UNIVERSITÁ POLITECNICA DELLE MARCHE
Department of Agricultural, Food, and Environmental Sciences

PhD School in agricultural, food and environmental sciences
(XV cycle)
Disciplinary sector: *AGR/12 – Plant pathology*

Low environmental impact strategies for the control of *Botrytis cinerea* and *Plasmopara viticola* of grapes

PhD student:
Dr. Andrea Servili

Academic tutor:
Prof. Gianfranco Romanazzi

Co-tutor:
Dr. Philip Elmer

Coordinator:
Prof. Bruno Mezzetti

INDEX

ABSTRACT

The aim of the thesis was to evaluate low environmental impact strategies against *Botrytis cinerea* and *Plasmopara viticola*. After harvest table grapes easily undergoes to fungal spoilage, mainly caused by *B. cinerea*. For this reason, in conventional agriculture, table grapes are repeatedly treated with fungicides during the season. Postharvest treatments with essential oils or storage at hypobaric conditions are alternatives to the use of synthetic fungicides that are welcome by consumers for their low impact on human health and the environment. The aim of this work was to test the effectiveness of exposure of table grapes to vapors of essential oils of *Rosmarinus officinalis*, *Mentha piperita*, and *Thymus vulgaris* in reducing postharvest decay of table grapes stored for 24 h either at atmospheric or hypobaric pressure. Treatments with vapors of rosemary essential oils at atmospheric or hypobaric pressure reduced the incidence and the McKinney's Index of table grapes postharvest decay compared to the control, while essential oil of mint was effective in reducing decay only when applied at hypobaric pressure.

Grapevine downy mildew (GDM) is one of the most serious diseases of grapevines. After the limitations in the use of copper-based products in organic agriculture imposed by the EU, alternative compounds are increasingly required for grapevine protection. A two-year field trial was carried out to assess the effectiveness of several natural compounds, applied as foliar sprays, in reducing GDM symptoms. Among alternatives to copper, chitosan proved to be the most effective compound, showing a good protection, under condition of both low and high disease pressure. Furthermore, it was able to contain the vegetative vigour of grapevines, a key factor to obtain high quality productions. Another two-year field trial was carried out to evaluate the control of GDM using formulations at reduced dose of copper on different cultivars and in different locations. In the first year, under low disease pressure, Bordoflow resulted in the lowest GDM incidence on leaves, while Coprantol Hi Bio in the lowest GDM incidence on grape bunches. In the second year, with higher disease pressure, Bordoflow confirmed the best protection against GDM on leaves, while on grape bunches Heliocuivre provided the best GDM protection. The data obtained by this experiment confirm that the cv. Pecorino is the least susceptible variety to GDM. Directive 128/2009 has made mandatory the application of IPM in the EU countries from January 1st, 2014, and aware of the toxic effects of copper on plants, animals and of accumulation in the soil, the use of safer alternatives could help to reduce, and at times replace, the applications of copper and synthetic fungicides.

RIASSUNTO

Strategie a basso impatto ambientale per il controllo di **Botrytis cinerea** *e* **Plasmopara viticola** *su vite*

Lo scopo della tesi è stato quello di valutare strategie a basso impatto ambientale per il controllo di *Botrytis cinerea* e *Plasmopara viticola*. Dopo la raccolta l'uva da tavola va incontro a marciumi causati da funghi fitopatogeni, e in particolare *B. cinerea*. Per questo motivo, in agricoltura convenzionale, l'uva da tavola è ripetutamente trattata con fungicidi durante tutta la stagione. I trattamenti postraccolta con oli essenziali e la conservazione in condizione ipobarica sono alternative all'uso dei fungicidi di sintesi e sono preferite dai consumatori per il loro basso impatto sulla salute umana e sull'ambiente. Lo scopo di questo lavoro è stato quello di verificare l'efficacia di vapori di oli essenziali di *Rosmarinus officinalis*, *Mentha piperita*, e *Thymus vulgaris* nel ridurre i marciumi postraccolta su uva da tavola conservata per 24 ore sia a pressione atmosferica, sia in ambiente ipobarico. I trattamenti con vapori di olio essenziale di rosmarino, a pressione atmosferica o in ambiente ipobarico, hanno ridotto sia la diffusione sia l'indice di McKinney della muffa grigia, mentre i vapori di olio essenziale di menta sono stati efficaci nel ridurre la muffa grigia solo in ambiente ipobarico.

