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Peptide analogs of a *Trichoderma* peptaibol effectively control downy mildew in the vineyard

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Abstract

Plasmopara viticola, the agent of grapevine downy mildew, causes enormous economic damage and its control is primarily based on synthetic fungicides. The European Union (EU) policies promote reducing reliance on synthetic plant protection products. Biocontrol agents (BCA) such as *Trichoderma* constitute a resource for the development of biopesticides. *Trichoderma* species produce secondary metabolites such as peptaibols, whose poor water solubility hampers their practical use as agrochemicals. To identify new bio-inspired molecules effective against *P. viticola*, some water-soluble peptide analogs of the peptaibol trichogin were synthesized. In grapevine leaf disk assays, various peptides at 50 µM completely prevented *P. viticola* infection after zoosporangia inoculation. Microscopic observations carried out with one of the most effective peptides showed that it causes membrane lysis and cytoplasm granulation of both zoosporangia and zoospores. Among the effective peptides, 4r was selected for a two-year field trial experiment. In the vineyard, the peptide administered at 100 µM (equivalent to 129.3 g/ha) overall reduced significantly disease incidence and severity on both leaves and bunches, allowing protection levels similar to those obtained with a cupric fungicide. In the second-year trial, reduced dosages were also tested, and results indicated that even by reducing the peptide concentration by 50 or 75%, a significant decrease in the disease level was obtained at the end of the trial. The peptide did not show any phytotoxic effect. Previously, peptide 4r had been demonstrated to be active against other fungal pathogens, including the grapevine fungus *Botrytis cinerea*. Thus, this peptide may be a candidate for broad-spectrum fungicide whose biological properties deserve further investigation.

Introduction

Downy mildews cause substantial yield losses and quality deterioration in several crops and ornamental plants worldwide (Chang et al., 2013; Cohen et al., 2017; Salgado-Salazar et al., 2018a, b; Spring et al., 2018; Keinath and de Figueiredo Silva., 2022). These disease-causing agents are obligate biotrophic pathogens and rely entirely on the host to complete their life cycle (Spring et al., 2018). They belong to the kingdom Chromista, subphylum Oomycota, family Peronosporaceae (Thines, 2014). Downy mildew is mainly caused by oomycetes belonging to the genus *Peronospora,* counting about 400 species, but other pathogens are members of additional 18 genera, including *Pseudoperonospora*, *Bremia*, *Plasmopara*, *Hyaloperonospora,* and *Sclerospora* (Thines and Choi, 2016). Among phytopathogenic oomycetes, *Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & De Toni affects grape production in all the viticultural regions around the world and, without protection, causes over 50% losses under disease favorable conditions (Agrios, 2005; Gessler et al., 2011; Leroy et al., 2013).

The first report of *P. viticola* as the causal agent of downy mildew in grapevines was in 1876; it is endemic in North America, and arrived in Europe in 1878 (Gessler et al., 2011), then spreading worldwide (Fontaine et al*.,* 2021). *Vitis vinifera* varieties are highly susceptible to downy mildew. Though some resistant varieties have been released (Pedneault and Provost, 2016), the most traditional wine varieties remain highly susceptible (Pertot et al., 2017).

The primary infections occur in the spring and result from zoospores released by zoosporangia differentiated after germination of resting oospores, whereas the secondary infections depend on zoospores released from sporangia evading from infected tissues (Gobbin et al., 2005; Kennelly et al., 2007). Stomatal openings are essential for zoospores germination, infection (Müller-Thurgau, 1911; Gessler et al., 2011), and mycelium sporulation; thus, all vegetative tissues are susceptible even if ontogenic resistance occurs in berries (Kennelly et al., 2005).

Disease management relies on chemical control with repetitive use of traditional copper-based products and/or synthetic fungicides (Agreste, 2021; ISTAT, 2011). While these substances effectively decrease disease pressure, they threaten the health of vine growers and populations living near the vineyards and contaminate the environment (EUROSTAT, 2007; Merz et al., 2015). In addition, the pathogen may become resistant, especially to more recent selective fungicides (Gessler et al., 2011).

European Union (EU) regulations provide for the sustainable use of pesticides by promoting Integrated Pest Management (IPM, Directive 2009/128/EC). The EU adopted stricter criteria for the authorization of Plant Protection Products (Regulation EC 1107/2009), discontinuing many active substances or including them in a list of candidates for substitution (ec.europa.eu/food/plant/pesticides/approval_active_substances_en). Copper compounds, the main ingredients of fungicides allowed in organic viticulture, are also candidates for substitution, and their application must not exceed 28 kg of copper per hectare over seven years (regulation EC 2018/1981). More recently, environmental and safety concerns have been incorporated into the EU Farm to Fork strategy (European Commission, 2020) through the revision of legislation of the Sustainable Use of Pesticides action which contemplates a 50% reduction in using pesticides by 2030. In this context, identifying and developing alternative molecules, mainly of biological origin, for the control of downy mildew is a research challenge.

Natural compounds derived from plants, animals, or microorganisms may be a source of antifungal molecules for crop protection (Copping and Duke, 2007). As an example, *Trichoderma* spp. represent an extensive reservoir of secondary metabolites for controlling phytopathogens (Mayo-Prieto et al., 2019, Zeilinger et al., 2016). As natural secondary metabolites, antimicrobial peptides (AMPs) attracted attention as candidates for plant protection products and have inspired the design of new semi-synthetic analogs (Montesinos, 2007). Among AMPs, peptaibols produced by *Trichoderma* gained interest from the scientific community for their bioactivity (Marik et al., 2019).

Peptaibols are peptides of 8–20 residues with non-proteinogenic amino acids that can aggregate, affect cell membrane integrity (Szekeres et al., 2005, Milov et al., 2016, Afanasyeva et al., 2019), and trigger programmed cell death in phytopathogenic fungi, like *Fusarium oxysporum* and *Botrytis cinerea* (Shi et al., 2012, Zhao et al., 2018). Moreover, the well-characterized peptaibols alamethicin and trichokonin also showed the capacity to induce resistance in plants (Leitgeb et al., 2007; Kredics et al., 2013, Li et al., 2014).

The peptaibol trichogin GA IV has bactericidal activity and remarkable resistance to proteolysis, but poor water solubility (De Zotti et al., 2009). Our previous work produced water-soluble analogs of the short-length peptaibol trichogin GA IV from *Trichoderma longibrachiatum* by solid-phase synthesis (De Zotti et al., 2020). Some of these peptides have significant fungicidal activity at a concentration lower than 50 μM. Peptides with higher *in vitro* antifungal activity reduce disease symptoms produced by *B. cinerea* on grapevine and tomato leaves, and grape berries (De Zotti et al., 2020; Baccelli et al., 2022). Selected peptides protect barley and rice from *Pyricularia oryzae* infection (Sella et al., 2021). However, few studies addressed peptaibols *in vitro* and *in vivo* biocidal activity against oomycete plant pathogens (Lederer et al., 1992, Otto et al., 2016). In this study, we investigated the efficacy of trichogin GA IV analogs in preventing *P. viticola* infection of grapevine leaf disks and their biocidal activity against sporangia and zoospores. Finally, we present the results of a two-year field trial to assess the efficacy of one of the most promising peptides in protecting the vineyard from downy mildew.

