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Study for the development of a rapid and non-destructive method for copper analysis in vineyards towards a precision fungal defense strategy

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Abstract. In the defense against downy mildew, copper is still widely used, particularly in organic management. At any rate, specific investigations are needed to significantly reduce the use of copper and minimize its environmental impact. This study, conducted in several farms in Friuli-Venezia Giulia (Northeast Italy), aimed to evaluate the concentration of copper on leaves in various climatic conditions, in order to create an important database for the development of a rapid control system through image analysis. The tests involved sampling leaves and grapes from bud break until the last treatment with copper-based products. After copper recovery using a nitric acid solution, the copper values were analyzed via ICP-AES analysis. Cuprotesmo imprints were also made on the same leaves to develop an application (APP) capable of quickly and non-destructively detecting copper on the leaf surface through image analysis. The analytical data revealed significant correlations between image analysis and copper values only in certain situations involving adult leaves. However, due to the variability in treatment situations, the use of the APP with image analysis for all copper defense scenarios is not yet feasible. Nevertheless, the results are encouraging and will serve for further extensive investigations to develop an image analysis system capable of detecting truly active copper and optimizing pesticide treatments for a precision defense strategy. This strategy aims to ensure low environmental impact, production sustainability, and grape quality.

1 Introduction

In grape production cycles involving grapes, wines, and musts, phytopathological control of plants is crucial to maintain high yield, quality, and wine production. Grapevines are subject to numerous pests and diseases [1], and among them, downy mildew (DM) caused by the oomycete *Plasmopara viticola* can be considered the most important [2]. DM is a severe disease in all temperate regions with high rainfall incidence and can infect all green tissues of the vine. Leaves are affected by a reduction in green area and assimilation capacity [3], while inflorescences, young shoots, and grapes may suffer significant damage, leading to a further loss of productivity and quality [4]. DM control is primarily carried out through the use of chemicals, with Copper (Cu) having been extensively used until now [5]. Copper is a preventive fungicide allowed in organic agriculture, which is active only at the application site, leaving new plant growth unprotected. Despite its unfavourable ecotoxicological profile and soil accumulation, Cu is one of the most important agrochemicals used in the organic sector due to its effectiveness against DM [6,7]. Additionally, Cu is an essential natural micronutrient in the plant's life cycle [8,9]. The worldwide use of Cu in viticulture has reached significant rates per year [10], and a large number of Cu-based compounds have been introduced and are now being sold and used. This has resulted in numerous problems regarding human health, environmental impact, and costs for farmers [11]. In this scenario, viticulture has had to consider the sustainability of agroecosystems and has been expanding rapidly worldwide [12]. However, it has become increasingly difficult to defend grapevines against DM, particularly in the organic sector, due to

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climate change, leading to higher temperatures, humidity, and rain events, all of which are fundamental in the development of DM [13]. Under these circumstances, Cu use should be improved to achieve sustainable use and waste reduction. Thus, it is crucial to be aware of the correct application time and quantity [6]), particularly the amount of this chemical required to guarantee an optimum protection level [14,15]. Nowadays, the use of copper fungicides in organic viticulture is restricted in Europe, as stipulated by the European Commission in 2002. In most European countries, including Italy, France, and Spain, the use of copper in organic farming is limited to 28 kg/ha in a period of 7 years, in accordance with CE 889/2008 and its derogations. Organic winegrowers place significant emphasis on disease management, following official advice and conducting field observations to protect their vineyards [16]. However, they may struggle to achieve both high yields and wine quality while maintaining the sustainability of their cultivation practices, especially considering the limitations on copper use [17]. While copper dosage, formulation, and application timing have been well studied and understood [7,18,19,20], the amount of copper cladding on leaves and grapes in the field necessary for adequate protection against downy mildew has not been thoroughly investigated.

