Eur J Plant Pathol
https://doi.org/10.1007/s10658-024-02845-w

The effect of copper reduction on the control of downy mildew in Mediterranean grapevines

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Accepted: 7 March 2024

Abstract The application of copper-based fungicides has led to the problematic accumulation of copper in the soil of many European vineyards. In this work, we tested the effectiveness of downy mildew control strategies that considerably reduce the amount of copper, through the application of other non-toxic compounds. The study took place in three different regions of Catalonia, by combining smaller and larger-scale trials for two growing seasons. Although variations among experiments were detected, the treatments used here may reduce the applied copper content by up to 77 percent. The cooper reduction-strategies, which alternated standard copper application of copper oxychloride with applications of products based on *Equisetum arvense* L., or with applications of products with heptagluconic acid (an alternative formulation with a very low-rate of copper), provided the most consistent protection against downy mildew. Moreover, these treatments reduced copper inputs by 63 and 47 percent as compared to conventional treatments based on repeated applications of copper oxychloride. This work underlines the possibility of reducing the amount of copper applied to control downy mildew in Mediterranean vineyards, through the use of newly-developed formulations.

Keywords Downy mildew · *Vitis vinifera* L. · Organic farming · Pesticide reduction

Introduction

The most common grape cultivars cultivated worldwide are highly susceptible to downy mildew, one of the most devastating diseases, caused by the oomycete *Plasmopara viticola* Berk. & Curtis (Dagostin et al., 2010). Since its discovery in the middle of the nineteenth century, this disease has spread from America to Europe and then to all grapevine-growing countries, leading to significant economic losses due to the lack of efficient disease control (Koledenkova et al., 2022). The environmental conditions that favour its appearance are warm, moist, and humid conditions during the growing season (Caffi et al., 2010; Rantsiou et al., 2020). The disease attacks vine leaves and berries, reducing grape yield. It also affects grape quality, by influencing the wine aroma and flavour. Its impact on the organoleptic properties of wine depends on the specific cultivar, the effect on

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s10658-024-02845-w.

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Published online: 26 March 2024

berry quality, and on the ageing potential (Pons et al., 2018).

The application of copper-based fungicides is the most widely used strategy to prevent and control this disease, especially in organic viticulture (La Torre et al., 2019). The continuous use of copper-based fungicides has entailed an accumulation of copper in the soil of many European vineyards. This accumulation is due to treatment washing after the application of the copper compounds to leaves, via precipitation and irrigation, decomposition of senescent vine leaves, or through mechanical wind action, especially in the upper soil layers (La Torre et al., 2019; Romanazzi et al., 2020; Rusjan et al., 2007). The main problem is that copper persists in the soil, as it cannot be metabolized by soil microorganisms, and its removal is negligible through leaching, run-off, or plant uptake (Lamichhane et al., 2018; Romanazzi et al., 2016; Torres & Johnson, 2001). The reiterated use of copper-based foliar sprays throughout the canopy has been shown to have reached toxic levels, which have affected soil organisms in some cases, as a result of microbiological and enzymatic alterations, and reduced soil fertility and pH, causing plant stress and reduced plant growth (Komárek et al., 2010; Wang et al., 2009).

In European agriculture, viticulture cropping systems apply the highest rates of plant protection products (Komárek et al., 2010; Mulholland et al., 2017). Consequently, the European Commission has applied restrictions, adding copper to the list of candidates for substitution (The European Commission, 2018b). The maximum copper dose has been limited from 6 to 4 kg/hectare/year spread over 7 years, starting in February, 2019 (The European Commission, 2018a). This problem is greatly important in several European countries (Dagostin et al., 2011; Herwig et al., 2023; Rusjan et al., 2007; Sereni et al., 2023). In Catalonia (Spain), the climatic conditions frequently favour disease development, depending on the vineyard characteristics. In Catalonia, 32% of planted vineyards are certified as practicing organic viticulture (Consell Català de la Producció Agrària Ecològica & Ministerio de Agricultura, 2021; Ministerio de Agricultura, 2020), and copper soil concentration is one of the highest in the country (Ballabio et al., 2018).

In this context, the present study assesses the possibilities to reduce copper dose in field treatments against downy mildew in organic vineyards in Catalonia, to ensure sufficient yield. The reduction in copper dosage can be achieved by reducing the number of applications through better spray positioning, but also through the application of other non-synthetic products effective against downy mildew.

Over the last decade, alternative strategies to control downy mildew on grapevines have been tested in different published research studies. Some of these strategies aim to reduce copper inputs with innovative formulation technologies, such as particle size reduction, or active substance micro-encapsulation (La Torre et al., 2019). Copper input can also be limited by dosage reduction. Thus, several studies have been carried out to identify the minimum effective copper doses in a single treatment (Dagostin et al., 2011). Furthermore, natural molecules have been identified that can be used in conjunction with copper or in alternate applications, to reduce copper metal dosage or to achieve a complete replacement. These substances are listed as products permitted for plant protection in organic crop production in Europe (The Commission of the European Communities, 2008). The use of plant defence elicitors to induce grapevine resistance to diseases is another strategy under investigation (Jacquens et al., 2022). Plant extracts from *E. arvense* were also tried for controlling downy mildew in grapevines (Marchand, 2016). These commercial preparations are rich in acids such as silicic, malic, oxalic, gallic, as they strengthen the cell wall structure of plants, and their effect against downy mildew is mainly physical.