La peronospora della vite è una delle più gravi malattie fungine della coltura. In seguito alle limitazioni nell'utilizzo di prodotti a base di rame in agricoltura biologica imposti dalla UE, composti alternativi sono sempre più richiesti per la protezione delle piante. Una prova di campo di due anni è stata condotta per verificare l'efficacia di diversi composti naturali nel ridurre i sintomi di peronospora. Il chitosano ha dimostrato di essere il composto più efficace, assicurando una buona protezione della vite, in condizioni di bassa ed elevata pressione della malattia. Inoltre, si è dimostrato capace di limitare il vigore vegetativo delle piante, un fattore chiave per ottenere produzioni di qualità. Un'altra prova di campo di due anni è stata svolta per valutare l'efficacia di prodotti commerciali a dosi ridotte di rame per il controllo della peronospora della vite. Nel primo anno, caratterizzato da bassa pressione della malattia, Bordoflow ha determinato la più bassa diffusione dei sintomi di peronospora sulle foglie, mentre sui grappoli Coprantol Hi Bio ha dato il più basso valore di diffusione della malattia. Nel secondo anno, caratterizzato da alta pressione della malattia, Bordoflow ha garantito di nuovo la migliore protezione sulle foglie, mentre su grappoli Heliocuivre ha fatto registrare i migliori risultati nella protezione da peronospora. I dati ottenuti confrontando le diverse cultivar confermano la cv.

Pecorino come la meno suscettibile alla peronospora della vite. La Direttiva 128/2009 ha reso obbligatoria l'applicazione della difesa integrata in tutti i paesi UE a partire dal 1 gennaio 2014, e considerando la tossicità del rame verso le piante, gli animali ed il suo accumulo nei terreni, il ricorso ad alternative a basso impatto ambientale può aiutare a ridurre, se non sostituire, l'utilizzo di rame e fungicidi di sintesi.

1 INTRODUCTION

In the last decades, agricultural production in Europe has been intensified. Intensive agriculture is characterised by a high productivity and involves high inputs, including pesticides, fertilisers and water, increased mechanisation (Le Féon et al., 2010) and more simplified cropping sequences (Stoate et al., 2001). Despite the advantages obtained in terms of higher production, the intensive agriculture has resulted in negative environmental consequences, such as increased soil erosion, decreased soil fertility, reduced farmland biodiversity, pollution of groundwater, eutrophication of rivers and lakes and negative global consequences including the emission of greenhouse gases (Matson et al., 1997). The effects of pesticides on the environment as well as their potential negative impact on human health have led the European Union to take measures to reduce the use of pesticides in agriculture. The EU Parliament has then adopted the Directive 2009/128/EC on "Sustainable use of pesticides", that made compulsory in the Member States the application of the integrated pest management (IPM) from January 1st, 2014.

Its overall objective is to establish "… a framework to achieve a sustainable use of pesticides by reducing the risks and impacts of pesticide use on human health and the environment, and promoting the use of IPM and of alternative approaches or techniques such as non-chemical alternatives to pesticides". One of the key features of the Directive is that each Member State should develop and adopt its National Action Plan and set up quantitative objectives, targets, measures and timetables, i) to reduce risks and impacts of pesticide use on human health and the environment, and ii) to encourage the development and introduction of IPM and alternative approaches or techniques in order to reduce dependency on the use of pesticides. In this Directive, IPM is defined as the ''careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimise risks to human health and the environment. IPM emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms". This definition stems from the consideration that only a combination of techniques, each providing partial control, can reduce the reliance on pesticides, and such an approach might lead to major changes in the nature of the cropping system (Mortensen et al., 2000; Vasileiadis et al., 2011).

Proper diagnosis in field is a key point of the IPM programs. Inaccurate diagnoses may lead to unnecessary fungicide applications. Additionally, management decisions can be affected by poor disease diagnosis. Until now, disease control in the field relied almost exclusively on the use of synthetic fungicides, but now this approach need to be reconsidered in view of the new regulations. New low-toxic, anti-polluting antifungal agents, that differ from the fungicides currently developed in their mode of action and chemical properties, will increasingly be required. Another problem in the use of systemic fungicides is that fungal plant pathogens can develop resistance, resulting in a more difficult disease management. Thus, there are increasing concerns regarding safer fungicides with low toxicological and environmental risks (Kim and Hwang, 2007).

