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COMBATTING FORMS OF RESISTANCE OF DOWNY MILDEW - *Plasmopara viticola*

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Abstract: Grapevine downy mildew, *Plasmopara viticola,* is the most damaging disease of grapes globally. Although the management of this disease involves the application of fungicides during the growing season, the resistance developed often makes chemical treatments ineffective. Most studies refer to combating the pathogen during the plant's growing season, although it continues to evolve during the vegetative rest period of the grapevine crop, in the leaf debris in the soil. In this study, laboratory research methods were used, as well as field research methods on the experimental plot. Five experimental variants and a control were used. Following the experiments carried out, it was found that the most effective variant was the 4-cymoxanil + copper hydroxide variant (13.33% attack degree). The aim of the experiments was to combat overwintering resistance forms of *P. viticola* in order to reduce the number of treatments during the growing season.

Key worlds: *downy mildew, resistance forms, chemical control, biological control.*

INTRODUCTION

Grapevine downy mildew caused by *Plasmopara viticola* has dimorphic reproductive forms, namely sexual and asexual forms, which are responsible for primary and secondary infections. Oospores represent the sexual stage of *P. viticola* and are formed after the fusion of an antheridia with an oogonium in the affected leaf tissue, from late summer to late autumn (Burruano, 2000; Rossi et al., 2013).

In autumn, September and October, due to conditions that gradually become more and more unfavourable for optimal and massive reproduction (changes in the cellular content of the leaves, as a result of their aging, inappropriate temperature and humidity), *P. viticola* moves towards an evolution that ensures the possibility of overcoming the harsh rigors of winter. Thus, following infections during this period, the fungus no longer tends to invade through its mycelium the entire area between and in the leaf cells and, therefore, to destroy the entire leaf, but is limited to areas between 1 and 3 mm. These autumn infections, restricted in surface area on the leaves, are externalized on the leaf surface by small, irregular, angular spots, which contrast with the rest of the green colour. This is the so-called mosaic foliar phase. If the fungus does not extend into the cellular structure of the leaf, instead, at the extremities of some filaments of the mycelium the sexual organs are formed, on some filaments the antheridia and on other filaments the oogonia. The antheridia are elongated, thickened at one end, and contain protoplasm in which numerous nuclei are included; the oogonia are spherical and also contain a protoplasmic mass with a large number of nuclei. The two sexual organs approach each other until they touch, the contents of the antheridia overflowing into the oogonium, the phenomenon

of this sexual fusion being known as plasmogamy. The antheridia are elongated, thickened at one end, and contain protoplasm in which numerous nuclei are included; the oogonia are spherical and also contain a protoplasmic mass with a large number of nuclei. The two sexual organs approach each other until they touch, the contents of the antheridia overflowing into the oogonium.

The result of the sexual process is the formation of the oospore in the mosaic leaf, a stage in which the fungus will survive over the winter. The oospores remain in the leaf litter above the soil of the vineyard and in the soil. In the following period, they complete their morphological maturation and overcome the vegetative dormancy, a physiological stage that does not allow them to germinate in winter, even if the climatic conditions are favourable. After their physiological development, in May and June, in spring-early summer, the oospores germinate in a macrosporangium that releases zoospores responsible for the primary infections with *P. viticola.* The formation of oospore does not require special temperatures, but seems to be affected by drought conditions (which prevent asexual sporulation) or by leaf senescence (Gessler et al., 2011). Oospores overwinter in leaf litter above the soil surface (Kennelly et al., 2007; Rossi et al., 2013).

During winter, oospores reach morphological maturity when the oospore wall thickens, the nuclei fuse, an ooplast is formed, and the large lipid globules separate into smaller ones (Vercesi et al., 1999). Germination of morphologically mature oospores is prevented by vegetative dormancy (Galet, 1977; Rossi & Caffi, 2007), a process regulated by the environment, nutrient permeability, and endogenous inhibitors. When vegetative dormancy is broken, oospores are considered physiologically mature and are able to germinate under favourable environmental conditions (Rossi & Caffi, 2007). Oospore germination ends with the formation of a macrosporangium containing zoospores (Galbiati & Longhin, 1984). Oospore germination requires a minimum temperature of 12-13°C, optimum being between 20 and 24°C (Laviola et al., 1986; Gessler et al., 2011) and also requires wetting of the leaves by precipitation or water flux from the atmosphere (Arens, 1929; Rossi et al., 2008a; Rossi et al., 2013). Drought conditions lead to longer periods of vegetative dormancy and can damage oospores if prolonged (Arens, 1929; Gessler et al., 2011). Some oospores remain dormant, but viable, for an entire season or even several years (Kennelly et al., 2007; Caffi et al., 2011).

