Study to optimize the effectiveness of Copper treatments for a low impact viticulture

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Abstract: Among all pathologies that afflict grapevine, Downy Mildew (DM) is the most important. Generally controlled by using Copper (Cu), recently European Commission confirmed its usage but limiting the maximum amount to 28 Kg per hectare in 7 years (Reg. EU 2018/1981). Anyway, in the grape growing context it is difficult to reduce the use of Cu and generally chemicals, due to climate conditions, as well as climate changing. For this instance, Cu treatments have to be improved. Hence, the aims of this work were to determine the possibility to reduce Cu using and evaluating the variation of Cu cladding on grapevine leaves and grapes, in relation to the phytoiatric status. The efficacy level of the Cu protection given to DM and the correlation among them was also assessed. Five organic vineyards located in north-eastern of Italy were selected as experimental sites. Leaves and grapes were sampled during vegetative season and analysed for determining the quantity of elemental Cu by the use of ICP AES. Spreading of DM in vineyards was evaluated as well and the correlation between DM and Cu quantity on leaves and grapes was determined. First results indicate that the mean level of Cu applied by farmers (range: 3.77 to 8.88 $\mu g/cm^2$ of Cu on leaves) during vegetative season is not enough to have an optimal protection against DM (diffusion on grapes and leaves: 40 to 50%), although at the maximum amount of Cu, the plant protection effect was quite good. Thus, Cu treatments have to be pondered on the basis of meteorological data and previous infection of DM, so that it will be possible to determine the right quantity of Cu to be applied in correlation to DM presence and weather. As future goals, data will be correlated with image analysis, in order to quickly study the best conditions for Cu application directly on field and having back a real time information of Cu cladding and risk classes of DM infection. In this way, it is possible to reduce inputs in plant defence and then guarantee a quality and sustainable production of grapes, wines and musts.