There are also other commercial products with a low content of complexed copper that are authorized as fertilizers, but are assumed to improve the plant's defences against diseases such as downy mildew. Accordingly, products based on copper complexed with heptagluconic acid have been proven to control downy mildew in vineyards in other studies (La Torre et al., 2011). This product acts with ascending and descending systemic absorption, on the hormonal system of the treated plants, in such a way that it favours the production of phytoalexins and, therefore, the natural defence of the plants against endoparasitic fungi. Furthermore, an innovative solution based on complexed zinc and copper microelements with a hydracid of citric acid has been recently developed. The application of this product produces a rapid and efficient absorption of these two essential microelements to guarantee an optimal performance of the enzymatic process. Therefore, the physiological state of the plant is improved and, indirectly, its natural defence system, thereby inducing better resistance to diseases caused by Oomycete such as *P. viticola* Berk. & Curtis.

In the studies described, these products were shown to be efficient in the control of downy mildew or were described to be an innovative solution with promising results.

Given the importance of finding alternatives strategies to reduce the amount of copper used, this work aims to demonstrate the effectiveness of downy mildew control strategies with a considerable reduction in the annual dose of copper. In field trials, we evaluated different formulations with minimal or no copper concentration, and certified for organic farming in Spain, in order to assess their efficacy and their effect on copper residues in soil and wine quality.

Materials and methods

Location and plant material

The present work studies alternatives for reducing the environmental impact of copper in Mediterranean Spanish vineyards, especially in Catalonia. The study took place in important Appellations of Origin in the Catalonian region, namely, 'Costers del Segre', 'Montsant', 'Penedès', and 'Cava' (Fig. S1). A 20-year-old 'Sauvignon blanc' (*Vitis vinifera* L.) commercial vineyard located in Raimat (41º39′47'' N – 0º30′9''E; Lleida, Spain) was studied during the 2019 and 2020 growing seasons (Zone A; Fig. S1). The vines were grafted onto SO_4 rootstock and planted with a 3.0×2.0 m, spacing in a North–South row orientation with a drip irrigation system. The canopy was trained using vertical shoot positioning, with a bilateral, spur-pruned cordon located 1.0 m aboveground. In this case, the orography of the land was flat and the vigour throughout the cultivar was very homogeneous. During the growing season, the vineyard was managed according to the wine grape production protocol of the'Costers del Segre' Appellation of Origin (Catalonia, Spain).

The 'Costers del Segre' Appellation of Origin is situated in the middle basin of the Segre River, between the Pyrenees and the Ebro River. Zone A, located to the East of the Appellation, has a mild relief at an altitude of around 300 m. The dry climate, away from the influence of the sea and marked by high insolation, scant rainfall, and persistent fogs of winter moisture, induces a risk for the contamination of grapes with fungi (Bellí et al., 2005). Moreover, 'Sauvignon blanc', the cultivar studied here, is highly sensitive to the downy mildew pathogen that is traditionally controlled with copper-based treatments.

During the 2019 growing season, an additional field experiment was carried out at Falset (41º9′12''N – 0º48′26''E) (Tarragona, Spain) (Zone B, Fig. S1) in a 'Grenache' vineyard grafted onto Richter 110 and planted in 1996 with a drip irrigation system. The distance between the vines was 1.2×2.6 m, with a Northwest-Southeast row orientation with a double Cordon Royat trained system. The management of the vines was uniform and in compliance with the 'Montsant' Appellation of Origin (Catalonia, Spain). This region is characterized by low rainfall, high temperatures in summer, cold winters, freezing winds from the North-Northwest, and by warm and humid winds from the East-Southeast (Portillo et al., 2016). In this zone, the cultivar studied was the red grape 'Grenache' which is also very sensitive to downy mildew.

A complementary field trial was performed at Gelida (41º25′53''N – 1º50′4''E) (Gelida. Barcelona, Spain) (Zone C, Fig. S1) during the 2020 season, with the 'Macabeo' cultivar. The vineyards were planted in 1998 and grafted onto Richter 110 with a spacing of 2.80×1.20 m, with a northeast-southwest row orientation. Vines presented a double cordon de Royat trellis system and the vineyard management was uniform at the experimental site. The vineyard belongs to 'Cava' and 'Penedès' Appellations of Origin (Catalonia, Spain). The emplacement for this Appellation of Origin is one of the most productive wine regions of Catalonia, and it is located at 196 m above sea level. This region is wide and covers a great strip of land between the sea and the mountains, with a typical Mediterranean climatology (mild and warm). The cultivar included in the study was 'Macabeo', susceptible to downy mildew infections, and one the most important white grape varieties for making wine cava in this region.

