Research Article

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Additive and synergistic antifungal effects of copper and phenolic extracts from grape cane and apples

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Abstract

Background: Organic viticulture seeks sustainable alternatives for eco-toxic copper fungicides to control downy mildew caused by *Plasmopara viticola*. (Poly)phenol-rich extracts of agricultural byproducts are known to possess antifungal activity, but high production costs often limit their actual implementation.

Results: We developed and produced novel ligninsulfonate-based grape cane extract (GCE) formulations and an apple extract on a pilot plant scale, including a detailed (poly)phenol characterization by high-performance liquid chromatography photodiode array mass spectrometry (HPLC-PDA-MS). Our GCE formulations alone reduced downy mildew disease severity in greenhouse trials by 29%–69% in a dose-dependent manner, whereas a standard application of the copper-based agent alone reached ∼56%. When applied together, disease severity was diminished by 78%–92%, revealing a synergistic effect that depended on the mixture ratio. Combining GCE formulations with the apple extract, additive effects were found (80% disease severity reduction).

Conclusion: The studied plant extracts are proposed to both substitute for and synergistically reinforce copper fungicides in grapevine downy mildew control.

Supporting information may be found in the online version of this article.

Keywords: biocontrol; biopesticides; downy mildew; natural products; *Plasmopara viticola*; polyphenols; stilbenoids; viticulture

1 INTRODUCTION

In recent years, agriculture has strived towards sustainable practices with reduced applications of agrochemicals for the production of both conventional and organic produce. In the US, the Environmental Protection Agency has expedited the 'Conventional Reduced Risk Pesticide Program' to promote sustainable alternatives to high-risk pesticides.[1] In Europe, European Union (EU) authorities have elaborated the European Green Deal with the 'Farm-to-Fork' strategy, stipulating a halving of pesticide usage in crop protection by the year 2030 as well as reaching a 25% share of organic farmland; a more than twofold increase from a 12% share in 2020.[2,3]

In this regard, one of the most important crops in Europe is grapevine (*Vitis vinifera* L.) with 3.5 million hectares in 2020, accounting for 50% of the global grapevine cultivation area.[4] Because of its high susceptibility to various plant diseases, grapevine requires a comparably high number of applications per season. For instance, the pesticide treatment index for grapes has been among the highest of all crops for numerous years in Germany, being 17.06 in 2020, topped only by apples with a pesticide treatment index of 28.24. Noteworthy, 95% of the applications on grapevines were attributable to fungicides.[5]

Because on-going climate change will result in more frequent extreme precipitation events, increased temperatures and prolonged seasons, a higher incidence of fungal diseases such as powdery and downy mildew, as well as accelerated pathogen evolution assisting pathogen survival is anticipated.[6] As a result, an increased pesticide demand in vines might be expected.

Downy mildew is caused by the biotrophic oomycete *Plasmopara viticola* (Berk. & Curt.) Berl. & De Toni (1888) and is regarded as one of the most destructive diseases in viticulture. In conventional viticulture, downy mildew is controlled by the use of various synthetic fungicides as well as copper-based agents. Although the former are not authorized for use in organic viticulture in the EU, copper-based agents are currently tolerated in organic farming (maximum dose 4 kg/ha/a, authorization expires 2025) despite

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their unfavorable eco-toxicological implications because of the current lack of alternatives.[7,8]

For environmental reasons and the resulting regulatory incentives, research has concentrated on the reduction of copper usage by implementing more eco-friendly plant protection products and biological control agents. For instance, partial substitution with chitosans led to an increased efficacy of copper in high-pressure disease seasons.[9] Further currently studied examples of bio-based antifungal extracts or compounds are laminarin from the brown algae *Laminaria digitata*,[10] *Inula viscosa* leaf extracts containing the sesquiterpene tomentosin,[10] and apple-based extracts comprising phlorizin.[11] Furthermore, stilbenoid-rich extracts from grape cane, a viticultural byproduct that is usually either burned or composted in the field after winter pruning, has gained some attention.[12,13] Examples of active principles in these extracts are resveratrol and its methoxylated, oligomerized or glycosylated derivatives.[14] The first *in vitro* studies with such extracts have demonstrated the fungitoxic activity of both grape cane extracts (GCE) and isolated stilbenoids against *P. viticola*, emphasizing a potent antifungal capacity of the oligomers ε-viniferin, vitisin B and hopeaphenol.[14–16] Based on these results, Richard et al.[17] confirmed the inhibitory effect of stilbenoid-rich GCE both in greenhouse (~80% reduction of infected leaf area) and, to a lesser extent, vineyard studies (~60% reduction of infected leaf area) using a maltodextrin-based GCE formulation (Vineatrol®). Likewise, a recently self-made crude GCE exhibited an approximate 40% reduction in downy mildew severity, as observed over three years on three cultivars.[18]

In the current study, we investigate a novel ligninsulfonate-based GCE formulation as both a partial and total replacement for copper-based agents, studying their practically relevant, potentially synergistic anti-oomycete activity against *P. viticola* upon co-application with either a currently used copper-based agent or a separately produced phenolic-rich apple extract in laboratory and greenhouse trials. To allow comparison with previous studies, we also included the commercially available maltodextrin-based Vineatrol®.