The application of synthetic fungicides remains the most common method to control postharvest rot of fruit and vegetables. However, for some commodities, the application of postharvest fungicides is not allowed, due to the normative restriction for fungicide use. Furthermore, the appearance of pathogens resistant to fungicides has dissuaded their repeated use. In addition, increasing public concern towards healthy food has contribuited to the development of alternative methods for controlling postharvest decay caused by pathogens on fruit and vegetables, which can integrate, if not totally replaced, the use of synthetic fungicides.

In organic agriculture, synthetic fungicides for the control of *Plasmopara viticola* have been replaced for many years by the use of massive doses of copper and has led to accumulation of this heavy metal in the topsoil in many European countries. Copper, as a result of canopy foliar sprays, lasts in wood residues and accumulates in the soil where it cannot be metabolized by soil microorganisms, and is only eliminated from the vineyard through leaching. This excessive accumulation of copper in the topsoil has resulted in decreases in the carabid and earthworm populations, caused microbiological and enzymatic alterations, lowered the soil pH and reduced grapevine growth. As a consequence, the use of copper fungicides in organic agriculture was restricted by European Union Regulation 473/2002. This Regulation limits the use of copper in organic farming to 6 kg/ha per year in the European countries, nad this threshold was recently adopted also in IPM. In response to these limitations, in recent years, studies on formulation with a low copper rate or alternative products, to reduce or eliminate copper-based formulations in organic agriculture, has been encouraged. Natural products can be an alternative to fungicides, like plant extracts, essential oils and other plant extracts, biocontrol agents, substances obtained from animal organisms e.g. chitosan or vermicompost extract (Dayan et al., 2009).

The aim of the thesis was to evaluate low environmental impact strategies against *Botrytis cinerea* and *P. viticola*. A topic of this thesis was to test the effectiveness of exposure of table grapes to vapors of essential oils of *Rosmarinus officinalis*, *Mentha piperita*, and *Thymus vulgaris* in reducing postharvest decay of table grapes stored for 24 h either at atmospheric or hypobaric pressure. The other aim of the study was the assessment of compounds at reduced dose of copper and natural compounds in limiting grapevine downy mildew. Overall, the thesis studied sustainable strategies for the management of two important diseases of grapes, both in the field and after harvest, providing evidence of treatments that can be applied to reduce losses and increase the quality of the production.

REFERENCES

Dayan, F. E., Cantrell, C. L., Duke, S. O. 2009. Natural products in crop protection. Bioorg. Med. Chem. 17:4022–4034.

Kim, B. S., Hwang, B. K. 2007. Microbial fungicides in the control of plant diseases. J. Phytopathol. 155:641–653.

Le Féon, V., Schermann-Legionnet, A., Delettre, Y., Aviron, S., Billeter, R., Bugter, R., Hendrickx, F., Burel, F. 2010. Intensification of agriculture, landscape composition and wild bee communities: a large scale study in four European countries. Agric. Ecosyst. Environ. 137:143–150.

Matson, P. A., Parton, W. G., Power, A. G., Swift, M. J. 1997. Agricultural intensification and ecosystem properties. Science 227:504–509.

Mortensen, D. A., Bastiaans, L., Sattin, M. 2000. The role of ecology in the development of weed management systems: an outlook. Weed. Res. 40:49–62.

Shtienberg, D. 2000. Modelling: the basis for rational disease management. Crop Prot. 19:747–752.

Stoate, C., Boatman, N. D., Borralho, R. J., Rio Carvalho, C., De Snoo, G. R., Eden, P. 2001. Ecological impacts of arable intensification in Europe. J. Environ. Manage. 63:337–365.

Vasileiadis, V. P., Sattin, M., Otto, S., Veres, A., Pálinkás, Z., Ban, R., Pons, X., Kudsk, P., van der Weidee, R., Czemborf, E., Mooneng, AC., Kiss, J. 2011. Crop protection in European maize-based cropping systems: Current practices and recommendations for innovative Integrated Pest Management. Agric. Syst. 104: 533–540.