Materials and Methods

Water-soluble analogs of trichogin. Trichogin and its water-soluble analogs were synthesized as previously reported (De Zotti et al., 2020). The peptide sequences used in this study are reported in Table 1 and, apart from the peptide K9r, they were previously described (De Zotti et al., 2020; Baccelli et al.,

2022). They are classified into full-length peptides, C-terminal modified analogs (carrying a C-terminal amide), and shorter analogs.

Plant material, *P. viticola* strain, propagation, and inoculum production. One to four-year-old potted plants of a white grapevine variety (*Vitis vinifera*, cv. Glera) grafted onto Kober 5BB rootstock were used for inoculum production and leaf disk assays (see below). One-year-old shoots of the dormant plants were pruned to 3-4 buds and, at different times from January to April of years 2017 to 2021, the plants were transferred into a climatic chamber (20-22 °C, 16 h photoperiod and about 75% RH). From about one month after bud opening, leaves were harvested as needed. Once the necessary leaves were collected, the plants were placed outdoors. About twenty plants were managed in total.

Sporangia of *P. viticola* were collected from infected grapevine leaves harvested at the beginning of June 2017 in a vineyard in the municipality of "Nervesa della Battaglia" in the Venetian region of Italy (45°49'23'' N, 12°12'21'' E.). The pathogen was maintained by weekly spraying a suspension of sporangia on the abaxial surface of detached fresh grapevine leaves. Leaves were arranged on moist towels in plastic trays and maintained at 22-25°C in the dark under humid conditions to allow infection and sporulation. After 6-9 days from leaf inoculation, sporangia were collected by washing with sterilized water. In absence of fresh leaves, sporangia vitality was preserved by storing air-dried sporulating leaves at -20 °C.

Inoculation experiments and peptide treatments. Grapevine leaves, from the 4th to the 6th from the shoot tip, were collected from different plants. Leaf disks of 1.7 cm diameter were excised by a cork borer and randomly distributed with the adaxial surface down on moistened sterile filter papers placed in 15 cm diameter Petri dishes (20 disks/plate).

Trichogin GA IV 1 mM stock solution was solubilized in 5% (v/v) of ethanol. Trichogin and its derivatives (Table 1) were dissolved in water at 50 µM, and about 0.1 ml of each peptide solution was sprayed on the surface of each of the 20 leaf disks with a 20 ml pump atomizer vial amber (Arco

Scientifica, Limena, Italy). After drying for 10 minutes in a laminar flow hood, the leaf disks were sprayed with sporangia suspension (about 0.75 ml of suspension per 20 disks) containing $4x10^5$ sporangia/ml (counted by a hemocytometer), and the plates were incubated in the dark at room temperature (22-23°C).

Different treatment sessions were carried out. Each session comprised one or more peptide treatments (one plate per treatment) and a control plate sprayed with water only. Twelve days post-inoculation (dpi), the disease incidence was calculated in each plate with the following formula: (the number of sporulating leaf disks/the total number of inoculated disks) x 100. Then, the effectiveness of each treatment was calculated with the following formula: [(disease incidence of the control plate - disease incidence of the treated plate)/ disease incidence of the control plates] x 100. Overall, two or three plates (replicates) were performed for the less active peptides and at least three or more replicates for the most active peptides.

To compare the effectiveness of peptides 4 and 4r in controlling downy mildew, we also assayed them at a concentration of 15, 20, and 30 µM. To establish the effectiveness of the peptides in comparison to a commercial fungicide, a tribasic copper sulfate fungicide (Tricopperland, ISAGRO s.p.a., Milano, Italy) was assayed against the pathogen. In a preliminary test, the fungicide administered at a field dosage (7.3 mM) of Cu metal provided complete sporulation inhibition (data not shown). To identify the Cu concentrations capable of giving effectiveness comparable to those of the peptides, the fungicide was assayed at a concentration of 50, 200, and 730 µM of copper metal. Data were statistically analyzed by applying the one-way ANOVA and Bonferroni-Holms test.

Microscopic observations. Aliquots of 200 µl of a water suspension containing $5x10^5$ sporangia were treated with peptide 4 at 50 µM, and, after 15, 30, and 60 min, sporangia were examined by optical microscope (Laborlux 12, Leitz). At least four optical fields with 50-100 sporangia each were analyzed, and sporangia with shape alterations were counted. At the same time points, untreated sporangia (negative control) were also examined and counted for morphological alterations.

A suspension containing $3x10^6$ sporangia/ml was incubated for 6 h in sterile deionized water to allow the release of zoospores (Islam et al., 2016). Then, peptide 4 was added, and the zoospores were examined after 15 and 30 minutes. At least four optical fields containing 40-80 zoospores were analyzed, and the lysed zoospores were counted. Untreated zoospores (negative control) were also analyzed. Each experiment on sporangia and zoospores was repeated three times. Data were statistically analyzed by applying a one-way ANOVA Tukey-Kramer test considering the treatment as a fixed effect and the experiment as a random effect.

Field trials. Field trials were performed in 2020 and 2021 on *V. vinifera* vineyards of Pinot noir and Moscato Bianco in Costigliole d'Asti (AT) (44°45'26,5"N 8°12'59,3"E), and in Vesime (AT) (44°37'04,7"N 8°12'38,8"E) municipalities of the Piedmont region (Italy), respectively. The plants, grafted onto Kober 5BB rootstock, were grown in a Guyot training system. Plants were spaced 0.8 m along the row and 4 m (Pinot noir) or 2.5 m (Moscato Bianco) between the rows. A randomized complete block design with four replicates was adopted, each parcel comprising 7 plants along the row. The treatments were performed in 2020 with a standard atomizer equipped with a conical nozzle, in 2021 with a motorized backpack sprayer, equipped with a five flat fan nozzle boom, approximately at a weekly time interval, from BBCH-stage 53 to 75 and from BBCH 15 to 78, in the 2020 and 2021 seasons, respectively.

In both years, the first and second treatments (Table 2) were carried out on the entire experimental fields with a commercial formulation of copper oxychloride (Zetaram Plus, Sipcam Italia s.p.a., Milano, Italy). Then, the successive treatments differed according to the experimental plans: control plots (C1) were treated with water, the peptide plots were treated with peptide 4r, and the fungicide plots were treated with the copper oxychloride fungicide. The peptide 4r was administered at 129.3 g/ha (equivalent to a 10^3 l of application volume with the peptide at the concentration of 100 µM; MW 1293) in 2020 and 129.3, 64.7, 32.3, 16.2 and 1.62 g/ha in 2021. The copper oxychloride fungicide was sprayed with 760

and 494 g/ha of pure metal in 2020 and 2021, respectively. The reduced copper dosage (494 g/ha) was aimed at reducing the metal pollution according to EU disposition (regulation EC 2018/1981) without losing efficacy (Cabús et al., 2017). In 2021, the commercial adjuvant Silwet L-77 AG (Momentive Performance Materials Inc., NY, United States) at 0.01% v/v was added as a surfactant to the peptide mixture to improve the uniformity of distribution and, therefore, additional control plots (C2) were treated with the adjuvant only. In both years, plots without any treatment were included in the experimental field (C0).