2 MATERIALS AND METHODS

2.1 Chemicals

For antifungal studies, Cuprozin progress (based on copper hydroxide) served as comparative substance and was purchased from Certis (Hamburg, Germany). Vivando was obtained from BASF (Ludwigshafen, Germany). Vineatrol was purchased from Actichem (Montauban, France) and represents a plant extract obtained from Cabernet Sauvignon and Merlot vine shoots from the Bordeaux region, provided in a powdery formulation with maltodextrin.[19] Further details concerning chemicals used in this study may be found in the Supporting Information.

2.2 Production of phenolic-rich grape cane and apple extracts

Grape cane of *Vitis vinifera* L. cultivars Pinot Noir and Accent was pruned in Geisenheim (Germany) in December 2020, then chopped and dried in an open shed to a constant weight and residual moisture of ~ 6%–8% (w/w) at ambient temperature for six months to allow post-pruning stilbenoid accumulation.[19] Subsequently, the material was milled (Fritsch Pulverisette 19, Idar-Oberstein, Germany) to an approximate average particle size of 1 mm. Extraction was performed by continuously stirring the grape cane material in denatured aqueous ethanol (80% vol., solid to solvent ratio 1:5 w/v) for 4 h at room temperature without light exposure on a technical scale (30 kg canes per extraction batch) as described previously by Besrukow et al.[20] After solvent evaporation to a total solids concentration of ~15% (Heidolph Hei-VAP Industrial, Schwabach, Germany), the crude extract was supplemented with sodium ligninsulfonate (30/70 w/w ligninsulfonate to extract solids ratio) and then fed into a laboratory-scale spray dryer (Büchi B-290, Flawil, Switzerland) operating at 150 °C inlet air temperature, 80–82 °C outlet temperature, 8 mL/min feed flow rate, and 5500 L/h air flow rate.

The apple extract was manufactured on a pilot plant scale by loading an absorber resin column system (PI 200/750 VOE-AB-FOG, Kronlab, Schermbeck, Germany) that contained a styrene-divinylbenzene copolymer (SEPABEADS™ SP70 Mitsubishi Chemicals, Chiyoda, Japan) with apple juice (cv. Boskoop, ~100 kg juice obtained from ~140 kg apples), purging the column with water, and subsequently eluting the absorbed polyphenols with ethanol (96% v/v). After solvent evaporation under reduced pressure at 50 °C, the crude extract was freeze dried (Beta 2-8 LD plus, Martin Christ, Osterode, Germany) to obtain the powdery apple extract (~300 g) used in this study.

2.3 High-performance liquid chromatography analyses

High-performance liquid chromatography coupled with a diode array detector (HPLC-DAD) analyses of stilbenoids in GCE formulations were carried out as described by Besrukow et al.[20] with a few modifications, using 0.1% (v/v) aqueous formic acid and acetonitrile for gradient elution on a Hypersil Gold RP-C18 column at 40 °C. Likewise, phenolic compounds present in the apple extract were characterized by ultra-high-performance liquid chromatography coupled with a diode array detector (UHPLC-DAD) after separation on a Luna Omega RP-C18 column at 40 °C using 2% (v/v) aqueous formic acid and acetonitrile/formic acid (98:2 v/v) as eluents. Further methodological details are described in the Supporting Information.

2.4 Antifungal tests against *P. viticola*

2.4.1 *Laboratory experiments*

Initial assessments of the antifungal activity of GCE formulations on *P. viticola* were performed *in vitro* by observing zoospore release and by motility tests using a previously described method with modifications.[21] In brief, potted vines were inoculated with a virulent *P. viticola* isolate mixture obtained from the strain bank of the Department of Crop Protection. Infected leaves were collected, placed on a water agar (1%) plate with their abaxial bottom side and then removed to transfer sporangia from the leaves to the agar surface. Then, 50 µL-droplets of aqueous solutions of our GCE formulations from cv. Pinot Noir or Accent canes [5, 10 and 20 g formulation/L, respectively; ≙ 0.5, 1.0 and 2.0 g stilbenoids/L (Pinot Noir) or 0.4, 0.9 and 1.8 g stilbenoids/L (Accent), respectively] or Vineatrol (2.5, 5 and 10 g/L ≙ 0.9, 1.8 and 3.6 g stilbenoids/L) were applied on the plate. Likewise, 50 µL-droplets of Cuprozin progress in water (3.2 mL/L ≙ 0.8 g copper/L) and pure water were used as a negative and positive control, respectively. Subsequently, incubation was conducted for up to 4 h in the dark at 25 °C. Zoospore motility, estimated by visual assessment as normal, reduced or completely impaired, as well as zoospore release, assessed by numbering amounts of empty sporangia in 100 counted cells, were observed hourly under a light microscope. Each treatment was replicated three times. The mean value (%) ± standard deviation of the affected spores in each treatment was calculated.

