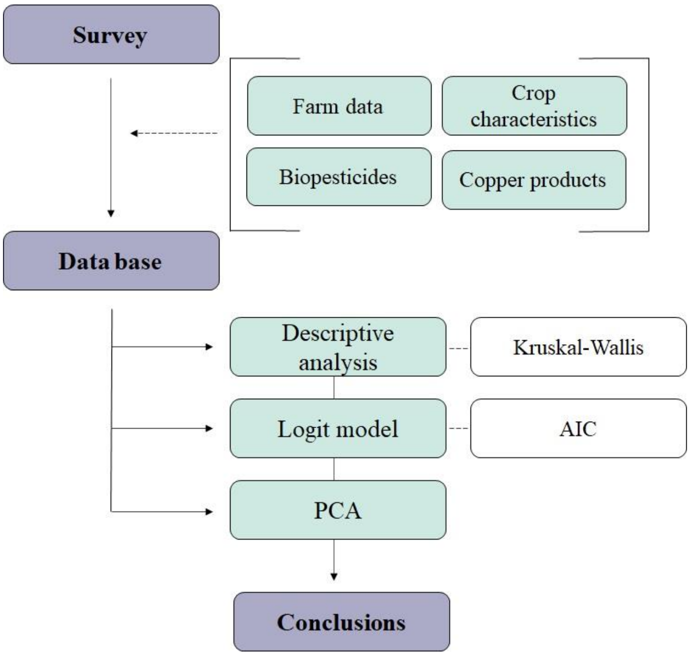

Figure 5.1. *Flow chart illustrating the methodology used in the investigation*

3. Results and discussion

According to the survey sample, 61% of respondents worked in the vineyards of a winery, while the remaining 39% owned vineyards without a wine cellar. The majority of these farms were smaller than 50 ha in size (59.6%), produced between 5000 and 10000 kg/ha of grapes (54.7%), or were not organic certified (62.3%) (Table 5.2). According to the Statistical Office of the European Communities (2020), 33% of farms were less than 10 ha, which corresponds with the average size of a vineyard in the EU, 1.4 ha in 2020. Therefore, both small-scale wine growers and large estates were represented (large estates were regarded as a cultivated area greater than 100 ha). In general, winegrowers cultivated redder (69.9%) than white grape varieties (30.2%) and were located near forests (36%) and watercourses, such as rivers (32%). Table 5.2 summarises the main variables in the collected data. the sample

Table 5.2. *Descriptive summary of the collected dataset in frequency and percentage.*

		Frequency	**Percentage (%)**
Country	Spain	74	77.1
	Portugal	24	22.9
Area (ha)	0–10	31	33.0
	11–50	25	26.6
	51–100	13	13.8
	101–500	14	14.9
	>500	11	11.7
Productivity (kg/ha)	600–1000	2	2.3
	1001–5000	27	31.4
	5001–10000	47	54.7
	>10000	10	11.6
Grape varieties	Tempranillo	34	19.9
	Garnacha	30	17.5
	Cariñena	13	7.6
	Syrah	12	7.0
	Other red	82	48.0
	Macabeo/Viura	17	26.2
	Albariño	6	9.2
	Other white	42	64.6
Crop characteristic	Trellis	69	75.8
	Gobelet	15	16.5
	Pergola	6	6.6
	Other	1	1.1
Geographical features	Near forest	33	36
	Near watercourses	29	32
	Near urban nuclei	4	4
	No geographical features	24	24
Organic certification	Yes	27	30.0
	No	56	62.3
	In process	7	7.7

3.1 Use of copper fungicides

Of the respondents, 79.0% were aware of the current legislation governing copper use in the EU, whereas 21.0% were not. Furthermore, nearly half of the respondents (47.0%) felt the present restrictions affected them personally. Copper-based products, primarily copper oxychloride (47.0%) and copper sulphate (28.0%), were used as fungicides against downy mildew by 93.0% of the farmers polled. Hydrochloride (17%), copper oxide (2%), and other materials (6%) were also used, but to a smaller extent. A large number of farmers (79.5%) applied less than five applications of Cu during the season, with three applications being the most typical (33.7%). Figure 5.2 shows that conventional farmers concentrated on high density coverage within an average of two to three treatments. Most organic farmers used the three treatments, with a median of 4, because there were farmers who carried out up to 15 copper applications. The Kruskal–Wallis test revealed significant differences (p = 0.0039) in the average number of treatments between conventional and organic growers, who had the most applications. This is consistent with Pořízka et al. (2021), who found that the concentration of copper in organic wine was nearly three times higher than that in wines from conventional viticulture due to the higher usage of copper fungicides in organic vineyards. According to Čuš et al. (2022) there were no statistically significant differences in Cu residues between organic and conventional production.