Sprays to control powdery mildew (*Erysiphe necator*) were carried out with sulfur and tetraconazole in 2020 and with fluxapyroxad, tetraconazole, metrafenone, cyflufenamid and boscalid in 2021. In both years, one treatment with acetamiprid was performed to control the leafhopper *Scaphoideus titanus*, the vector of the Flavescence dorée phytoplasma.

The grapevine downy mildew infection in each plot was evaluated on 100 leaves and 50 bunches. In 2020, leaf symptoms were recorded on five dates (May 26, June 11, 19, 30, and July 13, corresponding to the BBCH scale 57, 65, 69, 75 and 79, respectively), while symptoms on bunches were scored on July 7 (BBCH 79). In 2021, leaf symptoms were recorded on six dates (June 21 and 30, July 5, 10, and 20, corresponding to the BBCH scale 69, 73, 75, 77 and 79, respectively), and bunches were scored on August 5 (BBCH 81). Disease incidence was recorded as a percentage of infected leaves or bunches per total number of leaves or bunches. Disease severity was determined by visual inspection and classification of leaves and bunches according to the following percentage values of symptomatic area: 0% = healthy; 1.25% = 0 to 2.5%; 3.75% = 2.6 to 5%; 7.5% = 5.1 to 10%; 17.5% = 10.1 to 25%; 37.5% = 25.1 to 50%; 62.5% = 50.1 to 75%; 82.5% = 75.1 to 90% and 95% = 90.1 to 100%.

Disease incidence and severity data recorded in 2021 were also used to calculate areas under the disease progress curve (AUDPC). Data were statistically analyzed by applying a one-way ANOVA followed by the Tukey HSD test ($p<0.05$).

Results

The *in vitro* activity of the *Trichoderma* derived peptides against *P. viticola*. When assayed on leaf disks at 50 µM, trichogin GA IV and peptides 2, 3, 5, and 7 did not significantly reduce the number of sporulating disks compared with the untreated control. In contrast, peptides 1, 4, 6, and K9 were highly effective since they reduce downy mildew incidence by 80-90% (Fig. 1A). At this point further analyses with the expensive peptide 1 were abandoned while the peptides 4, 6 and K9 were also assayed in their rink version (r), in which the relatively expensive C-terminal -Lol moiety was replaced on Rink Amide resin by a -Leu-NH2 (leucine amide) residue. Results showed that the peptides 4r and K9r were as effective as their parental molecules in preventing *P. viticola* infection, while the peptide 6r was less effective (Fig. 1B). Previously (De Zotti et al., 2020), peptides 4c and 4c2, shorter and cheaper versions of peptides 4 and 4r, respectively, were demonstrated active against the fungus *B. cinerea*. By comparison, their activity was verified also against the oomycetes *P. viticola* and these short peptides appeared slightly less active than their parental peptides. The 4c1 peptide, i.e., a short version of peptide 4r, was relatively inactive (Fig. 1B). No phytotoxic effects were detected on leaves after treatment with any peptides (data not shown).

The effective peptide 4 and its cheaper analog 4r were also compared for their effectiveness in protecting the leaves from infection at doses lower than 50 µM (Fig. 2). At 30 µM, the peptides 4 and 4r prevented sporangia production to an extent not significantly different from the 50 µM doses ($p<0.05$). Their efficacy decreased by about 52% (peptide 4) or 77% (peptide 4r) at 20 µM. Thus, for both peptides, the half maximal inhibitory concentration (IC_{50}) value was comprised between 20 and 30 µM. To identify a copper concentration capable of giving an effectiveness comparable to those of the peptides, a copper fungicide was assayed. Compared to the untreated control, the copper fungicide, used at 730, 200 and 50 µM of copper reduced the disease incidence by 83.2%, 40.9%, and 14.3%, respectively (Fig. 2). Thus, a

similar level of protection (approximately 80% of disease incidence reduction) was obtained with 30 µM of peptide 4 and 4r and 730 µM of copper.

Microscopic observations. Microscopic observations were carried out on zoosporangia after treatment with peptide 4 at 50 µM. Compared to untreated sporangia (Fig. 3A), the treated ones lost their integrity and released cytoplasmic material after 15 minutes (Fig. 3B). This effect was more evident after 30 min (Fig. 3C and Table 3), and all sporangia exhibited abnormal morphology after 1 hour (not shown).

The same treatment performed on zoospores confirmed the detrimental effect of peptide 4. Compared to the untreated zoospores (Fig 3D), the membrane of most treated zoospores appeared completely disrupted after 15 min (Fig. 3E and Table 3).

Field trial experiments. Because of the possible industrial production as plant protection products, the peptide 4r was chosen for experimental trials carried out in the field in 2020 and 2021 aimed at protecting the vineyard from downy mildew. The peptide 4r was administered at 100 µM (129.3 g/ha) considering that the efficacy in the field is usually lower than that detected in the laboratory experiments.

In 2020, mild and frequent rain events throughout the experimental period and maximum temperatures below 30 °C until the second decade of June (Fig. 4A) were conditions suitable for downy mildew infection (Fig. 5A and 5B). Disease incidence increased from the first assessment date (11^{th}) of June to the end of the month (Fig. 5A). Both peptide and copper treatments decreased the disease incidence and severity. However, incidence values were not statistically different at every assessment date, and, at the last evaluation date, only copper was effective in reducing the incidence compared to the C1 control ($p<0.05$). The protective effect was remarkable considering the disease severity, which, at the last date, decreased by 53.5% and 57.5% in the treatments with the peptide and copper, respectively (Fig. 5B). Disease values on grape bunches mirrored those detected on the leaves. The effect of the peptide treatment on bunches was remarkable, with a 72.8% reduction in severity (Fig. 6).