2.4.2 *Greenhouse experiments*

Fifteen potted vines [cv. Riesling, BBCH 17–19, ~60 cm height, seven to nine leaves, pretreated with Vivando (0.4 g/L) against powdery mildew] per treatment were sprayed with 250 mL (equivalent to 155 L/ha) of the respective treatment solution using SprayLab application equipment (Schachtner, Ludwigsburg, Germany), a miniature replica of a pneumatic application commonly used in the vineyard. As treatment solutions, water (control), an aqueous solution containing 3.2 mL of Cuprozin progress per L (~500 mL Cuprozin progress/ha ≙ 124 g pure copper/ha) and our GCE formulations at 5, 10, and 20 g per L of aqueous spray solution were used. The same concentrations were used for the apple extract, whereas Vineatrol was applied at 2.5, 5 and 10 g/L. For interaction studies, Cuprozin progress solution was partly (25%, 50% or 75% v/v) replaced by the solution containing cv. Pinot Noir-based GCE formulations at 10, 20 and 30 g/L. The highest concentration of 30 g GCE formulation/L used in the substitution experiments was beyond the highest concentration in the experiments with the formulation alone, because we had initially expected the GCE formulation to be less effective than the copper-based agent being substituted. In parallel, the antifungal effect of a copper-free mixture containing the cv. Pinot Noir-based GCE formulations and apple extract at 10 g/L (ratio 50:50) was tested. Finally, aqueous solutions of the co-formulants sodium ligninsulfonate and maltodextrin were applied alone (without the aforementioned fungicidal components) at 10 g/L as present in the GCE formulations used at the highest dose (30 g/L). An overview of the treatments and concentrations applied in this series of experiments is listed in Supporting Information, Table S2. Water and Cuprozin progress were included in every experiment as the positive control and benchmark product, respectively. Treatments with the GCE formulations alone (Fig. 1(a–c)) were performed with a total of 30 plants per treatment, with two experiments of 15 plants conducted at two different times separated by ~4 weeks (May–June 2021 and July–August 2021). Studies with copper- and GCE-based mixtures (Figs 2(a–c) and 3) used 15 plants per treatment.

One day after treatment, the abaxial leaf surfaces of all leaves on each plant were inoculated with freshly prepared *P. viticola* (10^5 sporangia/mL), which had earlier been harvested from plants of *V. vinifera* cv. Riesling by washing sporangia from fresh sporulation leaf lesions using deionized water. Inoculated potted vines were covered with a dark plastic wrap for 24 h to create a favorable microclimate for infection and disease development. After randomization and incubation in the greenhouse for 7 days, plants were moistened with a hose with ~20 mL deionized water/plant and wrapped again overnight for 12 h to induce sporulation. Disease severity was evaluated visually by estimating the percentage of infected abaxial leaf surface according to the European and Mediterranean Plant Protection Organization (EPPO) scheme, including five of the youngest leaves after omission of the very youngest two apical leaves.[22] Disease incidence was calculated as the number of leaves with visible sporulation divided by the total number of leaves and was expressed as a percentage. Phytotoxicity was monitored according to EPPO guideline PP 1/135 (4).

2.5 Statistical analysis

Statistical processing of results was performed in Excel 2019 (Microsoft, Redmond, WA, USA). Results are expressed as means ± standard deviation unless stated otherwise. Significant differences of means were assessed by analysis of variance, applying the *post hoc* Tukey test.

Synergistic effects were calculated from the theoretically expected antifungal activity of the mixture $\left(a_{C_{\text{exp}}}\right)$ and the actually observed antifungal activity $\left(a_{C_{\text{obs}}}\right)$ according to the formula of Abbott[23] as follows. First, the theoretically expected antifungal activity of the mixture $\left(a_{C_{\text{exp}}}\right)$ was calculated according to Eqn 1:

$$a_{C_{\text{exp}}} = a_A + a_B - \left(\frac{a_A \times a_B}{100}\right) \quad (1)$$

where $a_{C_{\text{exp}}}$ is the theoretically expected activity, a_A is the observed activity of component *A* and a_B is the observed activity of component *B*.

The observed activities represent the leaf infestation level of the mixture in relation to that of the control ≙ $1 - \left(\frac{\text{Infestation level (after applying component A or B)}}{\text{Infestation level (after control (water) treatment)}}\right)$.

Subsequently, the synergy factor (SF) was calculated using Eqn 2:

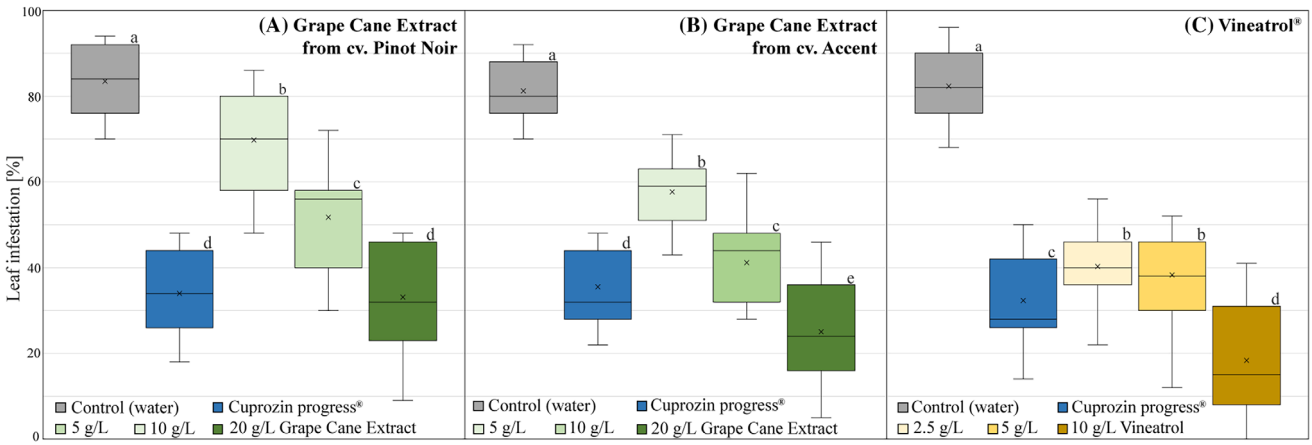

Figure 1. Downy mildew leaf infestation after treatment with grape cane extract (GCE) formulations from cv. Pinot Noir (a), cv. Accent (b) and Vineatrol (c), as well as with water (control) and Cuprozin progress as positive and negative controls, respectively. Infected leaf area declines with increased GCE concentration applied were comparable with copper treatment at 20 g/L GCE formulation (both cvs.) and 10 g/L for Vineatrol. Means (×) and medians (horizontal bar) are shown in the boxplots. Different letters indicate significant differences of means at $P < 0.05$.

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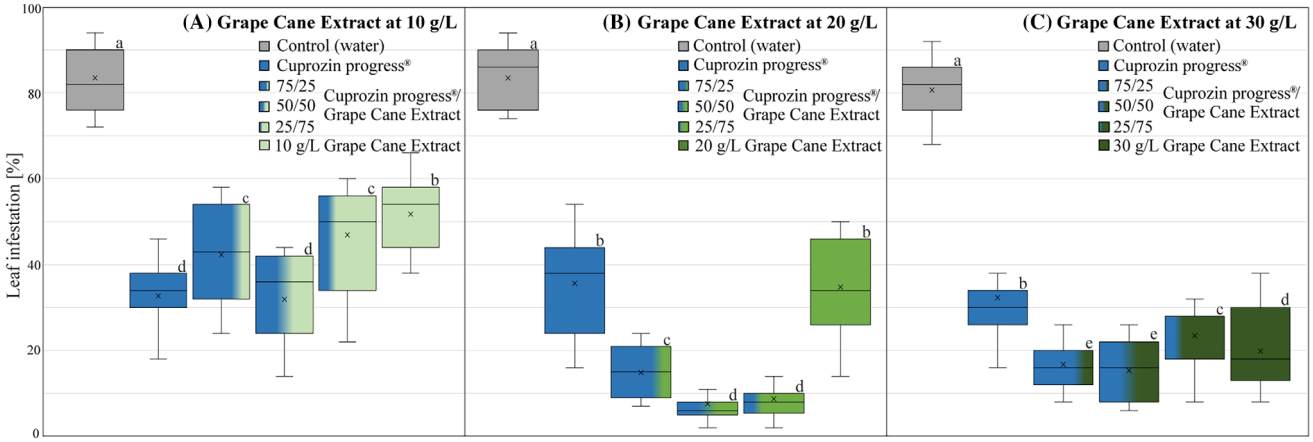

Figure 2. Downy mildew leaf infestation on potted vines after treatment with Cuprozin progress and grape cane extract (GCE) formulations (cv. Pinot Noir) at 10 g/L (a), 20 g/L (b) and 30 g/L (c), as well as mixtures with different ratios of these agents and water as a positive control. For mixtures of Cuprozin progress with GCE formulation at a potency of 20 g/L, inhibitory effects were significantly stronger than with singly applied substances, indicating a synergistic activity for both actives (Table 2). Means (×) and medians (horizontal bar) are shown in boxplots. Different letters indicate significant differences of means at $P < 0.05$.

$$SF = \frac{a_{C_{obs}}}{a_{C_{exp}}} \quad (2)$$

where $a_{C_{obs}}$ is the observed antifungal activity $\triangleq 1 - \left(\frac{\text{Infestation level (after applying mixture A+B)}}{\text{Infestation level (after control (water) treatment)}}\right)$ and $a_{C_{exp}}$ is the activity expected according to Eqn 1. The resulting SF indicated synergistic (SF > 1), additive (SF ≈ 1) or antagonistic (SF < 0.75) effects.[23]

3 RESULTS AND DISCUSSION

3.1 Laboratory zoospore release

In the etiology of grapevine downy mildew, the release of *P. viticola* zoospores from sporangia and the subsequent infection of receptive grapevine tissues through stomata play a key role in disease development.[24] Therefore, we investigated the inhibitory effect of stilbenoid-rich GCE formulations on zoospore liberation and motility, assessing their oomycidal potential at different concentrations *in vitro*.