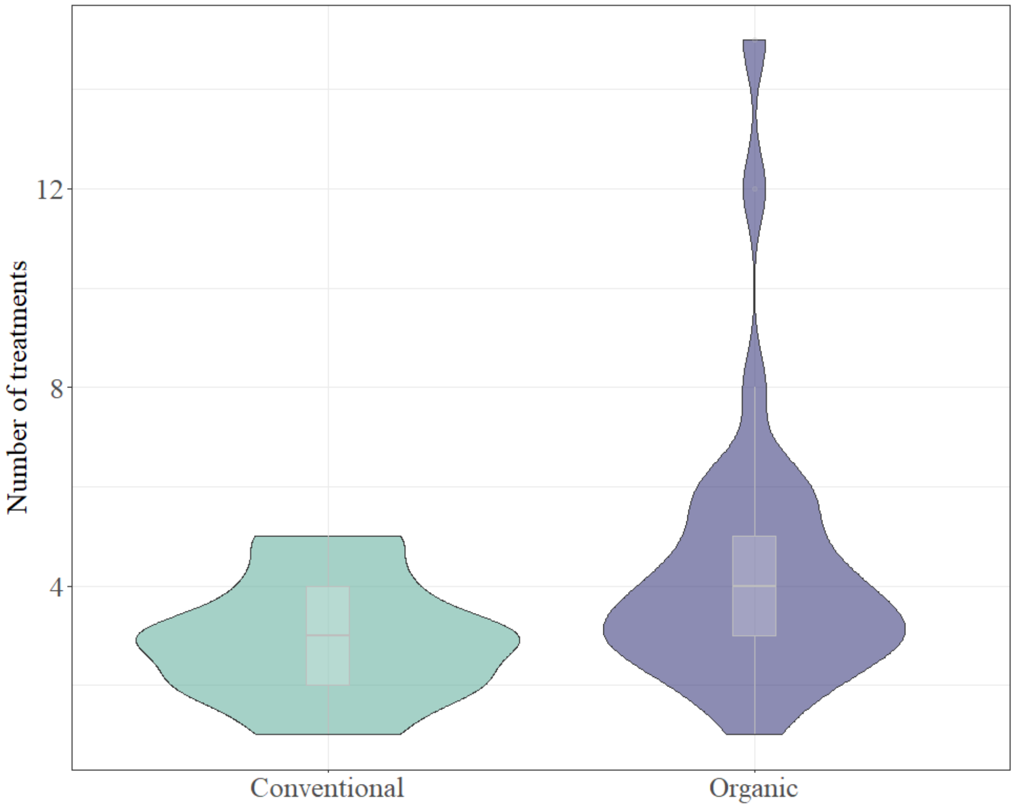

Figure 5.2: *Violin plot of the number of treatments administered during the campaign based on whether the farmers had an organic production certificate. (In process to be certificated = yes)*

Organic farming depends heavily on the use of copper in vineyards. According to Tamm et al. (2022), there is a correlation between the amount of copper used annually and the land used for organic production ($R2 = 0.941$, $p < 0.01$, Pearson), underscoring the reliance on these products in this type of agricultural system.

3.2 Use of biopesticides

Several commercial biopesticides can be used as alternatives to Cu in both conventional and organic production (la Torre et al., 2019). Although 69.0% of the respondents were aware of biopesticides, only 35.0% used them. Examining the data by type of management, 66% of conventional farmers and 74% of organic farmers were familiar with biopesticides. In terms of their use, the difference was much greater, at 41% in both cases, showing a 10% rise in biopesticides. The majority (71.0%) of farmers who used biopesticides considered them efficient against pests and diseases, whereas 29.0% did not think the results were satisfactory. In this case, the type of management did not affect the efficacy of the biopesticides. Regarding the type of active ingredient in the biological products used by farmers, 11.4% were based on fungi and 14.3% on oil products, while 25.7% were based on algal derivatives. The majority (48.6%) were other products, such as botanical extracts (Dagostin et al., 2010; Ramseyer et al., 2017; Thuerig et al., 2016; Thuerig, James, et al., 2018; Thuerig, Ramseyer, et al., 2018). Many of the respondents (48.0%) thought that the composition and dosage information on biopesticide labels was insufficient for proper use. The main criticism of the biopesticide labels' deficiencies centred on the dosage, optimal form, timing of application during the growing season, and the chemical's effects as a fungicide on other plant species. In terms of biopesticides application parameters, 30.6% of the farmers stated that they did not change the settings of any machinery for either biological or conventional products. However, 22.4% and 20.4% of farmers changed their travel speed and operating pressure, respectively, when applying only 12.2% to nozzles and 10.2% to air assistance.

Regarding farmers, the main criterion for comparing biopesticides with conventional agrochemicals is reliability. Nearly half of the respondents (47.9%) who do not use biopesticides doubted their effectiveness, which agrees with the conclusions of Acheuk et al. (2022). The cost of biopesticides was cited as a reason for non-use by 15.5% of farmers. Difficulty of application was a deterrent for 8.5% and the remaining growers (28.2%) did not use them for other reasons. If the use of biopesticides were expanded among farmers, 46.0% said they would use them, 46% said they might use them, and only 8.0% indicated they would not. The results reflect the intention to use biopesticides, however this ultimately does not materialise. Table 5.3 summarises the logit model analysis of the factors influencing biopesticide use. The data indicate that the variables with the highest level of significance and, consequently, the most influential on the likelihood of adoption of these types of products, are the level of technology (as a negative factor), the proximity to watercourses, and knowledge of the copper regulation (as positive factors). As illustrated in Figure 5.3, a higher level of technology is positively correlated with larger farms, higher productivity, and fewer treatments. An explanation for this might be that the economic impact increases with the degree of exploitation. Because a large vineyard has high production costs, adopting changes in the crop protection strategy is a potential risk, as it can more readily translate into losses.

However, because of the high risk of contamination, farms near watercourses must use additional caution when applying phytosanitary treatments, and buffer zones are mandatory in these situations (2009/128/EC). Given that farmers with these features in their fields are acutely aware of the risk of water pollution from pesticide application, it makes sense that it is a significant positive factor. The probability of choosing products with lower environmental risks increases when the surface water is closed (Table 5.3). The most influential variable in this model is knowledge of current legislation. Being aware of current regulations on the use of copper increases the likelihood of using biopesticides nearly 34 times more than not knowing, which agrees with Sacchettini et al. (2012), who argue that "raising awareness plays a key role in achieving the sustainable use of pesticides". Therefore, better knowledge of farmers' regulations ensures better compliance (Kvakkestad et al., 2021).

However, at a less significant level, the presence of a wine cellar in agricultural holdings is a positive factor for the use of biopesticides. Given that Cu can pass into rushed must and then into wine (Provenzano et al., 2010), it is reasonable that winery farmers are more careful with its use. Moreover, copper levels in wine are regulated; therefore, maintaining low levels of copper is required for wine commercialisation (Anastassiadou et al., 2020). In addition, the liability of downy mildew is a negative factor because farmers prefer to pretend to ensure proper production development and avoid taking a chance on new products. In line with the survey results, the farmers request field efficacy tests before using these products. It should be emphasised that the type of crop management (conventional or organic) was not a variable that contributed information to the built model, as it was discarded when the Akaike Information Criterion (AIC) was used to select the best model. Therefore, this had no effect on whether the farmers would be adopting biopesticides.