Introduction

Grapevine (*Vitis vinifera* L.) is one of the most widely cultivated crops. It has cultivated for human consumption for over 7000 years, present in both hemispheres and positioned in the highest compensated cultivated agrarian specie (Cambrollé et al., 2013) (Terral et al., 2010). The worldwide cultivation is estimated on about 7.6 million hectares, with 76 million tons of grapes produced and 259 million hectolitres of wine (OIV, 2019). Indeed, the world value stands at more than 260 billion euro, while in Italy, second worldwide after France, the value is around 13.2 billion euros (unioneitalianivini, 2017). In the production cycle of grapes, wines and musts, plant phytoiatric control is fundamental, to preserve yield, quality and wine production. In fact, grapevine is afflicted by numerous pests and diseases (Bois et al., 2017). Among all the pathologies that afflict grapevine, downy mildew (DM), caused by the oomycete *Plasmopara viticola*, may be indicated as the most important (Dick, 2005). DM is a severe disease in all temperate regions with high rainfall incidence. It can infect all green tissues of vine. On leaves, it reduces green area and the assimilation capacity (Moriondo et al., 2005). Inflorescences and young shoots can be attacked as well, but the major damage is on grapes, where DM could lead to a further loss of productivity and quality (Williams et al., 2007). Shortly, regarding the life cycle, oospores are formed during the vegetative period, by the fertilization of an oogonium (female organ) and an antheridium (male organ). During winter, oospores, which reach maturity in the autumn, remain dormant and their germination in the spring is influenced mainly by temperature conditions. At this point, oospores emit a sporangium containing zoospores responsible for primary infections, generally when certain conditions occur (temperature, bud length, amount of rainfall, humidity, leaf wetness). From these, secondary infections develop during all the growing season (Rumbolz et al., 2002). Pathology control of DM is mainly carried out by the use of chemicals (Caffi et al., 2012). For this, Copper (Cu) have been using intensively until now. Copper is a preventive fungicide, allowed in organic agriculture, which is active only at the application site, so that new plant growth results unprotected. Nevertheless, it is one of the most important agrochemicals used in the organic sector due to its effectiveness against DM (Pertot et al., 2017) (Provenzano et al., 2010). In fact, despite its unfavourable ecotoxicological profile and the accumulation in the soil, the use of Cu is strongly required in organic viticulture, in acknowledgment of its unique properties as a wide-spectrum fungicide and bactericide (Dagostin et al., 2011) (Flemming and Trevors, 1989). Moreover, Cu is an essential natural micronutrient in plant's life cycle (Lesnik and Vršič, 2013) (La Torre, 2011). Worldwide, in viticulture, Cu using have reached rates really high per year (Dagostin et al., 2011) and a great amount of Cu – based compounds have been introduced and are now being sold and used, with numerous problems regarding human health, environmental impact and costs for farmers (Komárek et al., 2010). In this scenario, viticulture had to consider the sustainability of agroecosystems and, for this instance, organic viticulture have been spreading a lot worldwide recently (Fragoulis et al., 2009). Though, whether it was quite easy to defend grapevine against DM some years ago, nowadays, it become difficult, especially in the organic sector, due to climate changing that led to a higher temperatures, humidity and rain events, all fundamental in the development of DM (Kennelly et al., 2007). In this situation, Cu using should be improved, to achieve both a sustainable use and reducing waste. Hence, it is important to be aware of the correct application time and quantity (Pertot et al., 2017), and especially which amount of this chemical is enough to guarantee an optimum protection level. Nowadays, the use of copper fungicides in organic viticulture is currently restricted in Europe (European Commission, 2002) and Cu used in organic farming is limited to 28 kg/ha in 7 year in most European countries, including Italy, France, and Spain (CE 889/2008 and derogation). Organic winegrowers pay a lot of attention on diseases: they follow official advices and conduct field observations to defend vineyards (Hofmann et al., 2008), but sometimes they are not able to put together a good production optimizing Cu effectiveness. Thus, by some years ago, the most important challenge in organic viticulture is to maintain both a good yield and final wine quality, respecting the sustainability of cultivation (Martínez-Zapater et al., 2010). While Cu dosages, formulations and timing of application are well studied and understood (Provenzano et al., 2010) (Romeu-Moreno and Mas, 1999) (Renan, 1994) (Romanazzi et al., 2016), which amount of Cu cladding on leaves and grapes on field that could lead to an adequate protection to DM is not well studied yet. For this purpose, this paperwork reports the results of the first year of experiment carried out in the north east of Italy, Friuli Venezia Giulia region (FVG), where five organic vineyards in different location were chosen and used as experimental sites. Leaves and grapes were taken during vegetative season, sampled weekly, and analysed to find out the quantity of Cu cladding on leaves and grapes. Phypatology data were collected of DM development as well. The aim was to evaluate which level of Cu present on plant organs is enough to explicate a phytoiatric control of DM, discriminating possible thresholds of Cu applied, and then correlating the Cu quantity to the mildew control. At least, the final aim of the whole research for the further years will be creating a simple mobile device, that; through imagine analysis; will give an information on Cu level on plant organs and a risk classes of infection, in real time on field. In this way, winegrowers and technician could have more acknowledgement about Cu using and its protection level in DM control, limiting yield and quality decrease, residues in musts and environment, and finally having a better vineyard management.

Materials and methods

Experimental set up

Five organic vineyards were chosen around FVG Region. In order, they were located at Corno di Rosazzo (UD); farm V and C, at San Giovanni al Natisone (UD); farm E, at Ruda (UD); farm P and the last one at Fagagna (UD); farm A. The vineyards variety under test were Pinot Gris. Every of them was cultivated with the same organic method, the same trellis system (Guyot) and then the same canopy management and soil fertilization. The difference among vineyards sites were the soil type and the quantity of Cu used during the growing season, as well as climate conditions.

Sampling and Cu analysis

During vegetative season, with a weekly timing, ten leaves per farm were collected. Specifically, leaves were taken in five selected rows per vineyard, choosing only those ones with a good exposure and located in the middle part of canopy. A number of bunches were taken as well. Once having collected samples, 30 cm^2 of leaves were washed, both the lower and upper leaf page, with 30 mL of a solution made with nitric acid HNO_3 (65%) 1%, to have an eluate corresponding of 30 mL for the lower page and 30 ml for the upper. All analytical solutions were prepared using ultrapure water (resistivity ≥ 18.2 MΩ cm) obtained from a Milli-Q® System (Millipore, Bedford, MA, USA). Indeed, bunches were immersed in the same acid solution, for ten minutes, with an amount of acid solution (mL) corresponding to the weight of bunches (1 ml was used for every gram of fresh weight). From the eluates, using a graduated syringe coupled with an acetate cellulose filter 0 20 diameter, 12 mL were filtrated, to carry out the next step of analysis. The concentration of Cu was then determined by using inductively coupled plasma - atomic emission spectrometry (ICP-AES) (5800 Agillent. Perkin Elmer Optima 3000) instrument, equipped with a crossflow nebulizer and an auto sampler. 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 given back from ICP AES instrument were expressed as the average of four different wavelength reading, expressed in part per million (ppm). Cu presence in solution was then transformed from ppm present in 30 mL to ppm per 30 cm^2 of leaves, and, at least, to µg/cm^2 of leaves. The same was done for bunches but reporting the final result as µg of Cu per fresh weight.