As shown in Table 1, 97.3% of zoospores treated with water in control media were released and showed normal motility after 4 h of incubation. In parallel, zoospore release and motility were completely inhibited when the copper agent was used as a treatment. Our ligninsulfonate-based GCE formulation from cv. Pinot Noir generally reduced zoospore motility. Furthermore, it reduced zoospore release to 57.3% at 5 g extract per L treatment solution (0.5 g total stilbenoids/L), to 33.7% at 10 g/L (1.0 g stilbenoids/L) and to 2.0% at 20 g/L (2.0 g stilbenoids/L). For our GCE formulation from cv. Accent, low-dose treatments led to 43.0% (at 5 g/L, i.e., 0.4 g stilbenoids/L) and 11.7% (at 10 g/L, i.e., 0.9 g stilbenoids/L) of emptied sporangia, whereas zoospore release was completely inhibited at the highest concentration (20 g/L, i.e., 1.8 g stilbenoids/L). Similarly, completely impaired zoospore release was observed at the highest concentration of Vineatrol used (10 g/L, i.e., 3.6 g stilbenoids/L), whereas zoospore release levels at 5 and 2.5 g/L were 15.7% and 24.7%, respectively (Table 1). The total phenolic amounts were 12.4% and 10.7% for the GCE formulations from cvs. Pinot Noir and Accent, respectively, and 36.6% for Vineatrol. Further details of the composition of the extracts can be found in the Supporting Information. The application of the formulation drying aids, i.e., maltodextrin (92.2 ± 3.0%) and ligninsulfonate (91.3 ± 4.7%), showed no significant inhibitory effect on zoospore release and no impact on motility when compared with the water control (data not shown).

These results confirm observations made in previous studies, in which GCE formulations inhibited zoospore release dependent on the concentration used.[17] However, complete inhibition was reported to be achieved at generally lower concentrations (0.3 g/L, i.e., 0.1 g total stilbenoids/L) by Richard et al.[17] than in our study (10 g/L for Vineatrol, i.e., 3.6 g total stilbenoids/L and 20 g/L for the GCE from cv. Accent, i.e., 1.8 g total stilbenoids/L). We believe these differing findings might be explained as follows. First, to reproduce high disease pressure, sporangia density in our experiment was set at a higher level (1×10^5 sporangia/mL) than in Richard et al.[17] (0.51×10^5 sporangia/mL), necessitating higher concentrations of stilbenoids for zoospore release inhibition. Second, when dispersing our testing agents in water, we refrained from adding solvents like acetone, which Richard et al.[17] used to enhance the dissolution of stilbenoids. Consequently, levels of stilbenoids, especially poorly water-soluble oligo-stilbenoids, might have been lower in our study. In our view, the application of solvents like acetone would complicate actual applications in agricultural practice by winegrowers.

As shown in Table 1, Vineatrol was more effective at lower dosages than our ligninsulfonate-based GCE formulations, presumably because of its higher total stilbenoid levels. In both GCE formulations from cvs. Pinot Noir and Accent, stilbenoid levels were more than three times lower than in Vineatrol (Supporting Information, Table S1). As a result, more empty sporangia were observed after treatment with GCE formulations from cv. Pinot Noir and Accent (57.3% and 43.0%, respectively) than after treatment with Vineatrol (15.7%) at the same applied dosage (5 g/L).

Vineatrol is a plant extract obtained from Bordeaux region Cabernet Sauvignon and Merlot grape cane formulated with maltodextrin.[17,19] Both cultivars are known for their high stilbenoid contents of >5000 mg/kg grape cane.[25] Similarly, cv. Pinot Noir used for extract production in our study is also known to be rich in stilbenoids (~5700 mg/kg grape cane).[25] Grape cane extracts from cv. Accent have been shown before to contain relatively low total stilbenoid levels (1905 mg/kg grape cane) with,

Table 1. Release and motility of zoospores from sporangia of *Plasmopara viticola* 4 h after treatment with Cuprozin progress, grape cane extract formulations of cvs. Pinot Noir and Accent as well as Vineatrol at different concentrations

Parameter	Control (water)	Cuprozin progress†	Grape cane extract from cv. Pinot Noir‡			Grape cane extract from cv. Accent§			Vineatrol¶		
			5	10 (g/L)	20	5	10 (g/L)	20	2.5	5 (g/L)	10
Zoospore motility	Normal	None	Reduced	Reduced	None	Reduced	None	None	Reduced	None	None
Zoospore release = empty sporangia in %	$97.3^{a} \pm 1.2$	0^{f}	$57.3^{b} \pm 2.3$	$33.7^{c} \pm 4.5$	$2.0^{ef} \pm 3.5$	$43.0^{c} \pm 5.3$	$11.7^{e} \pm 6.5$	0^{f}	$24.7^{d} \pm 3.5$	$15.7^{e} \pm 2.1$	0^{f}

Note: Different letters indicate significant differences of means at $P < 0.05$.
Note: Means are displayed as % of three experiments ± standard deviation.
† ≙ 0.8 g pure copper/L.
‡ ≙ 0.5 g pure stilbenoids/L at 5 g/L extract usage.
§ ≙ 0.4 g pure stilbenoids/L at 5 g/L extract usage.
¶ ≙ 1.8 g pure stilbenoids/L at 5 g/L Vineatrol usage.

however, a comparably high oligomeric share, as reported previously by our working group.[20] This could explain differences in zoospore release between the extracts from cv. Pinot Noir and cv. Accent in this work. Despite lower total stilbenoid levels, inhibition of zoospore release was stronger at all concentrations tested when the extract from cv. Accent was used, which might confirm observations made by Schnee et al.[15] that oligo-stilbenoids are more bioactive than mono-stilbenoids like resveratrol. For instance, at 5 g/L, zoospore release was diminished to 43% with the extract from cv. Accent and to 57% with the Pinot Noir-based extract (Table 1).