Experimental design and treatments

A large-scale experimental design was used in Zone A in a very homogeneous 'Sauvignon blanc' vineyard plot. The experimental design consisted of consecutive subplots with a single repetition per treatment. Each subplot contained nine rows of 170 vines along a row. These observational units were located within the 7 central rows, in four different sectors distributed from north to south inside the subplot. These sectors were considered as blocks for statistical analysis.

In Zone B and Zone C the experimental design consisted of a randomized complete block with four replicates per treatment. Each replicate contained 20 vines in Zone B and 15 vines in Zone C. Observations were made from the central vines of each treatment, where the 3 vines on each row end acted as guard vines.

Three commercial alternative products were selected and applied alternatively with copper oxychloride in all the experiments. These were products, two of them authorized as fertilizers and another as a basic substance (Table 1), innovative or with a proven effectiveness for the control of mildew in vineyards as well as authorized and certified for organic farming in Spain. We called these 3 treatments (T1, T2, and T3) "less Cu treatments". The commercial formulations applied were: Complexed zinc and copper microelements with hydracid of citric acid (in T1); preparation of *E. arvense* (in T2); and copper complexed with heptagluconic acid (in T3). All the products were used according to the manufacturer's instructions. These treatments were compared with the non-treated control (NTC) strategy, and T4, the standard copper oxychloride strategy. The order of the applications that appears in the strategy in Table 1 (odd–even) was not fixed, and could be modified during the trials depending on the disease pressure. Given the tightening of European legislation on the use of copper in agriculture, many copper products such as copper hydroxides or sulphates ceased to be found in the Spanish registry or authorized for use in viticulture, and the number of applications allowed in many of them was limited to one application per season. For this reason, we selected only one single formulation of copper oxychloride, in order to have replicability throughout the entire study, by using an authorized product allowed for several applications in the same season.

To avoid drift of products among plots, wind speed and direction were considered for treatment application. The field trials were conducted according to EPPO (EPPO, 2009) guidelines. The dosage applied throughout the season changed from the lowest, in the first application with less vegetation, to the highest in final applications with maximum vegetation. In the large-scale experiment (Zone A) the products were applied using a mist blower towed by a farm tractor distributing from 250 to 500 L/ha. In the other experiments, located in Zone B and C, the products were

Table 1 Treatment's summary and the tested products. The order of the applications that appears in the strategy (odd–even) was not fixed and could be modified during the trials depending on the disease pressure. The doses used in the application of each product were doses per litre, always applying the same amount of product per litre of water

Treatment		Strategy (odd applications + even applications)	Active ingredients (% w/v)	Product name/trade name (odd applications + even applications)	Dose (mL/L)
NTC	Non-treated		-	-	-
T1	Fertilizer + phytosanitary		Complexed zinc and copper (2% Cu) + copper oxychloride (70% Cu)	Hydracup/Biagro + Rebelde/Kenogard	2.7 + 1.5
T2	Basic substance + phytosanitary		*Equisetum arvense* L. + copper oxychloride (70% Cu)	Evasiol/Grupo Agrotecnología + Rebelde/Kenogard	9.0 + 1.5
T3	Fertilizer + phytosanitary		Copper complexed with heptagluconic acid (6% Cu) + copper oxychloride (70% Cu)	Idai Cobre/Idai Nature + Rebelde/Kenogard	3.5 + 1.5
T4	Phytosanitary + phytosanitary		Copper oxychloride (70% Cu) + copper oxychloride (70% Cu)	Rebelde/Kenogard + Rebelde/Kenogard	1.5 + 1.5

applied using a backpack sprayer, with the application of 300 to 600 L/ha (Table 2). Given that different spray volumes were applied, it is important to point out that the doses used in the application of each product were doses per litre, thereby always applying the same amount of product per litre of water (Table 1).

The timing of the applications was designed to protect the plant in phenological stages of higher sensitivity to fungal infection, intensifying the number of applications when the climatic conditions increased the disease pressure. The phenological stages and application dates are shown in Table 2, and the scale used to define the phenological stage was the extended BBCH scale (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) (Lorenz et al., 1995). Figures S2 to S5 show the application times within their climatic context in the following order: Vineyard A 2019 (Fig S2), Vineyard B 2019 (Fig S3), Vineyard A 2020 (Fig S4) and Vineyard C 2020 (Fig S5). These represent daily values of the most determinant climatic parameters in the development of the *P. viticola*: rainfall, relative humidity and temperature.

Weather measurements

The environmental parameters of temperature (ºC), relative humidity (%), and rainfall, were acquired from the weather station closest to each vineyard zone from the Meteorological Service of the Catalonian Government (Meteorological Service of the Catalonian Govenement, n.d.).