In 2021, the infection started at the beginning of June (not shown), then the rain scarcity (Fig. 4B) delayed the restart of the infection until the end of the month (Fig. 5C and 5D). The effect of the peptide and copper treatments in protecting plants from downy mildew was remarkable, as highlighted by the significant reduction of both disease incidence and severity (Fig. 5C and 5D). At the last assessment date, compared with their corresponding controls (C1 and C2), copper and peptide treatments significantly reduced the disease incidence by 63.6% and 52.5%, respectively (Fig. 5C). Similarly, the two treatments decreased by 84.6% and 73.8%, respectively, the disease severity (Fig. 5D). The treatment with the adjuvant alone (C2), displayed disease values not significantly different from those of the C1 control sprayed with water (Fig. 5C and 5D). On bunches, both incidence and severity were markedly reduced (78.0% and 94.7%, respectively). These values were comparable to those detected with the copper treatment (Fig. 6A and 6B). In 2021, treatments with concentrations of peptide 4r lower than 129.3 g/ha were also included in the trial. To summarize the effect of the peptide dosage on disease incidence and severity, the AUDPC values were calculated (Table 4). Although a reduction of the protective effect was observed by decreasing the peptide concentration, the protection levels on both leaves and bunches obtained with the peptide at 64.7 g/ha were similar to those obtained with 129.3 g/ha of peptide, or with the copper fungicide. At 32.3 g/ha, the protection was comparable to those of the higher peptide doses, except for a significant increase in disease incidence on bunches. At 16.2 g/ha, the protection significantly decreased compared to the higher doses of the peptide, but moderate protection was still evident as the AUDPC values were significantly lower than those recorded on the C2 control plants (Table 4).

It is important to point out that no treatments gave toxicity symptoms on leaves or bunches.

Discussion

To identify new bio-inspired molecules capable of preventing downy mildew infection, we explored the capacity of trichogin-derived peptides to control the grapevine pathogen *P. viticola*. Trichogin GA

IV is a poorly amphipathic 11-residue peptaibol produced by *T. longibrachiatum* with a helix structure able to interact with microbial membranes and a remarkable resistance toward proteolytic degradation conferred by three α-aminoisobutyric acid (Aib) residues (Yamaguchi et al., 2003). However, trichogin has poor solubility in water and is inactive against fungi (De Zotti et al., 2009 and 2020; Sella et al., 2021). Similarly, here we report trichogin is inactive against the oomycete *P. viticola* since it does not prevent sporulation on grapevine leaf disks. Conversely, some Gly-to-Lys substitutions in the trichogin sequence provide cationic properties, higher solubility in water, and the ability to prevent *P. viticola* infections.

Four of the eight full-length peptides (1, 4, 6, and K9) administered at 50 µM effectively prevented the grapevine leaf infection, while the remaining four peptides (2, 3, 5, and 7) did not significantly reduce the infection rate. The number of Lys substitutions alone does not explain the difference in activity, as a single Lys is present in the effective peptides 6 and K9, and in the ineffective peptides 2, 3, and 5, and double Lys are present in the effective peptides 1 and 4, and also in the ineffective peptide 7 (Table 1).

The C-terminal amide modification of the active peptides 4, 6 and K9, and of the shorter version of peptide 4 reduces the cost of synthesis. The observation that the full-length 4r and K9r peptides, as well as the short versions 4c and 4c2, are active against *P. viticola* while the 6r peptide is only partially active and the short version 4c1 is not active, demonstrates that modification or shortening of the N-terminal part of the sequence may also influence the activity of the peptides. Thus, the cheaper peptides 4r, 4c, and 4c2, previously demonstrated active also against the fungus *B. cinerea* (De Zotti et al, 2020) are interesting for their excellent activity also against *P. viticola*.

A comparison of peptide activity against *B. cinerea* (De Zotti et al., 2020) and *P. viticola* (this work) as a function of the Lys number and position reveals some similarities and differences between the results on these two pathogens. Among the peptides with a single Lys substitution, peptides 2 (Lys^2) and 3 (Lys^5) are poorly or not effective against both *P. viticola* and *B. cinerea* (Table 1), while peptide K9 (Lys^9) is

active against both pathogens. Moreover, the peptides 5 (Lys^6) and 6 (Lys^5 substitution with an additional Aib in position 6) were effective against *B. cinerea* or *P. viticola*, respectively (Table 1). Among the peptides with double Lys substitutions, peptides 4 and 4r (Lys^5 and Lys^6) and peptide 7 (Lys^2 and Lys^6) behave similarly against both pathogens, being active and inactive, respectively, while peptide 1 (Lys^2 and Lys^5) is active only against *P. viticola* (Table 1). Thus, in some cases, the position of the Lys residues seems to trigger the selectivity of the peptide towards the two pathogens.

The relative position of the two Lys in the 3D structure of peptides 1, 4, and 7 does not help to explain their activity against *P. viticola*. Previously, the spatial vicinity between Lys residues was argued as being responsible for diminished activity against the necrotrophic fungus *B. cinerea* (De Zotti et al., 2020). That explanation helped to justify the ineffectiveness of peptide 1 and the poor activity of peptide 7 against *B. cinerea*. However, this behavior does not fit with *P. viticola*, as peptide 1 affects zoospore infection. Peptide attitude to self-assemble, forming pores in the pathogen cell membrane, and the membrane composition may play an essential role in the effectiveness of the peptides. For instance, in model liposomes the presence of cholesterol stabilizes transmembrane peptide self-assembly. In fact, it was previously reported that trichogin insertion in cholesterol-containing membranes is accompanied by self-aggregation of parallelly aligned transmembrane peptide molecules, while in cholesterol-lacking membranes, the peptides are monomolecularly distributed (Syryamina et al., 2012). The membrane of the oomycete *P. viticola* differs from that of fungi. Indeed, sterols are absent in the membrane of oomycetes, while ergosterol, the sterol found in fungal membranes, is responsible for fungal membrane fluidity and resistance to stress (Block, 1983; Wise et al., 2014). Thus, we assume that the difference in membrane lipidic composition between fungi and oomycetes may affect the activity of some peptides. Microscopic observations also point to a different interaction of peptides with the plasma membrane of fungi or oomycetes. After treatment with peptide 4, the protoplast of *B. cinerea* conidia shrinks and the membrane detaches from the cell wall (De Zotti et al., 2020), while sporangia and zoospores of *P. viticola*

lyse. Finally, since we assayed the peptide activity on *B. cinerea* spore germination *in vitro* and against *P. viticola* on leaf disks, possible interference with the grapevine leaf surface can affect the availability of some peptides to interact with *P. viticola* zoospores. Further investigation may address these topics.

The 4 and 4r peptides had previously been shown to be equally effective in protecting plant leaves from the fungi *B. cinerea* and *P. oryzae* (De Zotti et al., 2020; Sella et al., 2021), with a negligible impact on the leaf metabolism (Baccelli et al., 2022). Particularly, the peptide 4r did not induce reactive oxygen species (ROS) production in tomato or *Arabidopsis* leaves, whereas ROS production induced by peptide 4 did not result in a significant modulation of plant defense genes (Baccelli et al., 2022), corroborating the observation that trichogin analogs are not phytotoxic for plants.

In the grapevine leaf disk assay, peptide 4r has been confirmed as effective as peptide 4 also against *P. viticola*, and both provide a 100% reduction of the disease incidence when used at 50 μM. The cost of synthesis of the peptide 4r is lower than that of the parental peptide 4; for this reason, the peptide 4r was selected in the field experiments.

To establish a suitable amount of peptide to be used in the field trial, we compared different dosages of the peptide with those of a cupric fungicide in the leaf disk assay. A similar level of protection was obtained with about 39 mg/l of peptide 4r (30 μM) and 46 mg/l of copper (730 μM).