Downy Mildew infections recording

During the vegetative seasons, at least 4 times, data collecting of DM spreading was performed. As soon as symptoms appeared, 50 leaves and 50 bunches per vineyard were chosen randomly and DM incidence, in terms of diffusion and severity damage class, were evaluated. Diffusion was described as the percentage (%) of infected grapes found in the whole sample. Indeed, damage severity was determined as the mean percentage between the analysed sample.

Statistical analysis

One-way analysis of variance was carried out using **R** Core Team (2020). Statistical analyse for the determination of significant differences between treatments was performed by using Student-Newman-Keuls test ($p \leq 0.05$). Furthermore, the correlation between variables under test, namely DM spreading and Cu quantity, was found by applying the correlation index (R^2) in a simple regression model. Finally, to discriminate thresholds of Cu in relation to DM infections, data were divided into percentile classes and the minimum and maximum level of Cu in relation to DM incidence performed.

Results

Firstly, Cu applied by winegrowers varied from 2 to 4.8 Kg/ha/year. Hence, this information was taken in consideration by us to correlate DM infections to Cu average used during vegetative season. In Table 1, it is reported the final amount of Cu sprayed by winegrowers, during 2020 study period, in each tested farm. Indeed, in Table 2 and 3, it is reported the amount of Cu cladding on leaves (µg of Cu/cm^2 of leaf) and grapes (µg of Cu/ g of fresh weight), respectively, at the different data of treatments, and the mean of Cu sprayed for each farm under test. Furthermore, given these data, in Figure 1, it is showed the total Cu cladding on plant organs in relation to the total Cu used by winegrowers during the study period, with a R^2 index that reached a value of 0.8626.

Farm	Cu applied (Cu/ha)
A	4.2
E	4.04
P	4.8
C	2
V	3.9

Table 1. Copper applied by every farm during 2020

μg/cm²	11-Jun	19- Jun	26- Jun	01-Jul	07- Jul	15- Jul	22- Jul	29- Jul	06-Aug	19-Aug	mean	St. Dev.
Farm A	1.54	2.72	2.93	2.74	2.33	2.43	5.33	5.32	5.15	7.24	3.77	1.75
Farm E	0.87	2.55	2.36	2.19	2.57	3.34	3.54	4.39	2.67	9.05	3.35	2.09
Farm P	0.86	3.17	6.27	2.36	5.23	6.56	9.28	7.72	16.22	11.62	6.93	4,35
Farm V	0.90	1.94	2.40	3.02	3.42	4.17	5.32	3.65	9.44	4.57	3.88	2.22
Farm C	3.07	11.79	9.93	3.02	9.80	6.36	13.60	7.96	2.14	21.14	8.88	5.51

Table 2. Cu applied by winegrowers at different data in the study period, expressed in μg of Cu/cm^2, on leaves, and standard deviation

μg/g FW	19- Jun	26- Jun	01-Jul	07- Jul	15- Jul	22- Jul	29- Jul	06-Aug	19-Aug	25-Aug	Mean	St. Dev
Farm A	1.92	4.61	3.25	2.30	3.19	5.52	4.61	4.02	4.64	2.63	3.67	1.12
Farm E	1.29	4.19	3.77	4.22	9.89	5.31	2.48	5.10	2.72	2.75	4.17	2.24
Farm P	0.74	5.82	2.36	5.26	4.25	4.87	4.53	3.16	1.84	7.32	4.01	1.89
Farm V	0.51	4.27	4.08	3.29	5.22	5.96	13.49	2.91	2.20	2.31	4.42	3.37
Farm C	4.47	26.04	23.10	11.38	5.23	16.14	5.50	10.65	5.60	4.76	11.29	7.57

Table 3. Cu applied by winegrowers at different date in the study period, expressed as μg/g FW, on grapes, and standard deviation