Downy mildew assessment

Downy mildew level was evaluated 2 times per growing season; the first before flowering (BBCH 57) and the second in the pea grain size phenological stage (BBCH 75). At each evaluation date, within 20 vines of each repetition, 50 leaves and 50 clusters were randomly selected to be examined. Disease incidence was assessed as the percentage of leaves and clusters with visible downy mildew symptoms, and the severity was evaluated as the percentage of the area of leaves and clusters covered by these symptoms, following the EPPO standard scale (EPPO, 2009). Subsequently, the percentage of infection (McKinney index), was calculated (McKinney H.H., 1923).

Table 2 Moments of applications of the treatments and spray volume of the applications. Within each vineyard, year of study and phenological moment, each application was made on the same date and using the same spray volume in all treatments

Year	Vineyard	Number of applications			Phenological timing, dates of applications and spray volume (S.V) in L/Ha											
					BBCH-scale[28]: 19		53		61		73		75		77	
		Total	Odd	Even	Leaves unfolded		Inflorescences visible		Flowering onset		Berries groat-sized		Berries pea-sized		Berries beginning to touch	
					Date	S.V	Date	S.V	Date	S.V	Date	S.V	Date	S.V	Date	S.V
2019	A	4	2	2	May 21st	300			Jun 4th	350			Jun 19th	400	Jul 11th	450
	B	3	2	1	May 23rd	330			Jun 3rd	370			Jun 18th	500		
2020	A	4	2	2	April 22nd	300			May 18th	350			Jun 2nd	400	Jun 17th	450
	C	6	3	3	April 30th	330	May 19th	350	May 29th	400	Jun 5th	550	Jun 15th	600	Jun 25th	600

Soil copper concentration

Before starting and after finishing the trials, soil samples were taken using a hand auger to analyse the copper concentration in the soil. At these two moments, a soil sample was taken for each treatment in each trial. The goal of copper quantification in soils in this study was to compare surface content before and after application, for the exact same soil. Soil samples were taken from different locations spread out along the treatment surface with a ratio of 10 locations / ha in the large-scale trials (Zone A), and a minimum of 4 points per replicate in the smaller-scale trials (Zones B and C). To achieve a high representativeness of the sample, the locations were distributed in a zig-zag pattern throughout each treatment, and throughout all the replicates (Zones B and C), and subsequently homogenized to obtain a representative sample.

The sampling locations were located 10–20 cm from the base of a plant, and the sampling depth varied between 5 and 30 cm. In 2019, a single sample was taken as the initial value for each location, while in 2020, a representative sample for each treatment was taken to assess initial copper concentration.

Copper soil concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Previously, the soil was dried at room temperature and then sieved with a 200 mm diameter, stainless, 2 mm sieve (164,768/1, Filtra Vibración S.L., Barcelona, Spain). The sieved soil was treated with 1 M ammonium acetate and 0.0127 M ethylenediaminetetraacetic acid (EDTA) extraction solution at pH 7.0, and stirred for two hours in a twister at 500 rpm at 25 ºC. The samples were settled for 45 min and filtered with a 0.22 µm syringe nylon filter. 2 mL of the residue was diluted to 10 mL with a 2.5% v/v HNO_3 aqueous solution in a polypropylene tube and analysed. All the analyses were carried out on an iCAP 7000 Series inductively coupled plasma optical emission spectrometer (Thermo Scientific, Ma, USA). The emission wavelength considered for copper was 324.754 nm. The stock solution contained 1000 mg/L Cu dissolved in 5% HNO_3 aqueous solution (Agilent Technologies, Ca, USA).

Harvest parameters and grape sampling

The harvest date of the experiments was determined according to the winery's objective criteria. For each treatment, 6 to 10 vines were randomly selected from two repetitions, in which the harvest was carried out manually, and the yield measurement parameters were: vine yield weight, number of clusters per vine, and cluster fresh weight, which was estimated by dividing the total yield by the number of clusters per vine.

Berry sampling was also performed at harvest. A sample of 100 berries was collected from two repetitions per treatment and transported to the lab under refrigerated conditions to be analysed.

Chemical analysis of grape juice

The berries from each treatment were crushed to obtain the grape must liquid. The soluble solids concentration (ºBrix) was measured using a refractometer (Palette PR-32, ATAGO, Tokyo, Japan) and fermentable sugars (glucose + fructose) (g/L) were evaluated by the enzymatic methods established by BioSystems S.A. (Barcelona, Spain), in accordance with the OIV methods by means of Y15-BioSystems equipment. Potential ethanol content (% v/v), titratable acidity (g/L) and pH were analysed with Fourier transform infrared spectroscopy (FTIR) by FOSS (Wine Scan™ SO_2, FOSS Iberia, Barcelona, Spain).

The grape juice obtained from 500 g of collected grape samples was diluted (1:5) in a 2.5% HNO_3 aqueous solution, filtered with a 0.22 µm syringe nylon filter, and directly analysed by the iCAP 7000 Series equipment (Thermo Fisher Scientific, Ma, USA) to determine copper content. The emission wavelength considered for copper was 324.754 nm. The stock solution contained 1000 mg/L Cu dissolved in a 5% HNO_3 aqueous solution (Agilent Technologies, Ca, USA).