In field practice, the recommended copper concentration for vineyard protection is between 0.5 and 1 g/l (Cabús et al., 2017), which is higher than the concentration that proved effective in our leaf disk assay. However, these high values consider the low solubility and gradual release of copper ions in the water film wetting the leaf surface and the need to ensure the persistence of the active ingredient in the presence of rain wash-off events.

Considering that peptide 4r is soluble in water, and may also be subjected to rain wash-off, a dose higher than that effective in the leaf disk assay (i.e. 129.3 mg/l against 64.7 mg/l, respectively) was used in field experiments. In this sense, it should be pointed out that the dose of fungicides recommended for

plant treatments in the field is higher than the minimum inhibitory concentration determined *in vitro* (Andrieu et al., 2001).

The field trials conducted in 2020 and 2021 underwent different climatic conditions influencing the onset and course of infections. In 2020, the climatic conditions were more favorable for *P. viticola* infections, as highlighted by both disease incidence and severity values in the untreated control plots (C0). As expected, in both years on the first survey dates, the disease level detected on leaves of the C1 and C2 (C2, only present in 2021) control plants was lower than that recorded in the untreated plants (C0) because the two copper treatments carried out at the beginning of the season. Those treatments delayed the rise of downy mildew symptoms. Later, the differences diminished, and, at the end of the experiment, the disease levels in the C0, C1, and C2 plants were similar (Fig. 5).

In both field trials, peptide 4r significantly reduced the disease quantity on leaves and bunches compared with the corresponding controls (C1 or C2). The effectiveness and duration of protection were remarkable and comparable to those obtained with a cupric fungicide with the same administration frequency. An uneven distribution of the peptide on the vine canopy, possibly determined by the absence of adjuvants, may explain the slightly higher disease incidence obtained in 2020 by the peptide treatment compared to the cupric treatment.

In the second-year field trial, the results showed that even lower doses of the peptide could effectively contrast the disease. This encouraging result highlights that there is room for reducing the cost of the treatment while achieving an excellent protection level by reducing the dosage or improving the peptide formulation.

The trichogin analogs increase the availability of effective bio-inspired linear AMPs. So far, AMPs have been mainly tested to contain some widespread bacterial plant diseases (Cabrefiga et al., 2017; Baró et al., 2020; Mariz-Ponte et al., 2021; Mendes et al., 2021). The efficacy of the trichogin analogs against some important plant bacterial diseases is the goal of one of our current studies.

In conclusion, water solubility, persistence, absence of phytotoxicity, and excellent fungicidal activity make peptide 4r an interesting new molecule for controlling filamentous pathogens in viticulture. If this peptide meets the approval criteria of Regulation (EC) No. 1107/200, it will be necessary to consider the issues related to the production and development costs and effective formulations.

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Tables

Table 1. Sequences of the trichogin analogs and efficacy in preventing *Plasmopara viticola* infection and activity against *Botrytis cinerea* conidia. Modified residues are highlighted in bold.

Peptide ID	Sequences[a]	Efficacy[b]	
Full-length peptides		*P. viticola*[d]	*B. cinerea*[e]
Trichogin GA IV	nOct–Aib–Gly–Leu–Aib–Gly–Gly–Leu–Aib–Gly–Ile-Lol	-	-
1	nOct–Aib–**Lys**–Leu–Aib–**Lys**–Gly–Leu–Aib–Gly–Ile-Lol	+	-
2	nOct–Aib–**Lys**–Leu–Aib–Gly–Gly–Leu–Aib–Gly–Ile-Lol	-	-
3	nOct–Aib–Gly–Leu–Aib–**Lys**–Gly–Leu–Aib–Gly–Ile-Lol	-	-
4	nOct–Aib–Gly–Leu–Aib–**Lys**–**Lys**–Leu–Aib–Gly–Ile-Lol	+	+
5	nOct–Aib–Gly–Leu–Aib–Gly–**Lys**–Leu–Aib–Gly–Ile-Lol	-	+
6	nOct–Aib–Gly–Leu–Aib–**Lys**–**Aib**–Leu–Aib–Gly–Ile-Lol	+	-
7	nOct–Aib–**Lys**–Leu–Aib–Gly–**Lys**–Leu–Aib–Gly–Ile-Lol	-	-
K9	nOct–Aib–Gly–Leu–Aib–Gly–Gly–Leu–Aib–**Lys**–Ile-Lol	+	+
C-terminal modified analogs			
4r	nOct–Aib-Gly-Leu–Aib–**Lys**–**Lys**–Leu–Aib–Gly–Ile–Leu-NH_2	+	+
6r	nOct–Aib–Gly–Leu–Aib–**Lys**–**Aib**–Leu–Aib–Gly–Ile–Leu-NH_2	-	nd
K9r	nOct–Aib–Gly–Leu–Aib–Gly–Gly–Leu–Aib–**Lys**–Ile–Leu-NH_2	+	nd
Shorter analogs			
4c	nOct–Aib– **Lys**–**Lys**–Leu–Aib–Gly–Ile-Lol	+	+
4c1[c]	nOct–Aib–Gly–Leu–Aib–**Lys**–**Lys-** –Leu-NH_2	-	+
4c2[c]	nOct-Aib- **Lys**–**Lys**–Leu–Aib–Gly–Ile–Leu-NH_2	+	+

[a] nOct, n-octanoyl; Aib, α-aminoisobutyric acid; Lol, leucinol
[b] + high antimicrobial activity; – partial or no antimicrobial activity
[c] shorter analogs carrying a C-terminal amide
[d] data obtained in this work
[e] data obtained by *in vitro* assays on *B. cinerea* (De Zotti et al., 2020), *in vitro* activity of the K9 peptide has not been previously published.

543 **Table 2**. Treatments schedule and correspondence with the plant development stage.

2020		2021	
Date[a]	**Plant developmental stage (BBCH)**	**Date**[a]	**Plant developmental stage (BBCH)**
12-may	53	8-may	15
20-may	55	15-may	19
27-may	57	22-may	53
01-june	60	29-may	55
10-june	63	6-june	57
15-june	68	13-june	63
22-june	71	20-june	69
30-june	75	25-june	71
		30-june	73
		5-july	75
		11-july	77
		17-july	78

[a]In both years, at the first dates, the plots were sprayed only with the copper oxychloride fungicide, on the remaining dates, each plot was treated according to the experimental plan.

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546 **Table 3**. Percentage of collapsed zoosporangia and zoospores of *Plasmopara viticola* after the treatment
547 with peptide 4 at 50 µM.

	Collapsed zoosporangia (%) after 30 min	**Collapsed zoospores (%) after 15 min**
Control	6.2 ± 3.2 a	7.2 ± 1.96 a
Treated	82.4 ± 19.9 b	87.3 ± 5.15 b

Data are the mean ± SD of values obtained from 3 separated experiments. Each experiment consisted in the counting of 4 microscopic fields each containing at least 40 zoosporangia or zoospores. Different letters indicate statistically significant differences according to the Tukey-Kramer test ($P<0.05$).