Table 5.3. *Logit model analysis of factors affecting the use of biopesticides ($p < 0.05$ ** and $p < 0.1$ *)*

	β	**Exp (β)**	**Sig.**	
Technology level	-5.14E-01	0.59	0.0135	**
Regulation	3.52E+00	33.68	0.0161	**
Watercourses	1.80E+00	6.03	0.0397	**
Downy mildew	-2.86E-01	0.75	0.0653	*
Winery	1.31E+00	3.70	0.0766	*

Soil	2.31E-01	1.25	0.1116
Area	-1.01E-03	0.99	0.1527
Productivity	-6.04E-06	0.99	0.4270
Hosmer and Lemeshow	(p-value = 0.5812)		

Knowing the law is the most important factor for using biopesticides (according to the built model). Figure 5.4 describes the farming profiles for both categories according to the knowledge of copper regulations. The main differentiating factor between the two groups was that large-scale farmers were aware of the legislation, whereas small-scale farmers were not. Another crucial parameter is the type of farming practice; farmers using more traditional farming technique are less aware of the legislation. With fewer differences between the compared groups, the profile of farmers who do not know the legislation shows that they apply more pesticide treatments even if the downy mildew problem is not severe. Additionally, these treatments are performed using a less advanced technology. Together, these factors indicate that these farmers' crop-protection activities are less precise. As a result, the target profile for knowledge transfer regarding the copper product regulations, as they are not aware of them based on these facts, should be small-scale traditional farming

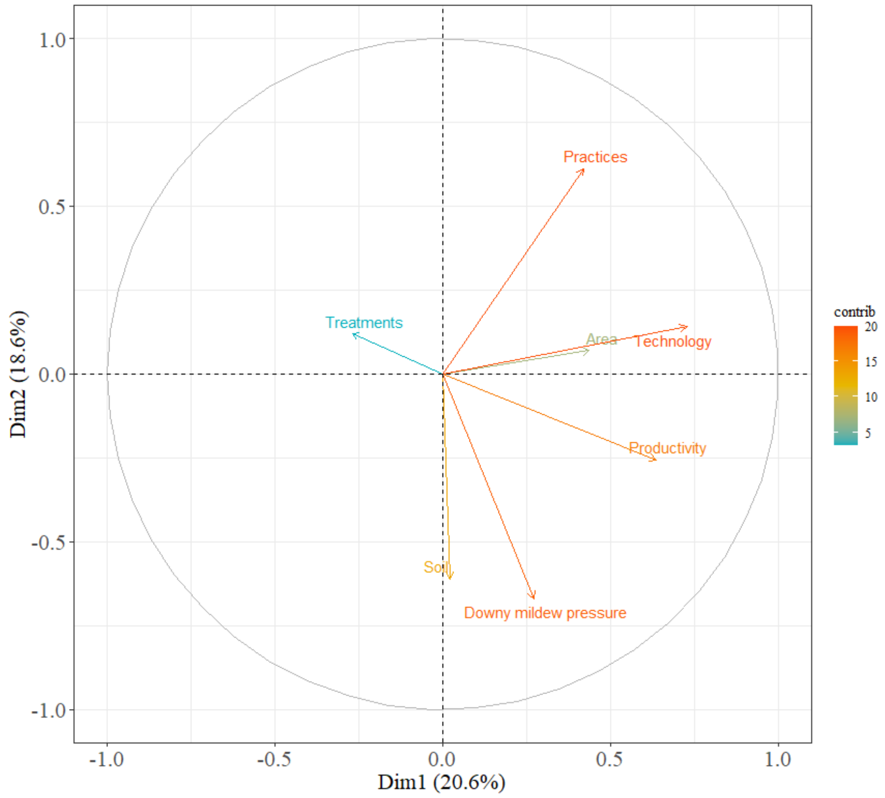

Figure 5.3. *Principal Component Analysis (PCA) variable correlation plot of the characteristics of grapevine fields (variables have been rescaled from 0 to 10). The colour legend expresses the variables' contribution to the principal components.*

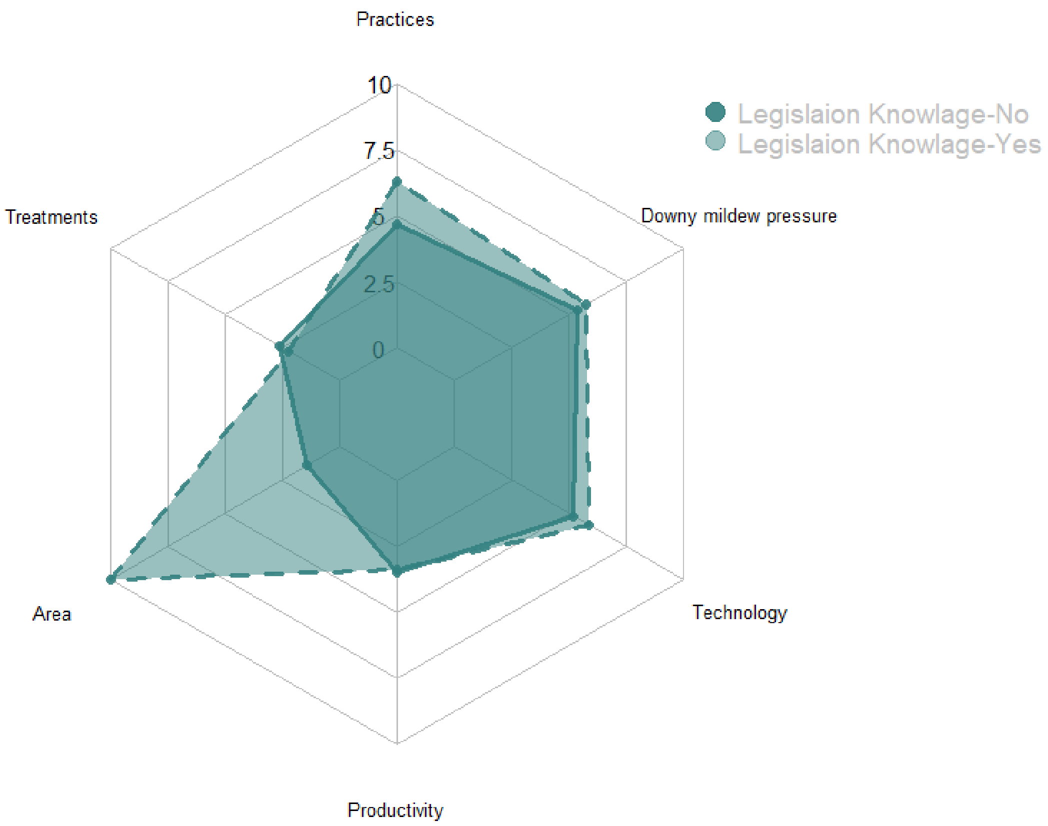

Figure 5.4. *Radar plot of the profiles of farmers that know and do not know the copper use regulation. The mean value of each variable is expressed for each of the two different groups plotted rescaled from 0 to 10 in order to be comparable.*

3.3 Applicability of EU copper policy

As mentioned in the Introduction, one of the primary goals of the European Green Deal is to promote organic farming and reduce the use of chemical and hazardous pesticides by 50% by 2030 (EC, 2020). In Spanish and Portuguese viticulture, however, there is strong reliance on the use of copper as a fungicide.