Oospores are the only source of inoculum for primary infections with *P. viticola* and were long thought to play a role only in the outbreak of the disease at the beginning of the grapevine growing season; the subsequent explosive growth of the disease was attributed to asexual reproduction and secondary infections (Blaeser & Weltzien, 1979; Lalancette et al., 1988; Lafon & Clerjeau, 1988).

The use of DNA microsatellites, which allow the identification of genotypes causing lesions on a single leaf, has shown that new genotypes of *P. viticola* continue the disease for most of the grape growing season, indicating that oospores continue to germinate throughout the season and that the primary inoculum not only triggers epidemics but also contributes to their progression (Kump et al., 1998; Gobbin et al., 2003; Rumbou and Gessler, 2004; Gobbin et al., 2005; Gobbin et al., 2006). Oospores constitute a very large and diverse inoculum reservoir, resulting in a pathogen population with high genotypic diversity. This diversity allows the pathogen to adapt to multiple microclimates and different host varieties (Rossi et al., 2013).

To detect the *P. viticola* oospores in grapevine leaf tissue, the following methods are mainly carried out: direct microscopic observations after cleaning and staining the leaves (Burruano, 2000; Díez-Navajas et al., 2007; Taylor, 2018) or after filtering the leaf tissue (Vercesi et al., 2000; Toffolatti et al., 2007); indirect estimation by a "floating grapevine leaf

disc" bioassay (Hill, 1998). In the latter method, leaf fragments containing oospores are immersed in water in the presence of uninfected floating leaf discs, so that zoospores originating from the oospores swim towards the stomata of the discs and cause infections, the severity of which is proportional to the number of oospores.

Other analyses are molecular biology methods, such as real-time PCR, which even allow the quantification of oospores (Ammour et al., 2020). With this method, the authors were able to find a relationship between oospores in plant residues and the dynamics of primary infection (Fedele et al, 2025), the method being able to be used for soil and plant residue management.

These methods have been used to study the processes of oospore formation and germination (Lehoczky, 1965; Vercesi et al., 2002; Vercesi et al., 2010), mating types (Wong et al., 2001; Taylor, 2018), fungicide resistance in oospore et al. (Bicong et al. 2001; Gisi et al., 2007 Toffolatti et al., 2011; 2018) and in the production of partially oospore-resistant grapevine varieties (Brown et al., 1999; Delbac et al., 2018). The methods have also been used to assess the effect of environmental conditions on oospore maturation and germination dynamics in vineyards (Hill, 2001; Pertot & Zulini, 2003; Toffolatti et al., 2004; Rossi et al., 2008a; Caffi et al., 2009).

No studies have been conducted to count oospores in leaf litter to assess the inoculum potential of *P. viticola* in a vineyard. One possible reason is that both microscopy and leaf disc bioassays are too expensive and time-consuming to assess the large numbers of oospores (up to several hundred per g of leaf residue) that may be present in leaf litter. Molecular methods that have been used to quantify inoculum in other pathosystems (Pavón et al., 2008; Li et al., 2010; Hussain et al., 2014; Van der Heyden et al., 2019) could be used for *P. viticola*.

Although polymerase chain reaction (PCR) and real-time PCR (Valsesia et al., 2005; Gindro et al., 2014; Ammour et al., 2020) have been developed for the detection and quantification of *P. viticola* in fresh grapevine leaves, no molecular methods have been developed for the detection and quantification of oospores.