These observations on zoospore release are consistent with previous work,[26] reporting that the primary mode of action for grape cane stilbenoids is the inhibition of zoospore liberation and motility, impeding the progression of fungal diseases. Noteworthy, different substrates are known to practically inhibit *P. viticola* zoospore release such as the quinone outside inhibitor famoxadone.[21]

3.2 Greenhouse experiments

3.2.1 Total substitution of copper by GCE formulations

Potted greenhouse grapevine plants treated with the ligninsulfonate-based GCE formulation from cv. Pinot Noir showed mean leaf infestation levels of 69%, 52% and 33% (16%, 38% and 60% disease reduction) at 5, 10 and 20 g/L (0.5, 1.0, and 2.0 g total stilbenoids/L), respectively, being significantly lower when compared with infestation levels for the control treatment with water only (84%). For the highest concentration used, infestation levels were found to be comparable with those noted for the copper-based agent (34% leaf infestation, i.e., 59% disease reduction) (Fig. 1(a)). Similarly, leaf infestation was reduced by applying the extract from cv. Accent to 58%, 41% and 25% (29%, 49%, and 69% disease reduction) at the aforementioned concentrations (Fig. 1(b)).

For Vineatrol, the infected leaf surface areas were 40%, 38% and 18% (51%, 53%, and 77% disease reduction) at 2.5, 5 and 10 g/L (0.9, 1.8 and 3.6 g total stilbenoids/L), respectively (Fig. 1(c)). Co-formulants maltodextrin (88%) and ligninsulfonate (77%) alone showed no significant inhibition of leaf infestation (data not shown).

In all experiments, disease severity on leaves was between 80% and 90% when treated with water, and between 32% and 35% when treated with the copper agent. Disease incidence was at 100% for all treatments because of the high infestation rate (10^5 sporangia/mL). No apparent phytotoxic effects were observed.

In agreement with our *in vitro* studies, both of our ligninsulfonate-based GCE formulations showed a concentration-dependent inhibition of downy mildew leaf infestation with a protection level comparable with the copper agent customarily used in organic viticulture. Disease reduction levels of 51%–77% observed in our study were slightly lower than those found in the literature[17] reporting protection levels of ~80% for Vineatrol at 5 g/L (53% and 77% at 5 and 10 g Vineatrol/L in our experiment, respectively). As mentioned above, we did not use an organic solvent like acetone to improve the dissolution of stilbenoids of Vineatrol. Future optimization of the GCE formulation for improved solubility or dispersibility warrants further efforts. A further reason for the aforementioned comparably low

disease reduction levels in our study might be that we exposed the potted vines to a higher sporangia density used for inoculation of vines, resulting in rather high infestation levels (80% leaf infestation in our control *versus* 60% in the control of Richard et al.),[17] hence, necessitating higher agent concentrations for similar plant protection.

Another factor accounting for the variability of antifungal efficacy might lie in the different origin of the GCE from different cultivars, ultimately relating to genotypic variations in stilbenoid profiles. Earlier studies have reported a rather similar antifungal activity (in *in vitro* bioassays) for GCE from different cultivars, which, however, had rather similar profiles of stilbenoids with similar degrees of oligo- or polymerization.[15] Therefore, we investigated the GCE of two different cultivars with distinct profiles: (i) cv. Pinot Noir with common shares of oligomeric stilbenoids (53%) and (ii) cv. Accent with higher relative shares of oligomeric stilbenoids like ε-viniferin (69%; Supporting Information, Table S1). Furthermore, plants of cv. Accent are well known for their high level of resistance to *P. viticola*,[27] possibly being related to their increased content of particularly antifungal oligomeric stilbenoids. In addition to higher relative shares of oligomeric stilbenoids, however, the extracts of cv. Accent in our study simultaneously had slightly lower total stilbenoid contents (8.9/100 g) compared with cv. Pinot Noir (10.3/100 g); however, both extracts exhibited very similar antifungal activity against *P. viticola* in our greenhouse experiments (Fig. 1(a,b)). The presence of higher amounts of oligo-stilbenoids might have compensated for the overall lower total stilbenoid levels in the extracts of cv. Accent.

3.2.2 *Partial substitution of copper by GCE formulations (cv. Pinot Noir only)*

In our experimental series striving to characterize potential interactions of copper hydroxide with our GCE formulations, our treatments of potted vines with the cv. Pinot Noir-based extract alone showed mean leaf infestation levels of 52% (38% disease reduction), 35% (58% disease reduction) and 20% (75% disease reduction) when applied at 10, 20 and 30 g/L, respectively, confirming the results described above. Likewise, application of Cuprozin progress alone led to infestation levels between 32% and 36% (57%–61% disease reduction). Control remained at ~81%–84% leaf infestation (100% disease severity in this experiment) (Fig. 2). Disease incidence was 100% for all treatments. No phytotoxic effects were observed.