The demand for these items, particularly for organic farming (Kuehne et al., 2017), contradicts current legislative trends. One possible solution for organic vine growers could be to reduce the amount of product applied using a higher level of spray application technology (Campos et al., 2019; Nackley et al., 2021). Furthermore, novel copper formulations may be another way to reduce the amount of this metal used for vineyard crop protection. Novel microencapsulated products can improve fungicide deposition; therefore, farmers can decrease the quantity of products needed for disease control (Weitbrecht et al., 2021). Cultivars that are resistant or tolerant to downy mildew may provide a solution for reducing fungicide crop protection applications (Casanova-Gascón et al., 2019). However, using these hybrids alters the traits of grapes, which in turn affects the traits of wine (Adam-Blondon et al., 2016). This might completely change the wine industry.

Biopesticides are therefore being considered as an interesting alternative for the EU, as they will aid in the implementation of this policy. These products can be used as partial substitutes in both organic and conventional copper fungicide production, thus reducing the environmental impact(Cohen et al., 2006; Dagostin et al., 2010; Mulholland et al., 2017). Despite this, only a small percentage of the farmers used them. A lack of awareness regarding the effective use of biopesticides and their advantages has caused uncertainty among farmers (Kandar, 2021). Understanding the current legislation is a key factor in motivating winegrowers to use biological products (Table 5.3). However, the results show that obtaining this information from traditional small-scale farmers remains problematic. This is set in the European global context, where it has been established that there is still a gap between farmers and the latest technological advances, whether in the shape of high-tech equipment or new biological control products (Gil et al., 2020). It should be noted that from the farmers' perspective, there is scepticism about how public administration intends to achieve its objectives. Moreover, there is uncertainty because European directives commit to becoming involved in organic farming while simultaneously decreasing the use of copper. Although farmers are more likely to use environmentally friendly products, they need to be effective in order to avoid taking any chances. This uncertainty is exacerbated by the fact that copper substitutes are not yet widely used by farmers because of a lack of faith in their biological efficacy.

The present study highlights the need to boost the efficacy of biopesticides so that farmers will consider them as viable alternatives to reduce the use of copper. In addition, it influences legislative knowledge, especially on small farms, which are the two main consequences of these results. The survey contained a variety of farmers from different regions and farm types, but still in a very specific area (Spain and Portugal). However, viticulture is relevant in many other countries, particularly in the Mediterranean region. The behaviour of biopesticides in additional grape growing locations should be studied to have a more comprehensive understanding of their application as a copper alternative.

4. Conclusions

A survey was conducted among Spanish and Portuguese winegrowers to examine the use of copper and biopesticides (against downy mildew), as well as their level of awareness and commitment to the relevant European legislation. In terms of copper, the results show that the vast majority are aware of the new European institution rules on copper restrictions, and that they carry out less than five treatments per year. It should be noted that organic farmers use copper treatments at a higher rate than the conventional ones. On the other hand, while most of the producers are aware of the existence of biopesticides, only one in three claims to use them. In this regard, a high use of technology in vineyard farms has a significant negative effect on the probability of biopesticide use, although proximity to water courses nearby or awareness of copper legislation are positive factors, with the latter being the most relevant variable. There is still a gap between the demand for European administration trends and the methodology used by growers, primarily traditional small farmers, as well as scepticism about the use of alternative bioproducts to copper. Increased efforts in training programs and public communication among farmers are required to reach the objectives set by the New Green Deal and the Farm-to-Fork Strategy.

CHAPTER VI. General discussion

Each of the strategies evaluated in this work focuses on different approaches to offer potential solutions to the common problem of contamination caused by fungicides in vineyard fields. The implementation of these measures may vary depending on the part of the process they target and the feasibility of their execution. Therefore, cover crops can mitigate the contamination caused by fungicides to ground and surface waters, especially after fungicide application. This approach represents a post action measure, and may be relatively easier to implement, as it often requires minor changes in standard farming practices. Additionally, cover crops have been demonstrated to provide other agricultural side-benefits, encompassing physical, chemical, and biological aspects. These benefits range from reducing nutrient leaching, enhancing soil microbiome and its diversity, and increasing enzymatical activities (Kim et al., 2020; Sürücü et al., 2014). They also contribute soil carbon sequestration (Jarecki & Lal, 2003; Paustian et al., 2016; van Kessel et al., 2013), erosion reduction (L. Chen, Rejesus, et al., 2022), and even fostering biodiversity in the field (Bengtsson et al., 2005). Although cover crops can increase production expenses, they can also generate indirect income (Snapp et al., 2005). Furthermore, the implementation of cover crops can potentially impact water resources, particularly in Mediterranean non-irrigated vineyards. Such impacts should be closely monitored and managed, for instance, by increasing mowing frequency, reducing the sward strip and/or choosing less competitive species, as suggested by Lopes (2016) . Due to the global warming, rainfall events are projected to became more concentrated and intense, resulting in water becoming a scarcer resource in these regions (Armon et al., 2022; Myhre et al., 2019). Nevertheless, cover crops can be plants of agricultural interest/value and therefore harvested by farmers or incorporated into the soil as organic matter source. Further research is needed in this area, from the point of view of the species and mixtures of crops to be implemented in cover crops as well as on the possibility to adding another product, such as biochar, in the soil that may result in the increase of crop productivity and fungicide sequestration, reducing the surface and groundwater pollution (Safaei Khorram et al., 2016).