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564 **Table 4**. Downy mildew area under the disease progress curve (AUDPC) values determined in the
565 2021 field trial. Different amounts of peptide 4r were compared with the cupric fungicide treatment and
566 three different controls (C0, C1, C2).

Treatment	Leaves		Bunches	
	Incidence AUDPC [a] (% days)	**Severity AUPDC [a] (% days)**	**Incidence AUDPC [b] (% days)**	**Severity AUPDC [b] (% days)**
Untreated control (C0)	1275 ± 106 a	355 ± 79 a	1491 ± 113 a	466 ± 156 a
Partially untreated control (C1)[c]	934 ± 110 ab	271 ± 55 ab	1225 ± 14 ab	413 ± 47 a
Adjuvant 0.01% (C2)	870 ± 153 bc	200 ± 26 bc	1148 ± 46 abc	379 ± 70 ab
Copper (494 g/ha)	152 ± 58 f	18 ± 7 e	175 ± 27 f	10 ± 5 d
Peptide 4r 129.3 g/ha	319 ± 96 def	63 ± 29 de	231 ± 35 f	20 ± 11 d
Peptide 4r 64.65 g/ha	377 ± 97 def	80 ± 22 de	315 ± 42ef	27 ± 17 d
Peptide 4r 32.3 g/ha	450 ± 62 def	119 ± 28 cde	441 ± 58 de	96 ± 47 cd
Peptide 4r 16.2 g/ha	577 ± 165 cde	135 ± 4 cd	511 ± 35 cde	156 ± 25 cd
Peptide 4r 1.62 g/ha	670 ± 193 bcd	161 ± 33 bcd	609.0 ± 47.8 bcd	219 ± 38 bc

[a] calculated on 100 leaves per plot sampled for 29 days (from June 21 to July 20). Means followed by the same letters in the same column are not significantly different by the Tukey's HSD test ($p<0.01$).

[b] calculated on 50 bunches per plot sampled for 28 days (from July 8 to August 5). Means followed by the same letters in the same column are not significantly different by the Tukey's HSD test ($p<0.01$).

[c] only the two initial treatments with the cupric fungicide were performed (see Table 2, this work).

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570 # Figure Captions

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572 **Fig. 1.** Efficacy of peptides in reducing *Plasmopara viticola* sporulation on grapevine leaf disks cv.
573 Glera. (**A**) full length peptides. (**B**) peptides 4, 6 and K9, their rink version (4r, 6r and K9r) and the short
574 versions of peptide 4 (4c, 4c1, and 4c2). The peptide treatments were carried out at 50 µM before
575 inoculation with *P. viticola* sporangia. Symptomatic disks present on each plate (20 disks/plate) were
576 recorded at 12 dpi and the percentage of disease incidence was measured. The effectiveness of the
577 treatment was calculated with the following formula:[(disease incidence of the control plate - disease
578 incidence of the treated plate)/disease incidence of the control plate] x 100. TRIC = Trichogin; number-
579 letter IDs correspond to peptides of Table 1. Bars indicate standard errors. Two or three replicate plates
580 were carried out for the less effective or the ineffective peptides 3, 5, 7, 4c1, and at least three replicate
581 plates for the other peptides. Data were statistically analyzed by ANOVA and Bonferroni Holm test: only
582 pairs relative to trichogin were simultaneously compared. ** indicates significant differences at $p < 0.01$.

583 **Fig. 2.** Efficacy of peptides 4, and 4r, or copper tribasic fungicide, administered at different
584 concentrations, in reducing *Plasmopara viticola* sporulation on grapevine leaf disks cv. Glera.
585 Symptomatic disks were recorded at 12 dpi and the percentage of disease incidence was measured. The
586 effectiveness of the treatment was calculated with the following formula: [(disease incidence of the
587 control plate - disease incidence of the treated plate)/disease incidence of the control plate] x 100. Bars
588 indicate standard errors. At least three replicate plates were performed for each treatment. Data were
589 statistically analyzed by ANOVA and Bonferroni Holm test by simultaneously comparing all pairs.
590 Different letters indicate significant differences at $p < 0.01$.

Fig. 3. *Plasmopara viticola* zoosporangia before **(A)** and after 15 **(B)** and 30 **(C)** minutes from the treatment with peptide 4 at 50 µM. Untreated empty zoosporangia and zoospores **(D)** and collapsed zoospores (**E)** detected 15 minutes after the treatment with peptide 4 at 50 µM.

Fig. 4. Rainfall and temperatures recorded during the field trials from May to July 2020 **(A)** and 2021 **(B)**, by weather stations located in the proximity of the experimental fields.

Fig. 5. Downy mildew incidence and severity on grapevine leaves determined in 2020 (**A** and **B**, respectively) and in 2021 (**C** and **D**, respectively). Each data point represents the mean±SE of values collected on 100 leaves for each plot. Untreated control (C0), partially untreated control (C1), adjuvant (C2, only in 2021), peptide 4r at 129.3 g/ha and cupric fungicide at 760 and 494 g/ha in 2020 and 2021, respectively. At each date, data were statistically analyzed by ANOVA and Tukey HSD tests. Different letters indicate significant differences at $p < 0.05$.

Fig. 6. Downy mildew incidence **(A)** and severity **(B)** on bunches at the end of the assessment period (July 07 in 2020 and August 05 in 2021). Data points represent the mean±SE of the values determined on 50 bunches for each plot. Untreated control (C0), partially untreated control (C1), adjuvant (C2, only in 2021), peptide 4r at 129.3 g/ha and cupric fungicide at 760 and 494 g/ha in 2020 and 2021, respectively. Statistically significant differences by the Tukey test (< 0.01) are represented by different letters.