Mixtures of Cuprozin progress substituted with 25%, 50% and 75% GCE formulation at the lowest potency (10 g/L), showed mean leaf infestation levels of 42% (49% disease reduction), 32% (62% disease reduction) and 47% (43% disease reduction), respectively (Fig. 2(a)). Here, substitution of copper by the plant extract resulted in similar or only marginally lower disease reduction rates (43%–62%) compared with treatment with copper alone (57%–61%). Substitution of the potent copper agent with the GCE formulation was expected to perform even worse because of the relatively low antifungal capacity of individual GCE formulation at 10 g/L (38% disease reduction).

When the GCE formulation was used at a higher potency of 20 g/L, the respective infestation levels of the copper–GCE formulation mixtures were substantially improved at all substitution levels (25%, 50%, 75% copper substitution); that is, to 15% (82% disease reduction), 8% (91% disease reduction) and 9% (90% disease reduction), respectively (Fig. 2(b)). All combinations yielded significantly better disease reduction levels than the respective agents alone, indicating a synergistic effect as seen in SF values > 1.0 (Table 2). Herein, disease protection was amplified even further by up to 30% when compared with Cuprozin progress or GCE formulation alone (91% *versus* ~60% disease reduction over control).

In parallel, at the highest tested potency of 30 g/L, copper substitution by GCE formulation at 25%, 50%, and 75% led to infestation levels of 17% (79% disease reduction), 15% (81% disease reduction) and 24% (71% disease reduction), respectively (Fig. 2(c)). Again, mixtures led to tendentially lower infestation levels when compared with single applications. At this potency, the respective antifungal activities were found to be additive rather than synergistic, with SF values around 0.9 (Table 2). This might be explained by the fact that the GCE formulation alone at 30 g/L already exhibited a comparably high efficacy (75% disease reduction), leading to high denominator (C_{exp}) values in the formula mentioned above (Eqn. 2) and, thus, relatively low SF values (<1). Nevertheless, both GCE formulation treatments at 30 g/L and mixtures of this agent with the copper agent led to tendentially higher disease reductions than the individual copper treatment.

In general, copper-based fungicides act as protectants on plant surfaces by inhibiting penetration of a pathogen into the plant and its development therein. Copper as a contact fungicide acts directly on the pathogen via various biochemical routes, e.g., by forming complexes with enzymes causing their inactivation and finally disrupting the general metabolic activities of the fungus.[28] Successful containment of pathogen proliferation is dependent upon the pathogen infestation rate, on the one hand, and the available active compound, on the other hand. It might be hypothesized, with regard to the findings in our study, that combined treatments of GCE formulations with copper-based agents lead to a higher availability of active copper for contact with its target site in *P. viticola* cells. Although different mechanisms are known to evocate such increased agent availability, e.g., perturbation of fungal cell membranes, disturbance of intracellular ion homeostasis or decreased efflux activity,[29] it is conceivable that a potential involvement of stilbenoids might be the perturbation of *P. viticola* cell membranes as reported previously.[15] This might have contributed to more efficient copper ion penetration into the fungal cells, possibly contributing to the enhanced protection levels observed in our study. In addition, a direct antifungal effect of stilbenoids, particularly against zoospore motility and germination, was evident in earlier studies and the findings herein. Thus, GCE might exert a dual mode of action against *P. viticola* pathogenesis when applied with a copper-based agent: (i) the direct inhibition of zoospore mobility and germination on leaves of potted vines; and (ii) an indirect action mediated by facilitating an effective introgression of copper into fungal cells through membrane perturbation. A similar mechanism has been recently suggested for the synergistic effect of copper nanoparticles with the synthetic fungicide thiophanate-methyl, investigated on *Botrytis cinerea*.[30]

In addition, stilbenoids have been reported to function as elicitors of plant defense reactions,[27] a third mechanism that might also have contributed to the synergistic effects observed in our study. Further systematic study is needed to scrutinize these hypotheses.

3.2.3 *Total substitution of copper by a mixture of extracts from apple and grape cane*

Apple extracts applied alone at 5, 10 and 20 g/L on potted vines yielded leaf infestation levels of 35% (55% disease reduction),

Table 2. Synergy factors as calculated from observed leaf infestations, observed antifungal activities and expected antifungal activities as derived from the substitution experiment (Fig. 2)

	Observed leaf infestation (%)			Observed antifungal activity (%) (C_{obs})[†]			Expected antifungal activity (%) C_{exp}[‡]			Synergy factor (C_{obs}/C_{exp})		
	As observed in the substitution experiments (Fig. 2) when using the GCE formulation alone at a concentration (g/L) of											
Treatment	10	20	30	10	20	30	10	20	30	10	20	30
Control (water)	84	84	81	—	—	—	—	—	—	—	—	—
Cuprozin progress alone[§]	33	36	32	61	57	60	—	—	—	—	—	—
GCE formulation alone	52	35	20	38	58	75	—	—	—	—	—	—
Cuprozin progress/GCE formulation (75/25)	42	15	17	49	82	79	76	82	90	0.65	1.00	0.88
Cuprozin progress/GCE formulation (50/50)	32	8	15	62	91	81	76	82	90	0.82	1.11	0.90
Cuprozin progress/GCE formulation (25/75)	47	9	24	43	90	71	76	82	90	0.58	1.09	0.79

Note: Synergy factors (SF > 1) indicate a synergistic effect of Cuprozin progress and the grape cane extract (GCE) formulation (cv. Pinot Noir).
[†] [1−(leaf infestation (treatment)/leaf infestation (control))] × 100.
[‡] Calculated according to Eqn 1.
[§] Copper-dose remained constant in all experiments.