Copper reduction, by using microencapsulation technology is another of the strategies assessed on this work, which efficiency has been demonstrated. It interferes into the application part, while the spray application is performed, so this strategy will not need any mentality change by the farmer's side and have a direct effect on the pollution production. This new type of product can increase the deposition, and for instance reduce the amount of copper needed for crop protection proposes, while at the same time it leach slower, potentially having less environmental impact. Despite this, it is important to note that this strategy requires further testing to confirm its biological efficacy and explore different application technologies. While some similar formulations have yielded positive results (Weitbrecht et al., 2021), more research is needed to validate this approach. In addition, this reduction method presents an additional problematic, which is that is not a commercial product yet. As a result, the acceptance and commercialization of a product for crop protection is a lengthy process for farmers. Additionally, the use of copper in this strategy presents a downside, as it is extracted from mines and its extraction process contributes to environmental pollution (L. Chen, Zhou, et al., 2022; Chileshe et al., 2020). Furthermore, copper is a finite resource, which raises concerns about future availability.

The use of biopesticides as a replacement for the copper fungicides can suppose the most challenging strategy to adopt by farmers, but at the same time is the most efficient against pollution. This strategy acts before the spray application process, by choosing the product not for its efficacy, but because of its less environmental impact. In this case, farmers have been positioned as not optimistic about the use of this type of product, most of the growers are also aware of the existence of biopesticides, but just one out of three declares that they use them. Further research is needed in other to improve the efficacy of the products while just be increased the training programs to mentalise farmers about the need of change for a sustainable agriculture.

Table 6.1. *Comparison of implementation for the strategies studied*

	Cover crops and buffer strips	Copper microcapsules	Biopesticides products
Secondary benefits	☑	☒	☒
Immediate applicability	☑	☒	☑
Low implementation cost	☑	-	☒☑
No change of mindset required	☒	☑	☒
No need for development	☑	☒	☒
High impact on pollution reduction	☒	☑	☒☑
Sustainability in the future	☒☑	☒	☑

Summarizing, cover crops can be useful as a first step strategy, since it is easy to apply, and helps reducing the contamination, protecting the water courses and the environment. However, it cannot be a long-term solution, or the only one, since it does not act on the pollution source, just in the one already produced, and does not stop it fully, so that the contamination will be still produced. At present, the microencapsulated copper fungicide is not yet available as it is still in the developmental stage. However, once perfected, it will become an effective way to combat downy mildew with less reliance on metal. It would be best to combine this solution with the third strategy, which is entirely sustainable. While the necessary research achieves the desired biological efficacy of the biopesticides, and its acceptance by farmers side, it may be supplemented with copper products of a similar nature. Biopesticides are a way to reduce the pollution, and can help to achieve a sustainable goal and in line with the Green Deal objectives and the consumers tendencies. Besides the encapsulation of other nanomaterials with biopesticides neds to be explored from the point of view of their effectiveness to combat downy mildew, but also on farmers acceptance. Even so, the factor that can really make a difference is the training. Knowledge of legislation has proven to be a key factor and focus of the EU in terms of achieving greater sustainability in the countryside. This can be seen in initiatives such as the INNOSETA project (https://platform.innoseta.eu/), which aims to bridge the gap between researchers and farmers. The project highlights the fact that knowledge often fails to reach its intended audience.

CHAPTER VII. Overall conclusions

The findings of this thesis suggest that decreasing fungicide contamination is feasible through various methods of action. The main conclusions drawn from the research conducted in this PhD dissertation can be summarized as follows:

- Cover crops have proven to be an effective strategy to protect against groundwater contamination resulting from fungicide application in vineyards. Results indicate that the duration of fungicide contact with the rhizosphere plays a pivotal role in minimizing fungicide leaching. Vegetation has been found to enhance the reduction of most fungicides by accelerating their kinetic rates.
- Soil type affects the capacity of cover crops to reduce fungicide leaching, making it a crucial factor to consider. Results indicate that there are no differences among various types of grasses. However, it has been noted that higher biodiversity within the grass species enhances their ability to reduce pollutants.
- Vegetated buffer strips have been proven to be effective in reducing the transport of fungicides, through run-off events, which could otherwise potentially contaminate watercourses. The presence of vegetation in buffer zones also speeds up the elimination of fungicides in soil, along with their transformation products.
- The microencapsulated product improved the deposition, demonstrating its potential capacity to reduce the amount of copper needed in crop protection treatments. Simultaneously, this microencapsulated product seems to potentially have lower environmental impact.
- Vineyard fields with high-technology or those with high disease pressure are disliked for to use of biopesticides. In contrast vineyard located close to watercourses are favourable to their use. The knowledge of the copper legislation is the most relevant variable on the introduction of biological plant protection products. This study also has highlighted there is still a gap between the European administration trends, that especially does not arrive to the small and traditional growers and the goals they mark.
- The different approaches examined in this work to reduce fungicide pollution can be combined to further reduce this type of contamination, like promoting the use of vegetation instead of bare ground (both buffer and intercropping zones), using new microencapsulated products able to reach the same deposition with less copper amount, and increasing the efforts in training programs and public communication among farmers.
- Training and the knowledge of the legislation, has proven to be a key factor in the adoption of new solutions by farmers.

CHAPTER VIII. Future research

After conducting the doctoral thesis, several scientific questions open research lines for further research. First and foremost, it is crucial to note that the first part of the studies conducted in this PhD were carried out under controlled indoor conditions (Chapter III). Therefore, to prove the effectiveness of using cover crops as a mitigation solution for fungicides, additional studies must be conducted in real fields to ensure the reliability of results. To this end, the project *CoberProtect* was presented to the Generalitat de Cataunya (under the Technological Transfer of the Rural Development Programme 2014-2020) in collaboration with real wineries, with the aim to demonstrate how the use of plant covers reduces contamination of fungicides in a real field. Additionally, delving into the interplay between microbiome and crop exudates and their role in mitigating the effects of fungicides in the soil-plant system holds considerable interest. Similarly, the buffer strip effect should also be analysed on a real field scale, and additional measurements of the organic matter content of the two strips of bare and grassed soil should be taken, along with balance calculations to better understand the process that occurs with the contaminant compounds. While the formation of transformation products has been tracked in both cover crops and buffer strips, a more profound understanding of the toxicity of these newly generated compounds is necessary.