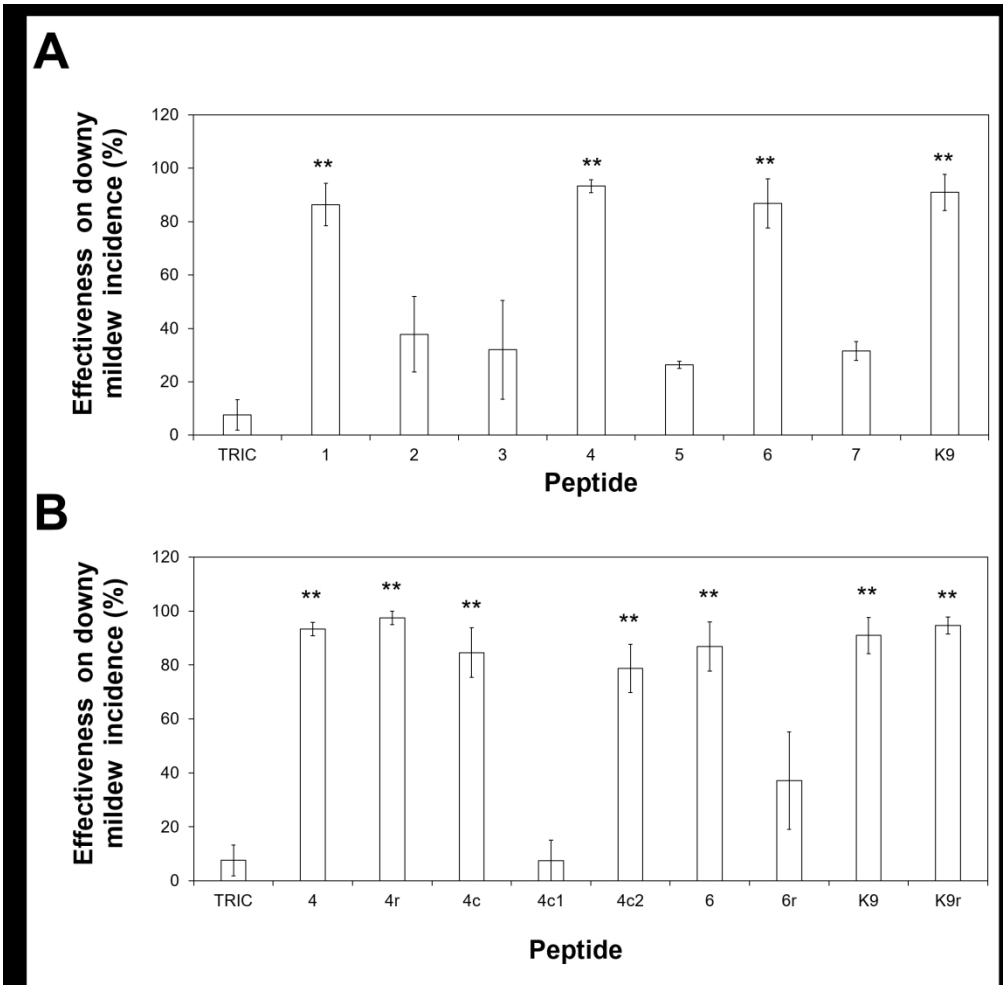

Fig. 1. Efficacy of peptides in reducing *Plasmopara viticola* sporulation on grapevine leaf disks cv. Glera. (A) full length peptides. (B) peptides 4, 6 and K9, their rink version (4r, 6r and K9r) and the short versions of peptide 4 (4c, 4c1, and 4c2). The peptide treatments were carried out at 50 µM before inoculation with *P. viticola* sporangia. Symptomatic disks present on each plate (20 disks/plate) were recorded at 12 dpi and the percentage of disease incidence was measured. The effectiveness of the treatment was calculated with the following formula:[(disease incidence of the control plate - disease incidence of the treated plate)/disease incidence of the control plate] x 100. TRIC = Trichogin; number-letter IDs correspond to peptides of Table 1. Bars indicate standard errors. Two or three replicate plates were carried out for the less effective or the ineffective peptides 3, 5, 7, 4c1, and at least three replicate plates for the other peptides. Data were statistically analyzed by ANOVA and Bonferroni Holm test: only pairs relative to trichogin were simultaneously compared. ** indicates significant differences at $p < 0.01$.

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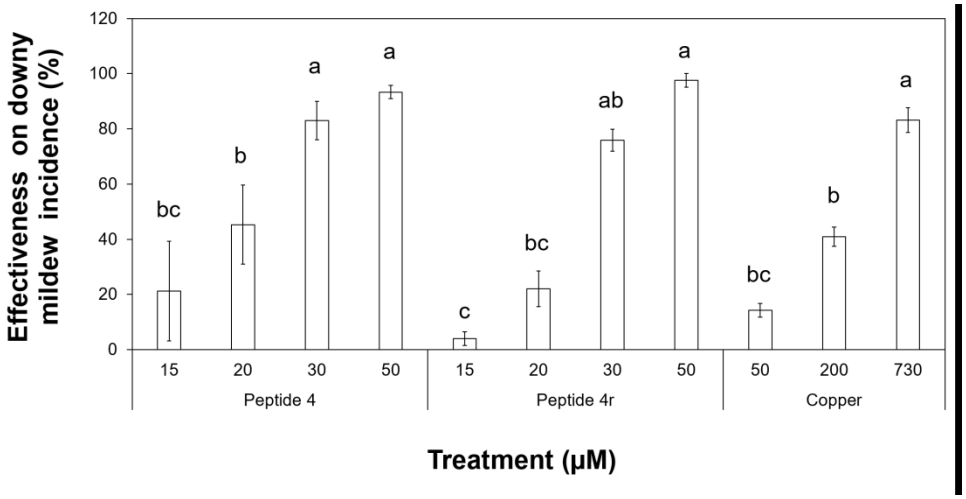

Fig. 2. Efficacy of peptides 4, and 4r, or copper tribasic fungicide, administered at different concentrations, in reducing *Plasmopara viticola* sporulation on grapevine leaf disks cv. Glera. Symptomatic disks were recorded at 12 dpi and the percentage of disease incidence was measured. The effectiveness of the treatment was calculated with the following formula: [(disease incidence of the control plate - disease incidence of the treated plate)/disease incidence of the control plate] x 100. Bars indicate standard errors. At least three replicate plates were performed for each treatment. Data were statistically analyzed by ANOVA and Bonferroni Holm test by simultaneously comparing all pairs. Different letters indicate significant differences at $p < 0.01$.

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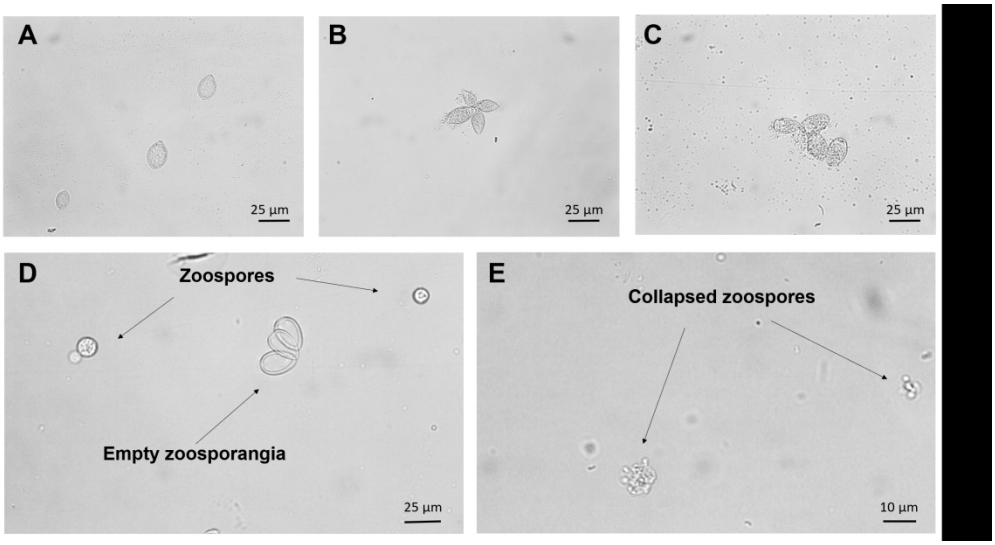

Fig. 3. *Plasmopara viticola* zoosporangia before (A) and after 15 (B) and 30 (C) minutes from the treatment with peptide 4 at 50 µM. Untreated empty zoosporangia and zoospores (D) and collapsed zoospores (E) detected 15 minutes after the treatment with peptide 4 at 50 µM.