In this context, the present study aims to develop a rapid in-field copper analysis system using image analysis to optimize the application of copper at the optimal time for maximum efficacy. The objective is to develop an app that can determine the active copper present on the vegetation in real-time on the field. Currently, there are no literature reports of such localized and rapid analytical applications for optimizing pest management treatments in specific situations. While there are interesting spectral analysis applications for environmental pollutant monitoring in agriculture [21,22], there are no specific and precise applications for the management of pest control treatments. This study seeks to contribute to the development of rapid and analytically robust field analysis systems that can assist in the management of low-impact precision defense strategies.

2 Materials and methods

Copper coverage surveys, aimed at developing an image analysis system, were conducted on five farms participating in the PSR-INTAVIEBIO project in order to gather information on very different scenarios such as viticultural areas and copper products used for downy mildew defense. During the 2020 growing season, leaves were sampled from late April to August for a total of 10 samplings, while grape clusters were sampled at four different times during their development until pre-veraision. Leaves were sampled in the central part of the observed vineyard, with 10 adult leaves per sampling, positioned proximal to the cluster. In order to recover copper for analytical purposes and to obtain information on the distribution of copper products used, the leaves were washed in the laboratory with a 1% nitric acid solution washing a specific surface of the upper and lower leaf page. Ten surveys were conducted weekly until August in order to collect a significant number of samples. Samples were also taken from the bunches at four different times during their development. The samplings were carried out under dry vegetation conditions in order to be able to manipulate and transport leaves and bunches without the risk of altering the copper coverage. The concentration of Cu was determined using inductively coupled plasma - atomic emission spectrometry (ICP-AES) equipped with a crossflow nebulizer and an autosampler. The analyses were carried out using the calibration curve method obtained by analysing seven standard solutions (range 0-20 µg/L) prepared after dilution of a stock standard multi-element solution (1000 mg/L). Data obtained from the ICP-AES instrument were expressed as the average of four different wavelength readings, expressed in parts per million (ppm). The presence of Cu in solution was then transformed from ppm present in 30 mL to ppm per 30 cm^2 of leaves, and at least to µg/cm2 of leaves. The same procedure was used for the grape clusters, but the final results were reported as µg of Cu per fresh weight. During the growing season, data on the incidence of downy mildew (DM) was collected four times from leaves and grape clusters. The assessments (diffusion and severity) were carried out in the same central area of the vineyard where the leaves were taken, using a precise sampling scheme that evaluated the entire vertical vegetative growth of the vines. When symptoms appeared, 50 leaves and 50 grape clusters were randomly chosen per vineyard, and the DM incidence, in terms of the percentage of infected grapes found in the entire sample, as well as the severity of the damage, were evaluated. The damage severity was determined as the mean percentage between the analysed sample. The data was then correlated with the copper values found in both the leaves and the grape clusters. The results from the first year of data collection allowed for some modifications to be made to the activities carried out in the second growing season of 2021. As the data from the two leaf pages was found to be redundant and correlated, only the lower part of the leaves was analysed in the second year of data collection. This allowed for an increase in the number of leaves analysed, specifically adult leaves (near the grape clusters) and young leaves (the third or fourth leaf from the apex). The samples were taken from the central part of the vineyards and provided interesting information, as the young leaves almost always represented the effect of a treatment. Furthermore, in a representative vineyard, samples were taken before and after each treatment. In the second year of data collection, the leaves were also analysed using an image analysis system to estimate the active copper. For this purpose, each leaf was washed on a known area on the left side relative to the central vein, while the right side of the same leaf was used to create an impression with Cuprotesmo paper for image analysis using an APP developed and calibrated using potted plants in a greenhouse with known and controlled copper doses. To estimate the total copper on the lower leaf page, Cuprotesmo paper was used, which is sensitive to copper and manifests as a pink colour intensity proportional to the concentration of copper on the leaf. To obtain comparable data with standard ICP analyses, each leaf was divided into two specular parts, one used for washing with nitric acid and the other for the copper absorption procedure on Cuprotesmo paper (Fig. 1). All Cuprotesmo impressions, after copper absorption, were acquired through digital photos using a dedicated VINIDEA - WENDA APP for the rapid estimation of total copper (Fig. 2).