Further research is necessary to study the behaviour of microencapsulated products during a whole crop protection campaign. To that end, the project *microÚs* was granted and financed by Generalitat de Catalunya (under the Technological Transfer of the Rural Development Programme. https://ruralcat.gencat.cat/web/guest/xarxa-i.cat/activitats-de-demostracio. 56 30150 2021 P4). The objectives of this demonstration project are to demonstrate that the use of microcapsule formulation with baking increases deposition on leaves in horticultural crops, and therefore, the amount applied can be reduced. To validate the increased persistence of copper on the leaf under different washing conditions. On the other hand, to demonstrate the biological efficacy of copper microencapsules against downy mildew and inform the different actors in the agricultural sector of the benefits of using a it.

Furthermore, the study conducted on biopesticides adoption as an alternative to copper presents interesting results. The research line to follow should include personal interviews and a stricter classification of some parameters to make them less subjective. It would also be interesting to increase the scale to other Mediterranean countries, as they are the main wine and grape producers in Europe.

Finally, a comprehensive overview of the impact of all these mitigation measures on crop health and yield can be explored in the future through the study of metabolomics and transcriptomics. These new tools offer a fresh perspective to investigate the response of plants to external stressors.

CHAPTER IX. Related activities

Throughout the development of the doctoral thesis, other activities have also been carried out to complement the study of pesticide contamination in a more general way.

1. *Participation in competitive projects*

- **INNOSETA** (H2020 EU Project): Accelerating Innovative practices for Spraying Equipment, Training and Advising in European agriculture through the mobilization of Agricultural Knowledge and Innovation Systems. Call: H2020-RUR-2016-2017 (Rural renaissance – fostering innovations and business opportunities). May 2018- April 2021. http://www.innoseta.eu/es/inicio/
- **OPTIMA** (H2020 EU Project): Optimized Pest Integrated Management to precisely detect and control plant diseases in perennial crops and open-field vegetables. Call: H2020-SFS-2016-2017 (Sustainable Food Security – Resilient and resource-efficient value chains). September 2018 - December 2021. https://optima-h2020.eu
- **NOVATERRA** (H2020 EU Project): Integrated novel strategies for reducing the use and impact of pesticides, towards sustainable Mediterranean vineyards and olive groves. Call: H2020-SFS-2018-2020. October 2020 – September 2024. https://www.novaterraproject.eu
- **COPPEREPLACE** – (EU INTERREG-SUDOE PROJECT) - Development and comprehensive implementation of new technologies, products, and strategies to reduce copper application in vineyards and remediate contaminated soils in the region SUDOE. Reference: SOE4/P1/E1000. November 2020 – February 2023.
- **BioUrban (**Barcelona city council project) - Implementation of biochar as a bioremediation tool in peri-urban areas: new model of responsible production for the Metropolitan Area of Barcelona. January 2022- January 2024.
- **Microús** (Catalonia government project) - Reduction of the use of copper in horticultural crops by means of the microencapsulation technique. July 2022 – June 2024

2. *Participation in other scientific publications*

- Salas, B.; Salcedo, R.; Ortega, P.; Grella, M.; Gil, E. Use of ultrasound anemometers to study the influence of air currents generated by a sprayer with an electronic control airflow system on foliar coverage. Effect of droplet size. *Computers and Electronics in Agriculture*, Volume 202, November 2022, 107381. https://doi.org/10.1016/j.compag.2022.107381

- Gil, E.; Salcedo, R.; Soler, A.; Ortega, P.; Llop, J.; Campos, J.; Oliva, J. 2021. Relative efficiencies of experimental and conventional foliar sprayers and assessment of optimal LWA spray volumes in trellised wine grapes. *Pest Manag Sci* 2021. https://doi.org/10.1002/ps.6276

- Campos, J.; Gallart, M.; Llop, J.; Ortega, P.; Salcedo, R.; Gil, E. On-Farm Evaluation of Prescription Map-Based Variable Rate Application of Pesticides in Vineyards. *Agronomy*, 2020; doi: 10.20944/preprints201911. 0306.v1.

- Salcedo, R., Pons, P. Llop, J., Zaragoza, T., Campos, J., Ortega, P., Gallart, M., Gil, E. 2019. Dynamic evaluation of airflow stream generated by a reverse system of an axial fan sprayer using 3D-ultrasonic anemometers. Effect of canopy structure. *Computers and Electronics in Agriculture*, Volume 163, August 2019, 104851. https://doi.org/10.1016/j.compag.2019.06.006

- Gil E., Campos, J., Ortega, P., Llop J., Gras A., Armengol E., Salcedo R., Gallart M. 2019. DOSAVIÑA: Tool to calculate the optimal volume rate and pesticide amount in vineyard spray applications based on a modified leaf wall area method. *Computers and Electronics in Agriculture*, Volume 160, May 2019, Pages 117-130. https://doi.org/10.1016/j.compag.2019.03.018

3. *Conference papers*

- Ortega, P; Sanchez, E; Tylkowski, B; Olkiewicz, M; Montornes,JM ;Gil, E. 2022. New method to increase pesticide deposition: Copper microencapsulation. In P. Balsari, S. E. Cooper, E. Gil, C. R. Glass, W. Jones, B. Magri, J. Van de Zande (Eds.), Aspects of Applied Biology 147, 2022 International Advances in Pesticide Application, pp. 157 - 164. Münster (Germany).

- Salas, B.; Ortega, P.; Berger, L.; Gil, E. 2022. Smart orchard sprayer to adjust pesticide dose to canopy characteristics. In P. Balsari, S. E. Cooper, E. Gil, C. R. Glass, W. Jones, B. Magri, J. Van de Zande (Eds.), Aspects of Applied Biology 147, 2022 International Advances in Pesticide Application, pp. 157 - 164. Münster (Germany).

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4. *International stays*

- Agricultural University of Athens (AUA), Greece. Laboratory of Plant Pathology, Department of Crop Science. From 01/09/2022 to 23/12/2022. Participation on research projects that concern the evaluation of biological and chemical management strategies for the control of ochratoxin A (OTA) produced by Aspergillus carbonarius in vineyards.