For the chemical control of grapevine blight, the International Organization of Vine and Wine found that approximately 70,000 tons of fungicides are used in Europe on 3.8 million hectares of grapevine crop (Compant et al., 2013). The recent European Union provisions on the registration and sustainable use of pesticides have direct consequences on the number of active substances that will be available in the future. It is therefore of paramount importance to maintain the efficacy of active substances available on the market, with good ecotoxicological characteristics, in particular when used against polycyclic pathogens capable of causing serious damage. Among the chemical active substances, cymoxanil and mandipropamid have been used successfully for over 30 years against grapevine leaf spot because they are rapidly metabolized.

Regarding biological control, tests with over 1,764 microorganisms showed that only 5% could inhibit the production of oospores and 08% of sporangiophores. In greenhouses, the effectiveness of some commercial strains such as *Bacillus subtilis*, *Trichoderma harzianum* T 39, *Clorostachis rosea* plus *Trichoderma* has been shown to be effective in combating grapevine wilt (Vecchione et al. 2007). Some bacteria (*Streptomyces viridosporum*, *S. violatus*) and fungi (*T. harzianum, T. viride*) have been tested for the control of *P. viticola* (El-Sharkawi et al. 2018). Studies on biological control showed that 303 bacterial strains were isolated from grapevine leaves and about 12 strains showed antagonism towards phytopathogens. One of them, more interesting, was the strain from the genus *Ochrobactrum* sp., which shows antagonism towards *P. viticola* and other phytopathogens (Zang et al., 2020).

Studies have shown that there are some bacterial strains that increase plant resistance to phytopathogen attack, such as bacteria that stimulate plants like *Klebsiella planticola* and some

that can inhibit growth like *Streptomyces alni* and *Pseudomonas fluorescens*. The list also contains strains of *B. subtilis*, *B. circulans*, *Serratia macrescens*, *Pantotea* and *Acinetobacter* which can also act against other phytopathogens (Compant et al. 2013). *Lysobacter capsici* AZ 78 is considered a good biological control agent because it inhibits the growth of *P. viticola* and, at the same time, is resistant to abiotic stress and certain concentrations of copper products, having a copper oxidase that reduces the attack of the pathogen as effectively as copper products (Puoppolo et al., 2014). *L. capsici* AZ 78 is as effective as the reference fungicide Kocide© 2000 and does not affect non-target organisms and yeasts for wine fermentation and can be developed as a commercial biopesticide (Markellou et al., 2022).

The endophytic fungal strain *Acremonium byssoides,* which colonizes the leaves of *Vitis vinifera* has been found in many grapevine varieties in Italy without obvious symptoms (Burruano et al., 2008). Its filtrate and culture extracts inhibit the formation of mould sporangia. Another traditional fungus used in biological control is *T. harzianum*, which induces plant resistance to downy mildew (Perazzolli et al., 2012). At the same time, abiotic stress has been found to reduce the induced resistance produced by this fungus (Roatti et al, 2013). It seems that *Trichoderma* strains produce VOCs, volatile organic compounds with antifungal effects against *P. viticola* (Lazzarzzara et al., 2021).

A superior fungus, *Agaricus bisporus,* in aqueous mycelial suspension of 10, 15 and 20% inhibited and reduced by 80% the germination of sporangiospores (Garcia et al., 2018).

Epicoccum nigrum, a general saprophyte (Kortenkamp, 1997) is considered to be able to control the *P. viticola* pathogen. Another fungus, *Fusarium* sp. (Ghule & Sawati, 2017), very questionable for use in biological control, would have some strains that, through their chitinase, glucanase and protease enzymes, contribute to the distortion of *P. viticola* sporangiophores. Some studies (Rouset & Jaquin, 2003) have shown that oospore maturation is related to climatic factors and the severity of the primary infection.

MATERIALS AND METHODS

The paper presents an analysis of the results obtained in combating overwintering resistance forms of grapevine downy mildew oospores, following treatments with chemical and biological substances applied in autumn at the end of the vegetation period, after grape harvest and before leaf fall.

Testing was carried out in 5 treatment variants: Bordeaux mixture (V1), dimethomorph (V2); mandipropamid (V3); cymoxanil + copper hydroxide (V4); biological product-*B. subtilis* (V5) to which a control lot was added.