Statistical analysis

Significant differences were evaluated with a one-way analysis of variance (ANOVA) and the Least Significant Difference (LSD) test. The statistical analysis was performed using the XLSTAT Package for Excel software, and the statistical significance was established at $P \leq 0.05$.

Results

Climate data

The study was performed in the 2019 and 2020 growing seasons. With respect to the evolution of the climatic conditions (Figs S6 and S7), March was the coldest month (10.3 to 11.8 ºC), and monthly temperatures increased, with July and August (24.1 to 25.6 ºC), being the warmest months. Regarding the recorded rainfall, in 2019 the most abundant rains took place during April and May (32.0 to 52.7 mm) (Fig. S6). In 2020 there was abundant rain during the initial months of the season (Fig. S7). In the same year, from March to June, the recorded rainfall was more than 40 mm/month, and the highest amount recorded was 149 mm in Zone C in April.

Copper applied by-products

Table 3 shows the total Cu content applied in each treatment for all locations and growing seasons. The total content was calculated according to the percentage of Cu in each treatment, which was mainly dependent on the quantity of copper oxychloride (70% w/v of Cu), and considering the number of treatments performed in each case (Table 2) The highest amount of Cu was applied in 2020 in Zone C, reaching a value of 2.1 kg per hectare. It should be noted that the European Commission limits the application of copper to 4 kg per hectare/year spread over 7 years, from February 1st, 2019 (The European Commission, 2018a). This value was due to the climatic conditions of this vintage (explained above), which favoured *P. viticola* proliferation, and therefore, the increase in the number of applications. On the other hand, the lowest amount of Cu was applied in 2019 in Zone A, with a total of 0.3 kg/ha.

A reduction in copper inputs was demonstrated in the "less Cu treatments" (T1, T2, T3), as compared to the standard treatment with copper oxychloride (T4). This reduction ranged from 45 to 72 percent in T1, 48 to 77 percent in T2, and 35 to 56 percent in T3 (Table 3).

Evaluation of grapevine downy mildew infection

Regarding the incidence and severity of downy mildew disease assessed on leaves and grape bunches, it

Table 3 Total copper applied in each treatment and cooper reduction compared to the standard treatment with copper oxychloride (T4)

Treatment	Zone A—2019		Zone B—2019		Zone A—2020		Zone C—2020		Average	
	Cu (g/ha)	Cu reduction (%)	Cu (g/ha)	Cu reduction (%)	Cu (g/ha)	Cu reduction (%)	Cu (g/ha)	Cu reduction (%)	Cu (g/ha)	Cu reduction (%)
NTC	0,0	100,0%	0,0	100,0%	0,0	100,0%	0,0	100,0%	0,0	100,0%
T1	461.7	72.1%	432.9	63.2%	691.7	45.1%	947.5	55.6%	633.5	59.3%
T2	377.2	77.2%	336.5	71.4%	651,0	48.3%	928.3	56.5%	573.3	63.2%
T3	721,0	56.4%	639.5	45.7%	816.7	35.2%	1152.5	46,0%	832.4	46.5%
T4	1652.8	0,0%	1177.9	0,0%	1260,0	0,0%	2136.1	0,0%	1556.7	0,0%

should be noted that for both, affection was lesser in 2019 than in 2020. This is associated with the climatic conditions recorded in those years, which revealed two different situations of disease pressure. The year 2019 had moderate rains and relative humidity during the first months of plant development (Figs. S2, S3 and S6), which resulted in conditions of low disease pressure, while 2020, with heavy rains and high relative humidity during the first months of development (Figs. S4, S5 and S7), presented conditions of high disease pressure. In 2020, affectation was similar for both leaves and bunches. Table 4 shows the efficacy results of the treatments against *P. viticola* calculated from data obtained after the evaluation of the degree of downy mildew infection in grape bunches on the date with the greatest affectation, with all the data taken in the second evaluation at pea size phenological stage (BBCH 75), with the exception of evaluations with maximum levels of incidence and/or severity (100%), where the comparison between treatments was not possible. This is the case of the Zone C trial in 2020, where the results shown in Table 4 are the data obtained in the first evaluation, before flowering (BBCH 57), as in the second evaluation the levels of incidence were 100% in all the treatments, with values of severity ranging from 82.6 to 90.5%, except for the conventional treatment (T4), which obtained a severity value of 67.3% (data not shown). The high levels of incidence and severity reached in this case are supported by the favorable climatic conditions of precipitation and humidity (Fig. S5).

Soil copper content

To assess the accumulation of copper into the soil due to the different treatment strategies, samples were taken at the onset and after finishing the trials in each location and year, except for Zone C. In that area, the experiment had to be interrupted, as the high disease pressure compromised the preservation of the vines. The lowest initial Cu content was obtained in Zone A for the 2019 vintage, with a concentration of 3.77 mg kg^{-1}. This concentration varied in the following 2020 season, between 4.62 to 6.59 mg kg^{-1}. In the case of Zone B in 2019, the initial value obtained was 5.69 mg kg^{-1} (Table 5).