- Central American Institute for Studies on Toxic Substances (IRET), Costa Rica. Laboratory of Ecotoxicology. From 30/01/2023 to 06/02/2023. Training on how to perform pesticide tolerance/sensitivity tests on *Draphnia magna*.

X. ANNEX: supplementary material

SM- Article 1: Use of cover crops in vineyards to prevent groundwater pollution by copper and organic fungicides. Soil column studies

Chemical and reagents

Analytical grade methanol, acetonitrile, hydrochloric acid (37% v/v), dimethomorph, zoxamide and acibenzolar-S-Metil were purchased from Merck (Darmstadt, Germany). Polytetrafluoroethylene (PTFE) 0.22 µm and Ø 47 mm filters were obtained from Frisenette (Knebel, Denmark).

Supplementary material about chromatographic conditions

A binary gradient elution programme consisting of mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid) was set as follows. Isocratic 0–1 min: 10% of B; 1–10 min: 10–100% of B; isocratic 10–15 min: 100% of B; 15–18 min: 100–10%; isocratic18-23 min: 10% of B. Linearity ranged from 2.5 to 2000 µg/L. Repeatability was lower than 10% for all the studied fungicides (n = 3).

Table 10.1.1-SM: *Laboratory analysis of the two soil types used in the experiment.*

	Loam soil	**Sandy soil**
Humidity (%)	< 1	1.17
pH	8.8	8.9
Electrical conductance ($dS\cdot m^{-1}$)	0.15	0.1
C content (% DMB)	0.95	< 0.29
Organic matter (%DMB)	1.6	< 0.5
Calcium carbonate equivalent (%DMB)	33	< 3
Active calcium (%DMB)	6	<3
N (%DMB)	0.12	0.077
P ($mg\cdot kg^{-1}$)	26.2	6.62
K ($mg\cdot kg^{-1}$)	208	40
Ca ($mg\cdot kg^{-1}$)	6605	2721
Mg ($mg\cdot kg^{-1}$)	204	95
Na ($mg\cdot kg^{-1}$)	16	16
C/N	7.7	< 0.29
Ca/Mg	32.3	28.5
Mg/K	1.0	2.4
Ca/K	31.7	67.5
Fe ($mg\cdot kg^{-1}$ DMB)	103	96

Cu (mg·kg^{-1} DMB)	4.8	21.3
Mn (mg·kg^{-1} DMB)	73	88
Zn (mg·kg^{-1} DMB)	7	3
Mo (mg·kg^{-1} DMB)	< 1.25	< 1.25

**DMB, dry matter basis*

Table 10.1.2-SM: *LOD and LOQ of the method for the tested compounds in ng·ml.*

Compound	LOD	LOQ
Oxathiapiprolin	6	7
Zoxamide	1	2
Dimethomorph	2	3
Acibenzolar-s-metil	4	6
Laminarin	1	2

Figure 10.1.1-SM*: close-up view of roots from grass covered soil columns*

SM- Article 2: Attenuation and soil biodegradation of fungicides by using vegetated buffer strips in vineyards during a simulated rainfall–runoff event

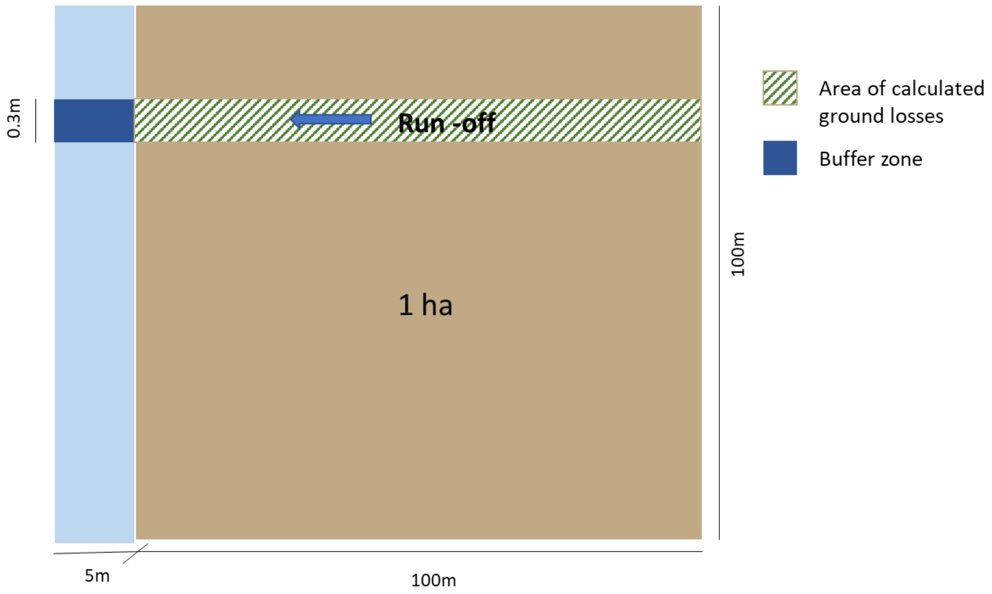

***Figure 10.2.1-SM**. Outline of the ground losses theorical calculation area*

***Table 10.2.1-SM**. Maximum label dose, expected product losses and expected product washed by the runoff of selected fungicides according to Gil et al. (2001).*

Commercial product name	**Label higher dose (kg·ha^{-1})**	**Expected product losses (µg·cm^{-2})**	**Amount of ground losses for the calculated area (mg)**
CODIMUR 50 (Copper oxychloride)	3	13.86	416
ZORVEC VINABEL (Oxathiapiprolin and Zoxamide)	1.37	6.33	190
FORUM (Dimethomorph)	2.5	11.55	347
BION MX (Acibenzolar-s-methyl)	0.3	1.38	42
VACCIPLANT (Laminarin)	2	9.24	277

Table 10.2.2-SM: *LOD and LOQ of the method for the tested compounds in ng·ml.*

Compound	**LOD** **Water** (ng·mL^{-1}) / **Soil** (ng·g^{-1})	**LOQ** **Water** (ng·mL^{-1})/ **Soil** (ng·g^{-1})
Oxathiapiprolin	6 / 27	6 / 28
Zoxamide	1 / 6	2 / 7
Dimethomorph	2 / 13	3 / 16
Acibenzolar-s-metil	4 / 6	6 / 7
Laminarin	1	2