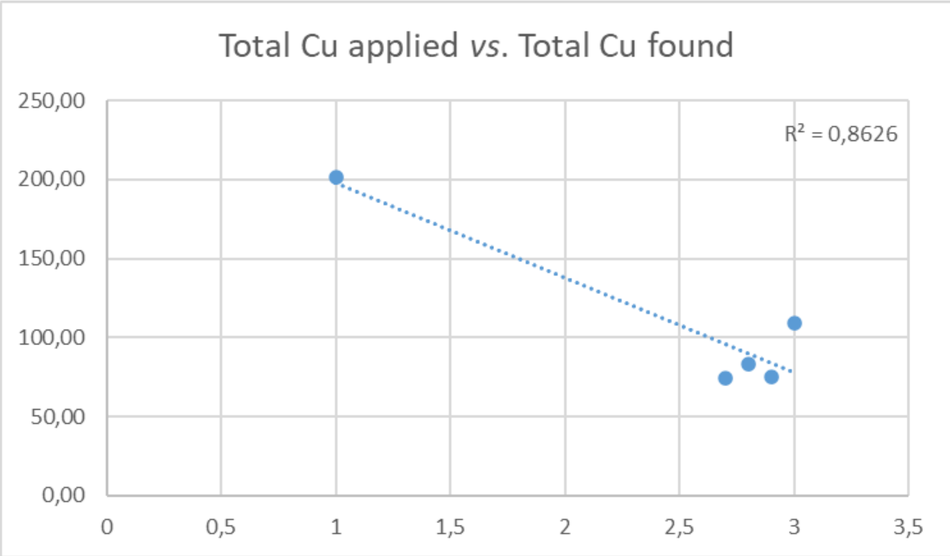

Figure 1. Total Cu applied by winegrowers in correlation to Total Cu, in the study period, found by analyses

At this point, we can report the final results of DM infections recorded at the end of vegetative season, in terms of diffusion and damage severity, for every vineyard tested (Table 4). Specifically, for leaves, in terms of DM diffusion the incidence was the highest in farm E and the lowest in farm C. Moreover, as severity of damage on leaves, the greatest attack was found in farm V and the lowest in farm C. Indeed, concerning DM diffusion on grapes, farm V showed the most spread attack and farm C the least. Finally, as severity on grapes, farm C was the most attacked, whilst farm P and C got the lower values.

	Leaves - DM		Grapes - DM	
	Diffusion	Severity	Diffusion	Severity
Farm A	46 a	6,79 a	36 a	2,32 a
Farm E	54 b	5,84 a	42 a	1,48 a
Farm P	47 a	4,12 ab	36 a	0,47 b
Farm V	47 a	7,46 c	52 b	5,79 c
Farm C	32 c	1.12 d	23 c	0.33 b

Table 4. DM infection, in terms of diffusion and severity of damage, on leaves and grapes for each Farm, recorded at the end of season. Different letters mean a statistical significance with $p \leq 0.05$

Cu mean (µg) and Downy Mildew (%) incidence on leaves and grapes

The next step was correlating Cu cladding on leaves and grapes to the phytoiatric status. In figure 2 and 3 are reported these correlations. It is possible to note how, on leaves, the correlation index R^2 reached a value of 0.7950 and 0.8858, concerning diffusion and severity, respectively. Furthermore, on grapes, the R^2 found was 0.6415 between Cu and Dm diffusion and 0.1552 between Cu and DM severity.