The variation in soil copper concentration after the treatments considerably differed in the same zone in different vintages, independently of the Cu

Table 4 Efficacy of the treatments against *Plasmopara viticola* on the disease incidence (DI), severity (SEV) and McKinney's Index [30] (MI) on grape bunches compared to non-treated control (NTC). These results have been calculated from the data of the evaluation of downy mildew level on the date of greatest infections. Treatment descriptions are reported in Table 1. Values showed mean values and standard deviations and different letters in the same column mean significant differences (*p < 0.05*)

Treatment	Zone A – 2019 (BBCH 75)			Zone B – 2019 (BBCH 75)			Zone A – 2020 (BBCH 75)			Zone C – 2020 (BBCH 57)		
	DI (%)	SEV (%)	MI (%)	DI (%)	SEV (%)	MI (%)	DI (%)	SEV (%)	MI (%)	DI (%)	SEV (%)	MI (%)
NTC	53.5 ± 2.2a	5.9 ± 0.5a	16.8 ± 1.7a	24.0 ± 4.8a	2.4 ± 1.2a	7.9 ± 2.4a	94.0 ± 1.8a	11.2 ± 0.8a	32.8 ± 1.7a	70.8 ± 20.8a	9.2 ± 2.6a	22.7 ± 6.0a
T1	37.5 ± 2.1b	3.3 ± 0.2b	11.4 ± 0.6b	17.5 ± 1.3a	2.4 ± 1.1a	6.4 ± 1.0a	74.5 ± 1.9a	5.6 ± 0.8bc	21.4 ± 1.4bc	42.2 ± 9.4ab	5.6 ± 1.4ab	13.4 ± 2.9ab
T2	38.0 ± 4.2b	3.7 ± 1.0b	12.5 ± 2.4ab	17.5 ± 5.2a	1.5 ± 0.9a	5.3 ± 2.0a	64.0 ± 7.3bc	4.5 ± 1.0bc	17.3 ± 2.5 cd	21.9 ± 13.1b	3.3 ± 2.0b	7.6 ± 4.3b
T3	38.5 ± 5.4b	3.2 ± 0.5b	11.6 ± 1.6b	16.0 ± 2.4a	0.5 ± 0.1a	4.4 ± 1.2a	77.0 ± 3.7b	7.4 ± 1.6b	24.1 ± 2.4b	31.9 ± 14.7ab	3.3 ± 1.4b	8.8 ± 3.7b
T4	45.9 ± 4.4b	3.7 ± 0.7b	14.2 ± 1.6ab	20.0 ± 1.8a	0.9 ± 0.1a	4.8 ± 0.5a	58.5 ± 4.5c	3.5 ± 0.4c	15.0 ± 1.2d	13.3 ± 7.2b	1.5 ± 0.7b	3.8 ± 1.7b

Data are means ± standard deviations. Values followed by different letters in the same column are significantly different (*p < 0.05*, Least Significant Difference test)

Table 5 Percentage of variation in soil copper concentration before and after treatments. NTC (non-treated control)

Year	Vineyard	Treatment	Cu (mg/kg)		%
			Initial	Final	
2019	A	NTC	3.77	3.51	-6.97
		T1		3.39	-10.04
		T2		4.64	23.07
		T3		3.68	-2.52
		T4		4.50	19.36
2019	B	NTC	5.69	5.34	-6.17
		T1		6.14	7.92
		T2		5.40	-5.06
		T3		6.20	9.00
		T4		10.93	92.06
2020	A	NTC	5.82	5.84	0.33
		T1	6.59	12.01	98.61
		T2	4.62	6.52	82.10
		T3	4.74	9.41	41.00
		T4	4.68	11.46	144.85

applied (Table 3). Nevertheless, the T4 treatment with copper oxychloride, where the highest copper amount was applied, obtained the greatest increase in soil copper in all observations, while the NTC presented minor variations.

Grape production and quality

Grape production in terms of yield and quality parameters were evaluated at harvest (Table 6). With respect to the yield parameters, the weight of the bunch was considered the best variable that could be used to correlate production with the values of incidence and severity of downy mildew evaluated in the grape bunches. Here, no significant differences were found in this parameter between treatments in the two cases studied (Zone B 2019 and Zone A 2020), despite this, a higher weight was observed in the grape bunches of T1 and T3 than in NTC. A lower yield (kg/vine) was observed in T1 vines as compared to the NTC in Sauvignon (Zone A 2020), with NTC obtaining the highest yield value, although this value was directly related to a higher number of bunches in the evaluated plants. Likewise, it may be surprising that, although higher weight was observed in grape bunches of some treatments, no significant differences were observed between treatments, as the NTC had the greatest affectation (severity and incidence), and the weight values of its grape clusters were expected to be significantly lower. This could be explained if we base ourselves on the non-progress of the disease from the second evaluation during the pea size phenological stage (never exceeding the severity value of 11.2% in the NTC Zone A 2020), and considering the abundant irrigation performed after this second evaluation in Zone A 2020, which contributed to the increase in the size of the berries (at a time of important growth