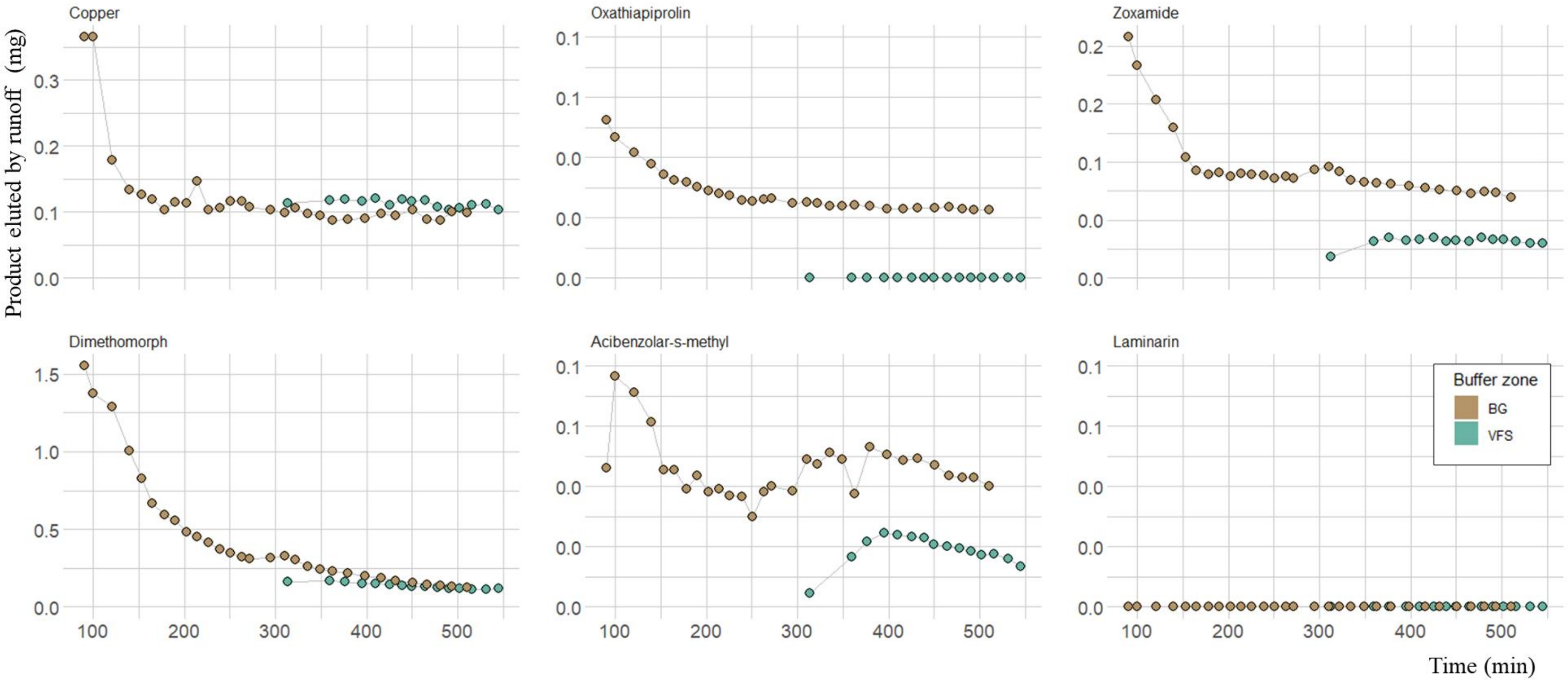

Figure 10.2.2-SM. *Pesticides eluted by runoff for the two tested strips, bare ground (BG) and vegetated (BS).*

Table 10.2.3-SM: *TPs detected into the soil*

Transformation product	**RT (min)**	**Molecular formula**	**Possible matching metabolite from literature**
Dimethomorph TP1	8.64	$C_{21}H_{22}ClNO_5$	-
Dimethomorph TP2	8.74	$C_{20}H_{20}ClNO_4$	Dimethomorph metabolite Z67: (4-[(E)-and(Z)-beta-(p-chlorophenyl)-3-hydroxy-4-methoxycinnamoyl]morpholine) Dimethomoprh metabolite Z69: (4-[(E)-and(Z)-beta-(p-chlorophenyl)-4-hydroxy-3-methoxycinnamoyl]morpholine)
Oxathiapiprolin TP1	8.64	$C_{24}H_{22}F_5N_5O_3S$	Oxathiapiprolin metabolite IN-RDT31: (1-(4-{4-[(5RS)-5-(2,6-difluorophenyl)-4,5-dihydro-1,2-oxazol-3-yl]-1,3-thiazol-2-yl}-4-hydroxypiperidin-1-yl)-2-[5-methyl-3-(trifluoromethyl)-1H-pyrazol-1-yl]ethanone) Oxathiapiprolin metabolite IN-RDG40: (1-(4-{4-[(5RS)-5-(2,6-difluoro-3-hydroxyphenyl)-4,5-dihydro-1,2-oxazol-3-yl]-1,3-thiazol-2-yl}piperidin-1-yl)-2-[5-methyl-3-(trifluoromethyl)-1H-pyrazol-1-yl]ethanone)
Oxathiapiprolin TP2	8.93	$C_{24}H_{22}F_5N_5O_3S$	
Zoxamide TP1	7.25	$C_{14}H_{17}Cl_2NO_3$	Zoxamide metabolite (RH-150721): (3*RS*)-3-amino-3-methyl-2-oxopentyl 3,5-dichloro-4-methylbenzoate
Zoxamide TP2	9.70	$C_{14}H_{17}Cl_2NO_3$	
Zoxamide TP3	8.54	$C_{14}H_{19}Cl_2NO_3$	-

Zoxamide TP3	9.95	$C_{14}H_{19}Cl_2NO_3$	-

Figure 10.2.3-SM. *Close-up view of the runoff assay*

Figure 10.2.4-SM. *Close-up view of the soil assay*

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