The effect of treatments with chemical and biological products in combating forms of resistance was analysed by the method of counting oospores under a microscope and the floating leaf disk method (Hill, 1998).

Microscopic determination. For microscopic determination of the number of oospore resistance forms, grapevine leaves from experimental and control variants were collected and analysed after treatments, in autumn before leaf fall. The leaves that presented a typical mosaic symptom were considered to be infected by the pathogen. For sample preservation, the leaves (60 leaves in total) were fixed in a solution containing 2.5% glutaraldehyde and 0.1 M phosphate buffer (pH 7.2) for 12 hours. The samples were then dehydrated in ethanol in increasing concentrations (20, 30, 40, 50, 60 and 70%), clarified with sodium hypochlorite (1%; 1:1) for 5 minutes (Lehoczky, 1956) and preserved in 70% ethanol at room temperature until further analysis under the electron microscope. Thirty leaves of the Italia variety, previously fixed and clarified, were selected for oospores identification. Three discs with a diameter of 12

mm were excised from each leaf, from the regions showing typical *P.viticola* lesions. The leaf discs were mounted on microscope slides in distilled water or anhydrous glycerol to visualize and count the oospores, in order to count the resistance forms existing on each variant.

Another method used to determine the number of resistance forms consisted of fragments of treated grapevine leaves of each variant finely ground in a glass Potter homogenizer, to which sterile distilled water was added. (Figure 1). The suspension was filtered twice through filter paper (pore size 100 and 45 μm) to remove leaf debris. The suspension drops containing oospores were analysed under a microscope and the resistance forms, oospores, were counted, by variants, using a Thomas cytometer, to observe the effect of the treatments (Figure 2).

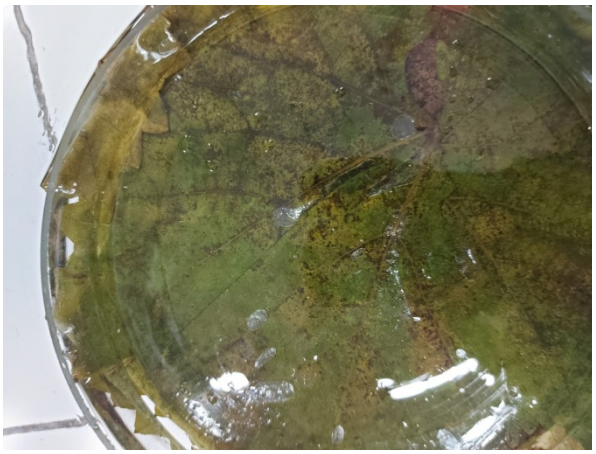

Figure 1 - Grapevine leaf with oospores resistant form of *P. viticola* (fixed).

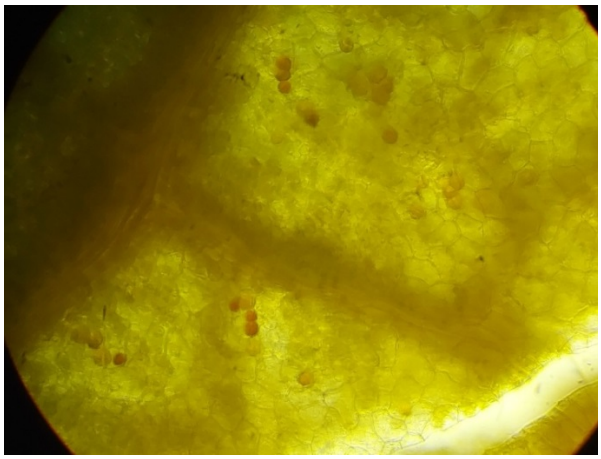

Figure 2 - Oospores resistance form of *P. viticola* visualized under a microscope.