Table 6 Yield and quality parameters of the harvested grapes. NTC (non-treated control)

Cultivar	Harvest date	Treatment	Yield (Kg/vine)	Cluster weight (g)	Soluble solids concentration (ºBrix)	Alcohol (% vol)*	pH	Titratable Acidity (g/L)	Cu (ppm)
‘Grenache’Zone B	19 September 2019	NTC	2.74ab	144.8a	25.2a	14.9a	3.35a	3.9ab	0.250b
		T1	2.80ab	155.5a	26.2a	15.7a	3.42a	3.8b	0.268b
		T2	3.62a	147.7a	25.5a	15.1a	3.35a	4.2a	0.252b
		T3	3.22ab	166.7a	26.6a	15.9a	3.39a	3.9ab	0.315b
		T4	2.43b	131.2a	27.7a	16.7a	3.41a	4.0ab	0.419a
‘Sauvignon blanc’Zone A	22 August 2020	NTC	3.70a	139.3a	21.0a	12.1a	3.29a	7.7a	2.08b
		T1	1.83b	147.2a	19.9a	11.3a	3.26a	8.4a	2.39a
		T2	2.13ab	138.7a	21.1a	12.2a	3.28a	8.0a	1.83bc
		T3	2.40a	142.1a	20.4a	11.7a	3.26a	8.1a	1.46d
		T4	2.71a	138.8a	21.4a	12.4a	3.30a	7.4a	1.69dc

[*] Potential alcoholic strength by volume

of the berries), and lastly, to the dissipation of the possible differences in weight in bunches that could exist in the second evaluation of the level of downy mildew in the peas size phenological stage. Few significant differences were observed, indicating that the treatments did not affect the oenological parameters of grapes. The soluble solids concentration, alcohol, and pH values were found to be homogeneous among treatments. However, small differences were observed in the titratable acidity in the grapevines from the T1 treatment, which was lower than the other treated 'Grenache' grapevines, which was not consistent with the results from the other variety.

Differences were found in the range of copper concentrations on the cultivars analysed, with these values being higher in 'Sauvignon blanc' cultivar (1.69 to 2.30 ppm of Cu) than in the 'Grenache' cultivar (0.250 to 0.419 ppm of Cu), which may be related to the higher amount of product application performed during the 2020 season. The results obtained in Zone B (Table 6) showed lower concentrations of copper in the NTC than in the treatment with oxychloride (T4), where the highest copper concentration was detected. The results obtained in Zone A 2020 can be explained by a possible secondary effect from the vicinity of the experimental vineyard, which showed normal values as compared with other studies in the Mediterranean area (Provenzano et al., 2010).

Discussion

The effectiveness of downy mildew control strategies to reduce the level of copper applied to vineyards was evaluated in this work. The climatic conditions during the years studied were favourable for the onset of downy mildew infection in spring, especially in 2020, when conditions favoured the severe development of the pathogen throughout the growing season. In 2019, with severity values of less than 6%, the incidence in the NTC vineyard was 53.5% in Zone A, and 24.0% in Zone B. In the latter, with a low disease pressure, there were no differences between the treatments and the control regarding to the disease incidence and severity assessments. However, in Zone A, despite having recorded low severity values, significant differences were observed in the incidence and severity values with respect to the control, while the percentage of infection, according to McKinney Index, was significantly reduced as compared to the control in T1 (microelements) and T3 (copper complexed with heptagluconic acid) treatments (Table 4).

The results obtained under conditions of high disease pressure showed that the alternatives tested significantly reduced the incidence and severity of the treated grapevines. Without taking into account the case of the Zone C trial in 2020, where the incidence and severity values achieved in the second evaluation were 100% but not comparable between treatments. In 2020, with severity values of up to 11%, significant differences were observed in the incidence and severity for treatments in front of control. The standard treatment with copper oxychloride (T4) obtained the lowest values of incidence and severity in both zones, and the same results were observed with the McKinney Index analysis (McKinney H.H., 1923). Even then, satisfactory outcomes were also obtained after the application of other alternatives, such as *E. arvense* (T2), which significantly reduced the disease incidence from 94 to 64% in Zone A, and from 70.8 to 21.9% in Zone C as compared with the control. In T2, the disease severity also decreased from 11.2 to 4.5%, and from 9.2 to 3.3% for Zone A and C, respectively. Moreover, the grapevines treated with copper complexed with heptagluconic acid (T3) showed significant reductions in disease incidence, from 94 to 77% in Zone A, and a reduction from 70.8 to 31.9% in Zone C. The assessment of downy mildew severity in this case showed a significant reduction as compared with the control, from 11.2 to 7.4%, and from 9.2 to 3.3%, for Zone A and C, respectively (Table 4).