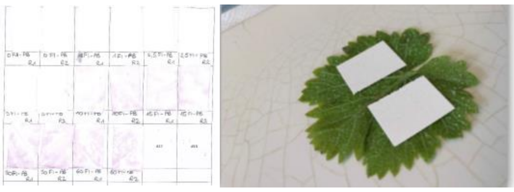

Figure 1. Cuprotesmo imprint and photo after copper absorption.

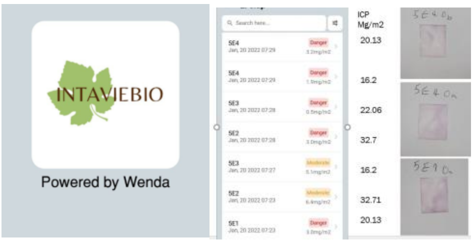

Figure 2. Copper data from the APP image analysis using Cuprotesmo imprint.

3 Results

The results of the first year of surveys have shown a substantial accumulation of total copper on the leaves, highlighting a possible progressive accumulation effect on adult leaves. The copper data detected by ICP-AES are in line with the literature, however, the values during the season also increase significantly due to a probable accumulation effect on leaves and clusters. The data from different companies, on leaves and clusters, showed significant variability, as the treatment protocols and copper products are not necessarily the same among different companies (Fig. 3).

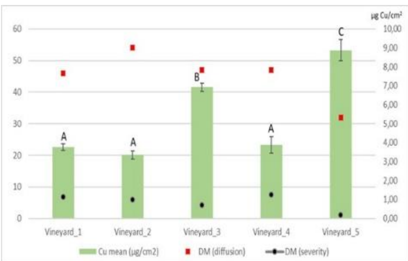

Figure 3. Copper and downy mildew variability in the different vineyards.

If the data are analysed overall in relation to downy mildew pressure, a positive correlation trend between copper and the incidence of downy mildew is observed, on both leaves and clusters. This is evidently the result of the accumulation of non-active copper on adult leaves, which shows an unreal relationship between infection and copper, a result influenced by the accumulated but non-active copper for downy mildew. This accumulation justifies efforts to seek rapid field systems to optimize copper treatment by reducing doses and maximizing the protective effect on downy mildew. Another interesting aspect that emerged from the surveys in the first year is the correspondence between the detected values on the two leaf surfaces. For this reason, a modified and optimized sampling and analysis criterion will be adopted in the second year of the survey to obtain additional information on the leaves with the same number of analyses. Regarding clusters, the information obtained did not lead to any interesting conclusions, so they were excluded from the second year of surveys. In the first year, surveys of downy mildew were conducted at four moments during the vegetative season, on leaves and clusters. The surveys (spread and severity) were carried out in the same central area of the vineyard where the leaves were collected, using a precise sampling scheme that involved evaluation of the entire vegetative vertical of the vine. The data were then correlated with the values of copper in the leaves and clusters themselves. As mentioned earlier, the aggregated data showed high variability among companies regarding pathogen pressure. Furthermore, the data showed a general positive correlation which is probably related to the accumulation of copper during the season (Fig. 4). This data could be related to accumulations with a good part of the copper remaining inactive.

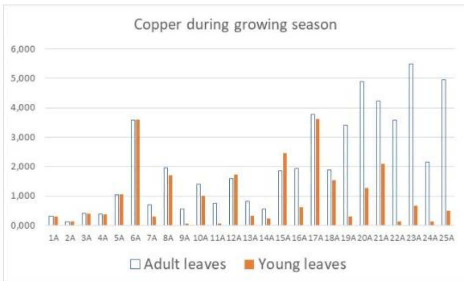

Figure 4. Accumulation of copper (μg/cm^2) on leaves (25 samples during growing season).