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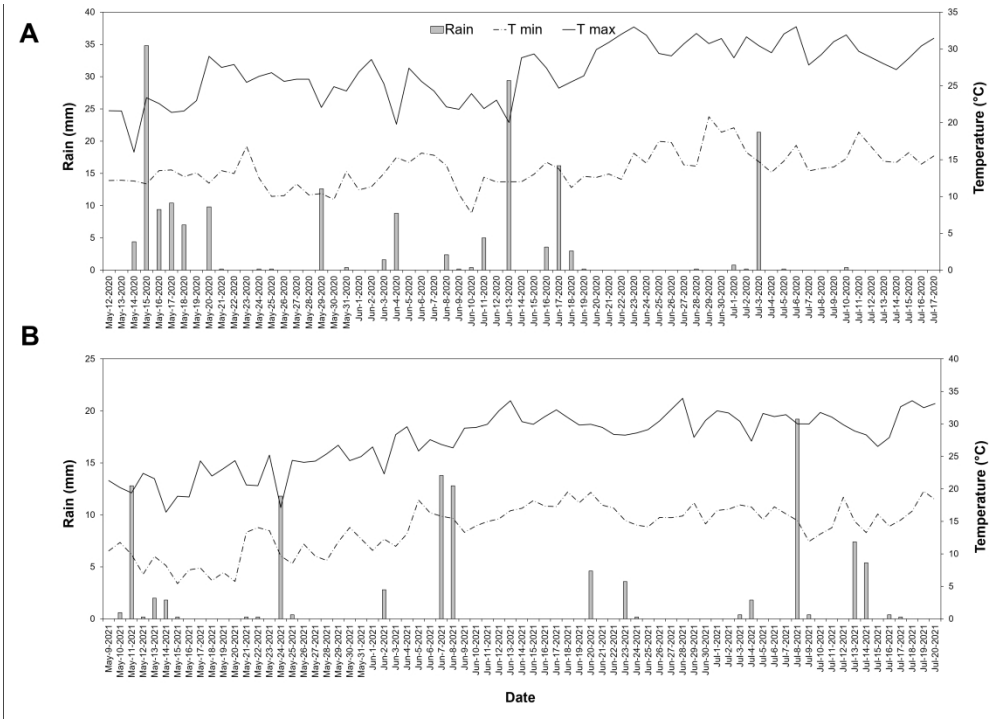

Fig. 4. Rainfall and temperatures recorded during the field trials from May to July 2020 (A) and 2021 (B), by weather stations located in the proximity of the experimental fields.

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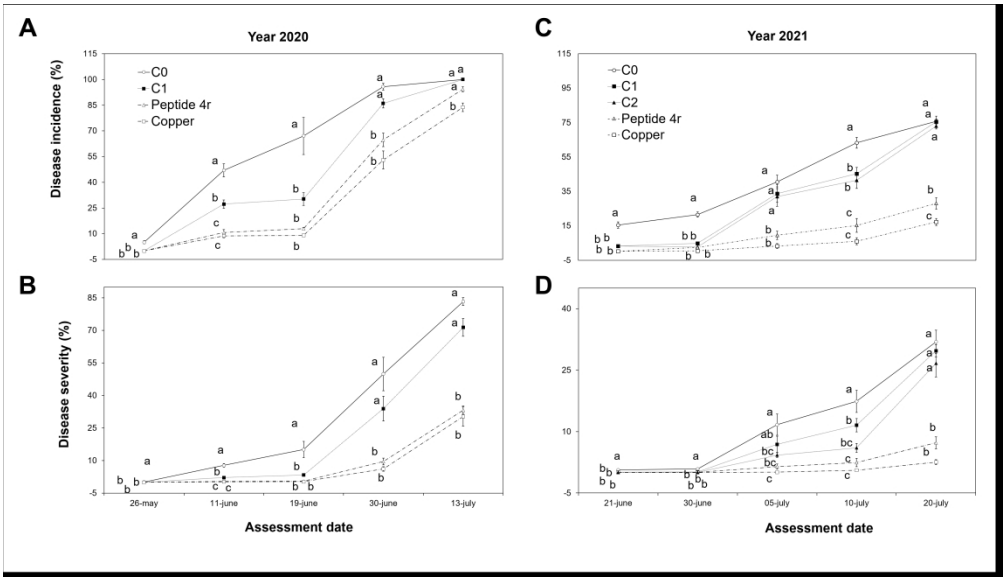

Fig. 5. Downy mildew incidence and severity on grapevine leaves determined in 2020 (A and B, respectively) and in 2021 (C and D, respectively). Each data point represents the mean±SE of values collected on 100 leaves for each plot. Untreated control (C0), partially untreated control (C1), adjuvant (C2, only in 2021), peptide 4r at 129.3 g/ha and cupric fungicide at 760 and 494 g/ha in 2020 and 2021, respectively. At each date, data were statistically analyzed by ANOVA and Tukey HSD tests. Different letters indicate significant differences at $p < 0.05$.

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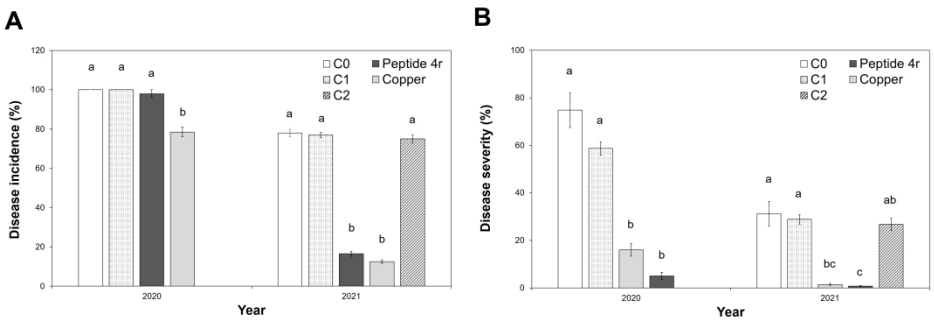

Fig. 6. Downy mildew incidence (A) and severity (B) on bunches at the end of the assessment period (July 07 in 2020 and August 05 in 2021). Data points represent the mean±SE of the values determined on 50 bunches for each plot. Untreated control (C0), partially untreated control (C1), adjuvant (C2, only in 2021), peptide 4r at 129.3 g/ha and cupric fungicide at 760 and 494 g/ha in 2020 and 2021, respectively. Statistically significant differences by the Tukey test (< 0.01) are represented by different letters.

746x274mm (150 x 150 DPI)