Zadoks, J. C. 1985. On the conceptual basis of crop loss assessment: the threshold theory. Annu. Rev. Phytopathol. 23:455–473.

2 HYPOBARIC STORAGE WITH ESSENTIAL OIL VAPORS REDUCED POSTHARVEST DECAY OF TABLE GRAPES

Abstract

After harvest, table grapes easily undergo to fungal spoilage, mainly caused by *Botrytis cinerea*, causal agent of gray mold. To reduce such losses, table grapes are usually treated with synthetic fungicides during the season, and cold stored in presence of sulfur dioxide. However, those applications are not allowed in organic agriculture, and there is a growing demand from consumers of fresh fruit free of pesticide residues, so alternatives to sulfur dioxide are desirable. Hypobaric treatments and applications of essential oils are promising alternatives to sulfur dioxide that have low impact on human health and environment. The aim of this work was to test the effectiveness of 24 h fumigation with essential oils of *Rosmarinus officinalis*, *Mentha piperita*, and *Thymus vulgaris*, alone or combined with hypobaric treatment at 0.5 atm, to control postharvest gray mold of table grapes. Fumigation with *R. officinalis* essential oil at room pressure and at 0.5 atm reduced incidence and the McKinney's Index of gray mold table grapes stored at room temperature for 9 and 5 days respectively, or cold stored at 4°C for 7 days then exposed to 3 days shelf life, while essential oil of *M. piperita* was effective only under hypobaric environment. A panel test revealed perception of essential oils soon after treatment or 24 h later on berries fumigated with vapors of *R. officinalis*, *M. piperita*, and *Lavandula × ibrida*, while 48 h after the treatment, the presence of essential oils of *R. officinalis* and *M. piperita* was not appreciated. The fumigation with essential oils of *R. officinalis* and *M. piperita*, alone or combined with hypobaric treatment, could be an innovative way to control postharvest gray mold of table grapes, but a proper period need to last between fumigation and exposure to the consumers.

2.1 INTRODUCTION

After harvest, table grapes are particularly perishable, being susceptible to drying, mechanical injury, decay and physiological disorders. Gray mold, induced by *B. cinerea*, is the most economically important postharvest disease of table grapes (Lichter and Romanazzi, 2017). Gray mold is a major obstacle to long-distance transport and storage, since the disease can develop at low temperatures (-0.5 °C) and spreads quickly among berries. During the last 50 years, the use of fungicides or chemicals such as sulfur dioxide (SO_2) was the main mean of *B. cinerea* management (Rosslenbroich and Stuebler, 2000;

Romanazzi et al., 2016). However, sulfur dioxide can cause bleaching of the table grapes berries, and it is responsible for decline in flavor quality and induction of adverse reactions in health consumers (Montaño García, 1989). In addition, SO2 and fungicides are not allowed for use in organic produce as a postharvest treatment (Mlikota and Smilanick, 2001). For these reasons, alternatives to SO2 in controlling postharvest decay of table grapes are needed (Romanazzi et al., 2012).

Essential oils, being biologically active, represent a rich potential source of alternative and environmentally more acceptable disease management compounds as compared to traditional synthetic fungicides. Since decades, the general antifungal activity of essential oils is well documented (Deans and Ritchie, 1987) and there have been several studies on the effects of essential oils on postharvest pathogens of fruit (Cindi et al., 2016; Mari et al., 2016). The advantage of essential oils is their bioactivity in the vapour phase, a characteristic which makes them attractive as possible fumigants for protection of stored produce, since fruit manipulation is almost avoided (Triphati et al., 2008). On the other side, treatments of fruit with essential oils should be carefully considered since they could change the fruit organoleptic characteristics and compromise consumers' acceptability (Guillén et al., 2007).

Storage of fruit under hypobaric condition can be considered as an alternative to synthetic fungicides in reducing postharvest decay of fruit (Apelbaum et al., 1977; Romanazzi et al., 2001; Hashmi et al., 2013). Under hypobaric pressure, vaporization and distribution on the surface of the fruit of essential oils increases.