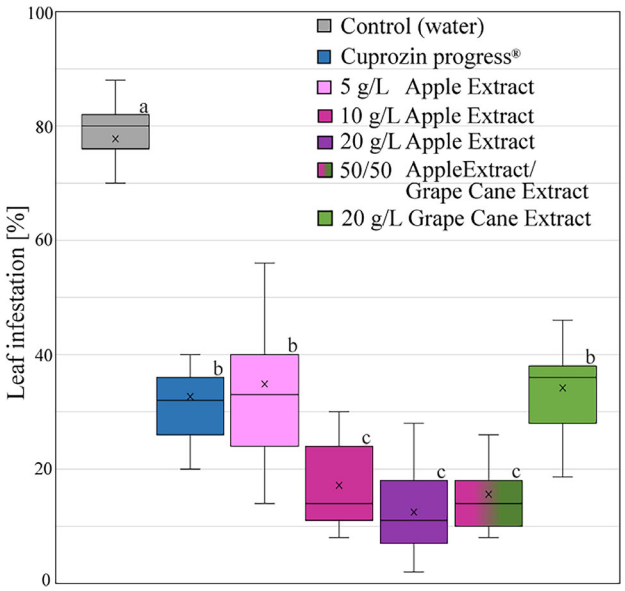

Figure 3. Downy mildew leaf infestation after treatment with apple extract at 5, 10 and 20 g/L and grape cane extract (GCE) formulation from cv. Pinot Noir at 20 g/L, as well as a combination of these (50/50 of 10 g/L apple extract and 20 g/L GCE formulation), and water and Cuprozin progress as positive and negative controls, respectively. Leaf infestation declined with increased apple extract concentration applied. At an apple extract dosage of 5 g/L, efficacy was comparable with that of the copper treatment. A combination of the apple extract and GCE formulation showed an additive antifungal effect. Means (×) and medians (–) are shown in boxplots. Different letters indicate significant differences of means at $P < 0.05$.

17% (78% disease reduction) and 13% (84% disease reduction), respectively, compared with 78% leaf infestation with water treatment serving as a control (Fig. 3). Although no data are available on the impact of apple extracts on *P. viticola*, an earlier *in vitro* study of Oleszek et al.[11] reported significant inhibition of *Botrytis* sp. growth when exposed to a crude apple pomace extract, attributed to the antifungal capacity of quercetin and phloretin derivates therein. In light of the large amounts of these flavonoids in our apple extract (Supporting Information, Fig. S1, combined quercetin and phloretin derivates = 65 mg/g extract) and high disease protection levels at comparably low apple extract concentrations applied (Fig. 3; 55% disease reduction at 5 g/L apple extract), these compounds might act as potential antifungal principles.

Equal amounts (50:50, v/v) of grape cane (20 g/L) and apple (10 g/L) extracts led to an infestation level of 16% (80% disease reduction). This yielded a SF value of 0.9, indicating an additive effect of these extracts. Sung et al.[31] suggested that chlorogenic acid, the main phenolic component in our apple extract (211 mg/g extract), exhibits antifungal effects by disrupting the fungal cell membrane of the studied *Candida* sp. The same mechanism might explain the improved protection against *P. viticola* when combining grape cane with apple extracts, whose chlorogenic acid might have helped to enhance the penetration of further active components (e.g., stilbenoids, quercetins, phloretins) into the pathogen. Furthermore, flavan-3-ols with antifungal properties (e.g., catechin, procyanidin B1)[32] being present in both the apple and the GCE formulations might have also bolstered disease protection in our study. A full decipherment of grape cane polyphenols, including their contribution in disease protection, is however lacking to date and requires further systematic studies.

In this context it is also important to scrutinize a potential plant-protecting impact of the adjuvant ligninsulfonate, which has been reported to possess antifungal properties against different (human-pathogenic) *Candida* species.[33] We did not find any antifungal effect on the release and motility of *P. viticola* zoospores on agar plates or on infected potted vine leaves in greenhouse studies. This might indicate a genus specific activity of ligninsulfonates.

4 CONCLUSION

This study confirmed that both stilbenoid-rich GCE formulations and flavonoid-rich apple extracts can act as antifungal agents to partially or fully replace copper-based agents for downy mildew control in organic viticulture. For the first time, GCE formulations were shown to boost the antifungal capacity of copper fungicides in a synergistic way when used at adequate concentrations. These findings might help us achieve environmentally important targets

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regarding the reduction of pesticide usage as well as the recycling of waste material in the future. The implementation of such extracts beyond organic farming into conventional viticulture as well as further pathosystems (e.g., apple/*Venturia inaequalis*, potato/*Phytophthora infestans*, hop/*Pseudoperonospora humuli*) should be experimentally explored because of the important ecologic and economic potential. We emphasize that our insights relate to *in vitro* and greenhouse trials only. Large-scale field trials are needed to corroborate or disprove our findings under open field conditions.

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DATA AVAILABILITY STATEMENT

The data that support the findings can be made available upon request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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