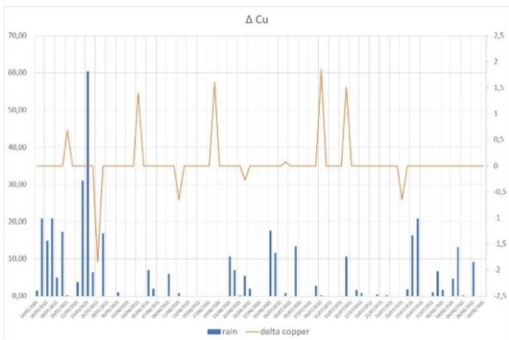

Figure 5. Copper value calculated as difference post-pre application.

The data from 2021 confirm the high variability among different companies, similar to what was observed in the first vegetative season. The accumulation on adult leaves is confirmed, while in young leaves, the data show copper values derived almost exclusively from a treatment, which is why the average values do not show accumulations. The same data are reported for the representative company as a difference between a treatment and the previous one, and the values show the copper actually applied with the last treatment. Negative values correspond to the effect of rain that washed away part of the copper present on the lower surface of the leaf (Fig. 5). The values detected before and after each treatment are also interesting, as they highlight the accumulations on adult leaves and the real contribution of the treatment on young leaves. The data from the differential between treatments on adult leaves and the values of young leaves are in the realistic range close to 5 mg/m2, a value considered as a standard for good efficacy against downy mildew. In general, from the data of the second year, high variability among companies is evident, as well as high variability among individual leaves, which is evidently related to the training system and the type of spraying machine used. The accumulation on adult leaves is confirmed (Fig. 6), and this is a very significant result as it could be related to high amounts of non-active copper present on the leaves. Therefore, it would be interesting to evaluate possible techniques to activate all the copper present in order to optimize treatment and at the same time reduce the total amount of copper, while ensuring effectiveness against downy mildew.

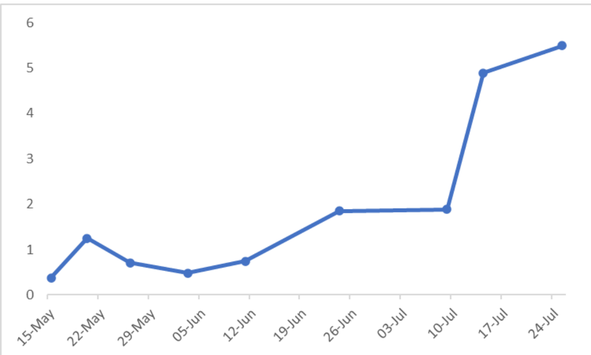

Figure 6. Copper accumulation (mg/m^2) on adult leaves during growing season.

Another interesting aspect of the obtained data is that even with copper quantities below the limit of the organic farming regulation, good defence and production can be achieved, if optimal vineyard and treatment management conditions are met. A positive relationship between copper quantity and downy mildew percentage is confirmed overall, further confirming the accumulation of total copper on the leaves. Only a currently unknown portion of this copper is actually active (accumulation graph). This last consideration could be a starting point for further scientific investigations on the management of effectively active copper.

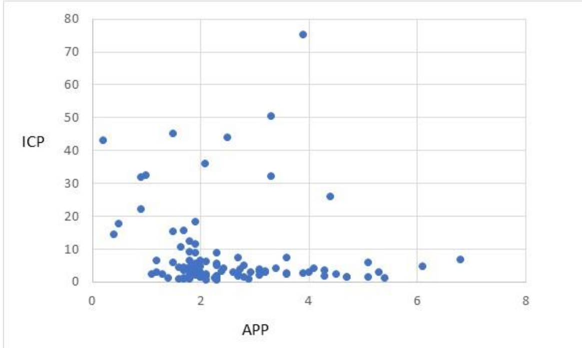

Figure 7. Copper data by ICP-AES (*y* axis) and APP image analysis (*x* axis).