The aim of this work was to test the effectiveness of vapors of essential oils of *R. officinalis*, *M. piperita*, and *T. vulgaris* in reducing postharvest decay of table grapes stored at room pressure or under hypobaric environment. In addition, a panel test was carried out at different times and conditions after exposure to verify if consumer perceive differences on table grapes fumigated with *R. officinalis*, *M. piperita*, and *Lavandula* × *ibrida* essential oils, being last one particularly perceivable by consumers.

2.2 MATERIALS AND METHODS

2.2.1 Fruit

The experimental trials were carried out on table grapes (*Vitis vinifera*) cultivar 'Italia' grown organically and harvested in commercial orchards. Fresh table grapes were transferred to the laboratory where portions of bunches of 10 berries each were selected according to homogeneous size, shape, color, weight and absence of injuries.

2.2.2 Treatments

The bunch portions were placed in small plastic trays, which were placed for 24 h in airtight boxes. Within each airtight box there were seven replicates of plastic trays containing each four bunch portions of table grapes. In addition, the airtight boxes contained flasks with 5 ml of water or essential oils of thyme (*T. vulgaris*), rosemary (*R. officinalis*), or mint (*M. piperita*) whose chemical compositions, shown on Table 1, were analyzed by gas chromatography (Perkin Elmer Clarus 500 GC/FID/MS) (kindly provided by Flora Srl, Pisa, Italy). In the first trials, these airtight boxes were kept at atmospheric pressure, while in the following trails, within the airtight boxes, a hypobaric pressure of 50 kPa was created with a vacuum pump. After 24 h the plastic trays containing the table grapes were moved out of the airtight boxes and placed into large covered plastic boxes. To create humid condition of storage, a layer of wet paper was placed in the bottom of the plastic boxe where the table grapes were stored at room temperature. In addition, the trials carried out at hypobaric pressure were repeated storing the table grapes into large covered plastic boxes for 7 d at 4 °C and then exposing them to shelf life. During the storage at room temperature and during the shelf life, decay evaluation was carried out as reported later.

Tab. 1 Chemical composition of essential oils (%)[a]

Lavandula × hybrida	*Rosmarinus officinalis*	*Mentha piperita*	*Thymus vulgaris*
α-Pinene (0.8)	a-Thujene (0.51)	a-Thujene (0.51)	Thymol (51.83)
β-Myrcene (1.34)	a-Pinene (10.26)	Pulegone (1.78)	Cymene (20.26)
α- Terpinen (0.14)	Camphene (4.15)	Menthol (34.71)	γ-Terpinen (6.91)
b-Ocimene (1.76)	Sabinene (0.26)	Neo-Menthol (3.51)	Linalool (4.45)
γ-Terpinen (0.19)	b-Pinene (8.55)	Menthofuran (3.77)	Carvacrol (2.80)
3-Octanol (0.43)	b-Myrcene (1.40)	3-Octanol (0.16)	
α-Terpinolene (0.95)	a-Terpinen (0.48)	Menthone (27.44)	
Linalool (34.4)	p-Cymene (1.07)	Iso-Menthone (4.31)	
Limonene (1.8)	Limonen (2.19)	Limonen (2.49)	
1,8-Cineol (6.88)	1,8-Cineol (48,24)	1,8-Cineol (5.08)	
Camphor (6.28)	g-Terpinen (0.88)	Menthyl acetate (4.92)	
Lavandulol (0.6)	Terpinolene (0.39)	b-Cariophyllene (2.48)	
Borneol (3.32)	Linalool (0.67)	t-Sabinene hydrate (0.34)	
4-Terpineol (2.21)	Camphor (8.66)		
α-Terpineol (1.16)	d-Terpineol (0.32)		
Linalyl acetate (25.8)	Borneol (1.85)		
Lavandulyl acetate (2.16)	Terpinen-4ol (0.71)		
Germacrene (0.52)	a-Terpineol (1.42)		
Farnesene (0.83)	a-Copaene (0.18)		
p-Cymene (0.21)	b-Cariophyllene (3.83)		
Cariophyllene (1.47)	a-Humulene (0.4)		
b-Pinene (0.48)	d-Cadinene (0.17)		
Neryl acetate (0.39)			
Sabinene (0.13)			