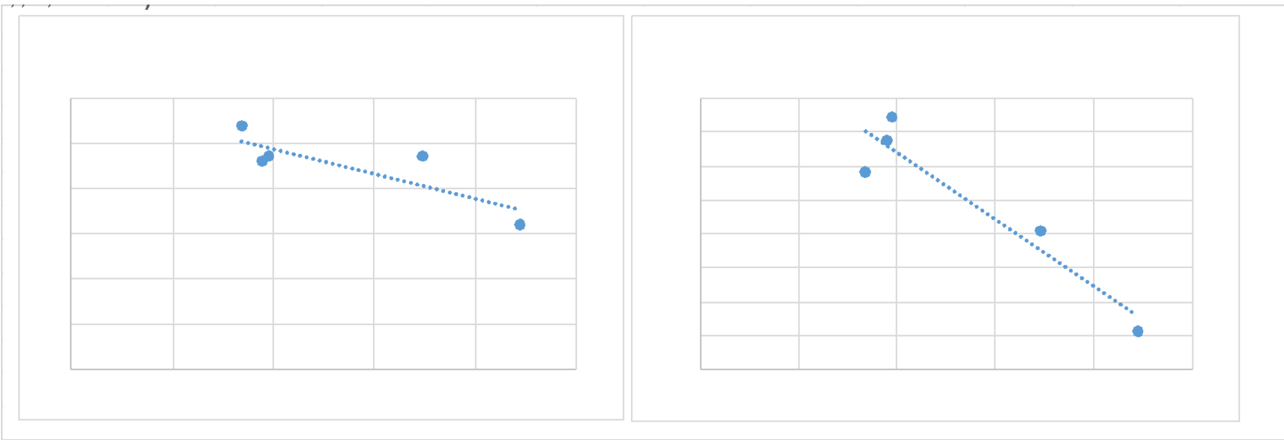

Figure 2. Correlation between variables (DM diffusion, severity, and Cu cladding) on leaves

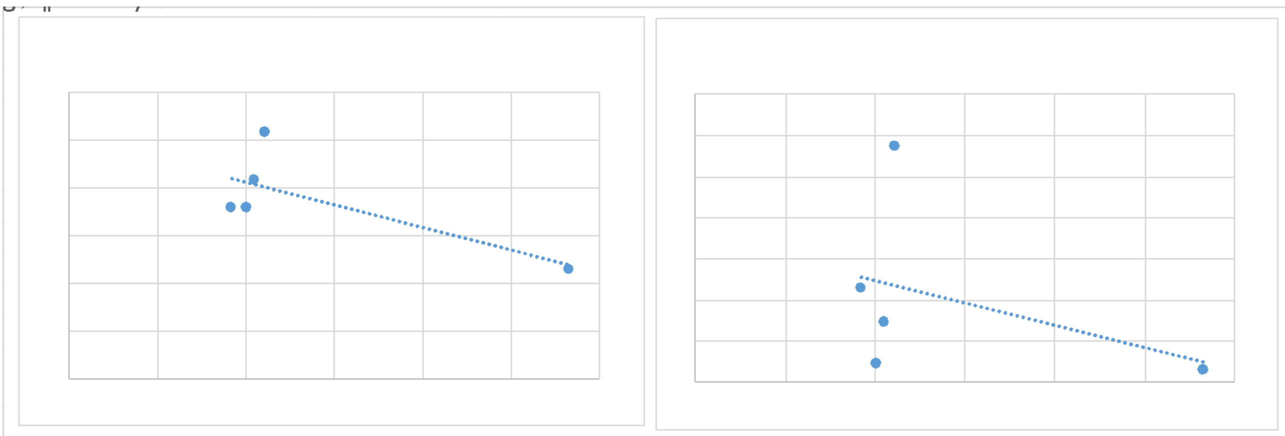

Figure 3. Correlation between variables (DM diffusion, severity, and Cu cladding) on grapes

First found thresholds of Cu cladding on leaves in correlation to DM infection

Table 5 reports the first found thresholds in one year study. Copper applied on leaves varied from 3.35 to 8.88 μg/cm^2, whilst on leaves the range was between 3.67 and 11.29 μg/g of FW. Moreover, by Table 5, we report how at the minimum quantity of Cu used, it was found the greatest DM incidence in terms of diffusion and severity (on leaves). Indeed, on grapes, this evidence was not the same, apart from the maximum amount of Cu used, where less symptoms of DM were recorded.

		Cu mean (μg/cm^2)	DM (diffusion)	DM (severity)
Leaves	**Farm A**	3.77	46.00	6.79
	Farm E	3.35	54.00	5.84
	Farm P	6.93	47.00	4.12
	Farm V	3.88	47.00	7.46

Farm C	8.88	32.00	1.12
MAX	8.88	54.00	7.46
MIN	3.35	32.00	1.12
Range	5.53	22.00	6.34
	Copper range (min – max)		
	3.35 – 8.88		

		Cu mean (µg/g of FW)	DM (diffusion)	DM (severity)
	Farm A	3.67	36.00	2.32
	Farm E	4.17	42.00	1.48
	Farm P	4.01	36.00	0.47
	Farm V	4.42	52.00	5.79
Grapes	**Farm C**	11.29	23.00	0.33
	MAX	11.29	52.00	5.79
	MIN	3.67	23.00	0.33
	Range	7.62	29.00	5.46
		Copper range (min – max)		
		3.67 – 11.29		