On the other hand, the treatment with microelements (T1) showed the lowest effectiveness, with a significant reduction of the incidence, compared with the control, from 94 to 74.5% in Zone A, and a reduction from 70.8 to 42.2% in Zone C, as well as a significant decrease in the severity of the disease from 11.2 to 5.6% in Zone A, and from 9.2 to 5.6% in Zone C (Table 4).

As previously stated, with the case of the second evaluation performed in 2020 in Zone C, in the scenarios where the severity of downy mildew obtained values higher than 80%, it was difficult to control the disease, even with the use of the standard copper oxychloride product.

Thereby, alternative strategies tested reduced the content of copper applied by 35 to 77 percent, as compared to the conventional copper oxychloride treatment, depending on the product used and the number of treatments needed in each vintage. Based on the field trials described, it was found that the treatment which alternated applications of standard copper with applications of a formulation based on *E. arvense* as a copper-free compound, and the treatment which alternates standard copper applications of copper oxychloride with copper complexed with heptagluconic acid, as an alternative low copper formulation, provided the best plant protection against downy mildew in grapevines under a situation of high disease pressure. On grapevines, Marchand and co-workers also observed a reduction in the affectation of downy mildew with the *E. arvense* treatment (combined with a low dose of copper), as compared to the untreated control (Marchand et al., 2018). However, in our study, the *E. arvense* treatment showed a better performance than in the Marchand et al. study, in which a significant increase in the affectation was observed with this treatment as compared to the conventional treatment with copper. Other researchers have also evaluated the efficacy of a product with copper complexed with heptagluconic acid, as an alternative to copper for the control of downy mildew in vineyards (La Torre et al., 2011). In that study, the product was used as a standalone treatment without it being combined with another copper product. As in the present study, significant reductions in cluster incidence were observed as compared to the untreated control, as well as reductions in severity, although not significant in the latter. Moreover, the treatment with heptagluconic acid in the study by La Torre provided results that were similar to our study when compared to the conventional treatment with copper, as in both of them, the conventional treatment showed lower levels of incidence and severity in the clusters, with these differences being significant in some of the cases. Llamazares and co-workers studied other products in combination with *E. arvense*, with satisfactory results, showing that natural products obtained from plants could be a sustainable alternative to conventional treatments in the vineyard (Llamazares De Miguel et al., 2022).

Although several factors may influence the determination of Cu in the soil, the increase in copper concentration may be linked to the application of plant protection products with a high Cu content, as observed in previous studies (Gómez et al., 2015; Iñigo et al., 2020). This was also observed in our study, where the greatest increase in soil copper was observed in the treatment in which a greater amount of copper was applied. This increase was more evident in the years in which the number of treatments was greater, such as 2020. According to the values obtained for soil copper concentration, our values, between 3.4 and 11.4 mg kg^{-1}, were within the range cited in other studies carried out in Spain (Gómez-Armesto et al., 2015; Iñigo et al., 2014; Navarro-Pedreño et al., 2018). Furthermore, the concentration of copper found in this study did not reach the reference level for potential toxicity of 85.28 mg kg^{-1} of copper defined by Iñigo and co-workers (Iñigo et al., 2020). In this sense, the evaluated soil copper content in this study may not induce toxicity in the soil, affect soil fertility, or become a potential risk to human health. However, a correlation was found with the levels of copper in grapes, according to the formulations of the products applied (in Grenache, 2019), as observed by other authors (La Torre et al., 2011).

The differences observed in oenological and yield parameters were not consistent between cultivars. Therefore, it is considered that these parameters were not affected by the treatments, as in other studies that also used alternative fungicides (Rantsiou et al., 2020; Romanazzi et al., 2016). Regarding the quality of the grapes, some authors reported that the application of conventional fungicides to control downy mildew can be negatively observed in the quality of the wines (Briz-Cid et al., 2018), in favour of biocontrol, which does not seem to have a remarkable impact on grape quality. Markellou and co-authors showed that microbial biopesticides to control downy mildew were equally effective to conventional fungicides, and did not observe negative effects on grape quality and yeast population dynamics in fermenting musts (Markellou et al., 2022).

Conclusions

In the organic vineyards evaluated in our study, the alternative product formulations based on *Equisetum arvense* and copper complexed with heptagluconic acid could be considered as proper strategies to control downy mildew disease in combination with conventional copper formulations. Nevertheless, further larger-scale experiments are still necessary to test the efficacy of these alternative strategies, and to evaluate their effects on harvest parameters. According to the reductions in the copper dosage achieved in our study, it is possible to reduce the amount of copper applied to control downy mildew, by using newly-developed formulations, although fully replacing copper is still not yet possible.

Acknowledgements The authors would like to acknowledge manufacturers for supplying products for field trials.

Funding Open Access funding provided thanks to the CRUE-CSIC agreement with Springer Nature. This work was funded by the Government of Catalonia with the Demonstration Activities Technology Transfer of the Rural Development Program 2014–2020 (N° 56 30021 2018 P4). The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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