Figure 8a. Copper data by ICP-AES (*y* axis) and APP image analysis (*x* axis).

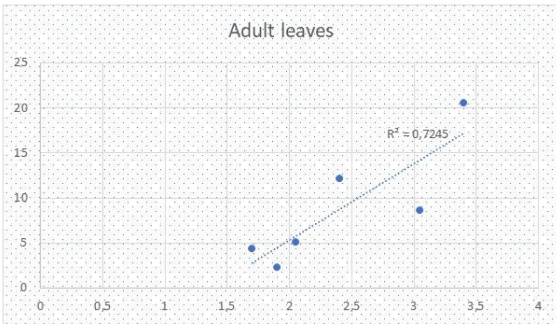

Figure 8b. Copper data by ICP-AES (*y* axis) and APP image analysis (*x* axis).

All the acquired data were used to develop an app aimed at the rapid monitoring of copper on leaves in order to optimize defense strategies from a precision farming perspective. The correlations between ICP-AES results and the APP are not significant when analysing the data as a whole or even for individual samplings (Fig. 7). Only in some situations of adult leaves do interesting correlations (Figs. 8a, b) appear between the data returned by the app and the ICP analysis. It is evident that further statistical analysis and experimentation are necessary in order to make a correct estimation of the amount of copper using the app. Moreover, the data highlight a great analytical variability of the app's data, similar to that found with ICP analysis (Fig. 9), confirming the sensitivity of the rapid analysis system in a wide range of copper deposited on leaves and therefore in all real treatment situations.

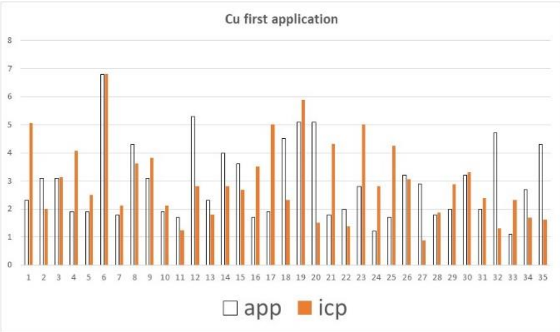

Figure 9. Variability of copper data (mg/m^2) ICP-AES and APP image analysis.

Furthermore, when evaluating the data by vineyard or sampling, the app data are much lower than those of the ICP, particularly in adult leaves. This aspect indicates the need for further implementation and practical application of the system in different defence situations, eliminating the possible factor related to the type of copper-based product used. From the accumulation data, it is evident that further investigations will be necessary to arrive at a correct estimation of the amount of copper that is actually active, rather than the total amount of copper, in order to reduce product doses and guarantee defense against fungal pathogens and, consequently, completely sustainable production.

4 Conclusions

From the data obtained in the past two years of trials, there is a high variability both among companies and within individual leaves. This variability is evidently related to the form of cultivation and the type of spraying machine used. The accumulation on mature leaves is confirmed, which is a significant result as it could be related to high amounts of inactive copper present on the leaves. Therefore, it would be interesting to evaluate any techniques to activate all the copper present in order to optimize the treatment while reducing the total amount of copper, ensuring its effectiveness against downy mildew. Another interesting aspect of the obtained data is that even with copper amounts lower than the BIO limit, good defence and good production can be achieved in optimal vineyard and treatment conditions.

In general, there is a positive relationship between copper quantity and downy mildew percentage, further confirming the accumulation of total copper on leaves. Only a part of this copper is currently known to be active. This last consideration could be a starting point for further scientific investigations on the management of active copper. Although the practical results of the APP are not complete and applicable in all situations, the approach seems promising in the perspective of having a rapid and non-destructive field analysis system for copper optimization in situations of actual need, in the minimum dose necessary to ensure downy mildew defense and the quality and quantity of the grape.

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