Table 5. Thresholds of Copper applied by winegrowers in each tested vineyard in each farm, during 2020, DM diffusion and severity

Discussion and Conclusion

Copper is an essential natural micronutrient, and it is widely used as plant defence agrochemical for the already known effectiveness. However, copper used as a plant protection product may have long-term consequences due to its accumulation in the soil and impact on human health (Jung et al., 2013) (Coelho et al., 2020). Hence, the optimization of copper application to control *P. viticola* in the whole viticulture, but especially in the organic sector, is a very important issue since Cu is the central component of management systems. In fact, frequent and prompt applications are required, in order to avoid yield and quality losses. Nowadays, in the world, there are almost 380.000 ha of organic grapes (La Torre et al., 2019). In the last years, in the light of new developments and evidence to a better management of the agroecosystem, the European Commission fixed a maximum amount of 4 kg per hectare and year of copper in plant protection (on average). This restriction of copper using caused further difficulties to organic farmers in the disease management. The development of optimizing strategies able to reduce or improve copper application is therefore a pressing need. Several research projects have been funded to find appropriate dosage and Cu coformulations on field (Aziz et al., 2006) (Torre and Spera, 2008)(Kuflik et al., 2009), but only a few focused on Copper cladding on plant organs in relation to DM (Cabús et al., 2017). For this instance, our purposes were to find out the correlation between Cu applied on field and Cu cladding on plant organs, by analyses made by ICP AES, to test the goodness of this method. Furthermore, define the correlation between Cu cladding and the phytoiatric status and, finally, determine ranges of Cu presence and thresholds of its effectiveness. Firstly, we can state how the Cu usage performed by winegrowers was on average in this territory (between 2 and 4.8 Kg/ha), where the annual rainfall incidence is greater than the rest of Italy, anyway, allowed by EU law. Secondly, Cu applied on field and Cu found out by using ICP AES was in a good correlation, in fact; R^2 reached a value of 0.8626. This in spite of the fact that there were many variables at stake (atomizer make, Cu formulations, workers, climate, etc.). Therefore, we report how this evaluation method worked well and will be used by us for further analyses within this project. Thirdly, we recorded DM infections with the main aim of using this data to correlate Cu presence to the phytoiatric status. For this, we found a correlation index between Cu applied on leaves, and DM diffusion and severity, of 0.795 and 0.8858, respectively. As well as previously, although in the biological system the variables were many, R^2 was appreciable. Indeed, not the same value was found on grapes. Here, the R^2 was less strong, with 0.6415 and 0.1552 between Cu, and DM diffusion and severity, respectively. Anyway, we should underline how in these last correlations on grapes, if only one farm is erased from the analyses, R^2 is subjected to a hard increase that makes it catch up leaves' correlation. Hence, we can state how some external errors could have been occurred for this data. At this point, we defined the maximum and minimum use of Cu. We can report how the range of Cu found was between 3.35 – 8.88 µg/cm^2 on leaves, and 3.67 – 11.29 on grapes µg/g of FW. More detailed, from the minimum to the maximum there was a gap of about 6 micrograms on average. Moreover, these thresholds corresponded to the higher and lower DM incidence, although DM infection strongly varied between thresholds. Nevertheless, we can say how at the maximum amount of Cu cladding on leaves and grapes, there was a very good plant protection effect, while at the minimum the effectiveness was really lower; about 2-fold less concerning diffusion till 5-fold less regarding severity.

These were the first evidence of one study years. We just determined that ICP AES method, to correlate Cu sprayed and cladding level on plant organs, is a good tool. Not only, the correlation between DM incidence and Cu used, was, at least, quite good. Furthermore, despite between the minimum and maximum threshold DM incidence varied a lot, the lowest and highest level of Cu cladding corresponded to the worse and better action against DM, respectively. These are the first reported information in this subject and will allow us to carry out further analyses. In fact, given this evidence, our future goals are to narrow down the range of thresholds, for being able to define different risk infection classes, taking into account DM incidence more in detail within the found ranges. At least, we want to correlate copper presence on plant organs performed using ICP AES to imagine analyses, with the final aim to have a simple portable device that analyses copper on leaves and grapes directly on field, giving back an information in real time about CU cladding and the correlation to the infection risk. This will be a useful tool for better managing treatments, improving grapes and wine yield and quality, and finally having a less residues in must end environment.

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