Floating disk method (Hill 1998) - % attack per variant. From each treated variant, leaf fragments containing viable or dead oospores were immersed in water in Berzelius cylinders, in the presence of uninfected floating leaf discs, so that zoospores from the oospores swim to the stomata of the uninfected discs and cause an infection, the severity of which is proportional to the number of viable oospores. From isolated greenhouse-grown grapevine cuttings, uninfected with *P. viticola*, 15 leaf discs (30 mm in diameter) were cut for each variant taken in the experiment. Only fully developed leaves, from the fourth to the sixth node from the top, were selected, since younger leaves generally give more reliable results than the most mature, thickened ones. The effect of the chemical and biological treatment was determined on the variants by determining the degree of attack on the leaf discs.

RESULTS AND DISCUSSIONS

Following testing the effect of treatments for combating overwintering resistance forms of grapevine scab, by microscopic counting, the results are presented in the figure. The average number of oospores/cm² of vine leaf, obtained after choosing 3 microscopic fields for counting, varied depending on the product applied, and was as follows: control - 1560 oospores/cm²; V1 - 980 oospores/cm²; V2 - 440 oospores/cm²; V3 - 280/cm²; V4 - 180 oospores/cm²; V5 - 680 oospores/cm².

Variant 4 has the lowest oospores survival rate by using the mixture of cymoxanil and copper hydroxide and variant 1 (Bordeaux solution) has the highest oospores survival rate. It

can be seen that the effect of the treatment with the biological product based on *B. subtilis* (V5) is of interest in combating overwintering resistance forms of *P. viticola*.

Floating disk method (Hill, 1998) - Percentage of oospores per variant. After establishing the degree of attack, the following results were obtained: control - 99.33%, V1 - 81.66%, V2 - 55%, V3 - 58.33%, V4 - 13.33% and V5 - 69.33% (Figure 4). From the analysis of the results obtained by the floating disk method, it is found that the best efficacy of the treatment was also in variant 4, represented by cymoxanil + copper hydroxide treatment.

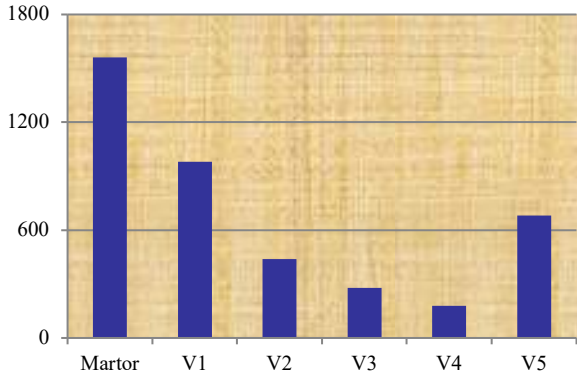

Figure 3. Oospore content determined by microscopic counting

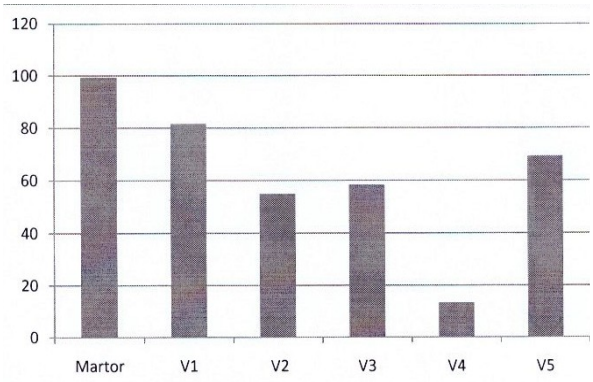

Figure 4. Degree of attack after treatments, determined by the floating disk method (Hill 1998)

Control, V1. Bordeaux mixture, V2. Dimethomorph, V3. Mandipropamide, V4. cymoxanil + copper sulfate, V5. *Bacillus. subtilis-based* biological product

CONCLUSIONS

The paper presents a synthesis of the results obtained in the chemical and biological control of overwintering oospores of grapevine downy mildew, using the counting method under the microscope and the floating disk method (Hill, 1998).

It was found that the most effective (88.5% effectiveness, 13.33% degree of attack) was the chemical variant with cymoxanil + copper hydroxide, both by counting and the floating disk methods.

Carrying out treatments on the vine in the fall, at the end of the growing season, during the period of oospore formation, to combat them, is a way to reduce the reserve of overwintering resistance forms of the vine borer and the number of treatments required during the growing season to achieve quality production.

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