[a]Data kindly provided by Flora Srl, Pisa, Italy

2.2.3 Postharvest decay evaluation

Table grapes postharvest decay was recorded daily. The diseases incidence was expressed as the percentage of infected berries. Disease severity was also recorded according to an empirical scale with six degrees: 0, healthy fruit; 1, 1–20% fruit surface infected; 2, 21–40% fruit surface infected; 3, 41–60% fruit surface infected; 4, 61–80% fruit surface infected; 5, more than 81% of the table grapes surface infected and showing sporulation (Romanazzi et al., 2000). The empirical scale allowed the calculation of the McKinney's Index, expressed as the weighted average of the disease as a percentage of the maximum possible level (McKinney, 1923). Specifically, it was calculated by the formula: $I = [\Sigma(d \times f)/(N \times D)] \times 100$, where d is the category of rot intensity scored on the sweet cherry and f its frequency; N the total number of sweet cherries examined (healthy and rotted) and D is the highest category of disease intensity occurring on the empirical scale (Romanazzi et al., 2001).

2.2.4 Essential oils vaporization

Under hypobaric environment, the essential oils can vaporize more than at room temperature. Therefore, containers with 5 mg essential oils of thyme, rosemary and mint were exposed at room pressure (100 kPa) and hypobaric environment (50 kPa) for 24 h, then weighted again.

2.2.5 Sensory analysis

The technique of triangle discrimination tests (Meilgaard et al., 1999; Lawless and Hildegarde, 2010) was employed to determine whether there was a detectable difference in the flavor of table grapes exposed to vapors of essential oils as compared to table grapes stored with water. Table grapes were exposed in airtight boxes for 24 h at hypobaric condition of 50 kPa in presence of container with water (control) or essential oils of lavender (*Lavandula × hybrida*, whose chemical composition showed on Table 1), rosemary, or mint, as previous described. Once table grapes were moved from the airtight boxes, they were i) immediately arranged to be submitted to untrained panelists; alternatively, they were placed in large covered plastic boxes at ambient temperature for ii) 24 h or iii) 48 h before to be submitted to the untrained panelists. At the moment of the analysis, each panelist was presented with three sets of three berries each. The first set was composed by three berries that were exposed to vapors of water or rosemary essential oils. The second set was composed by three berries that were exposed to vapors of water or mint essential oils. The third set was composed by three berries that were exposed to vapors of water or lavender essential oils. In each set, two of three berries had the same treatment, while one was different. Samples were presented in a random order and assigned three-digit codes to reduce influencing the decisions of panelists. Per each set, the panelists were asked to taste the berries and circle the sample number that they were determined was the different berry. The analysis was performed under controlled temperature and lighting conditions in individual booths.

The three tests were carried out on three different days, each day at 10 AM.

2.2.6 Statistical analysis

The data were analyzed statistically by one-way ANOVA, followed by Tukey's honestly significant difference (HSD) tests, at $P \leq 0.05$ (Stat-soft, Tulsa, OK, USA). When the range of percentages was greater than 40%, the percentage data were arcsine transformed before analysis, to improve the homogeneity of the variance. The actual values are shown. The experimental trials were repeated at least twice. Data from two or more trials were pooled, and the statistical analysis to determine the homogeneity of the variance was tested using Levene's test.

To analyze the data obtained from the sensory evaluation panelists, the correct answers of the triangle tests were compared to tabulated critical values (Lawless and Hildegarde, 2010).

2.3 RESULTS

2.3.1 Postharvest decay evaluation after treatments with essential oils

The main cause of postharvest decay recorded on table grapes was gray mold. Other rots caused by *Penicillium* spp., *Rhizopus stolonifer*, and *Aspergillus* spp. occurred very rarely. In the trial where table grapes were exposed to essential oils at atmospheric pressure and then stored at ambient temperature, only treatments with vapors of rosemary essential oils reduced incidence and the McKinney's Index of table grapes postharvest decay by 63% and 69%, respectively (Table 2). On the contrary, treatments with essential oils of thyme and mint did not reduce incidence and the McKinney's Index of postharvest decay of table grapes. The severity of decay was the same between table grapes stored with essential oils and table grapes stored with water.

Table 2. Incidence, severity and McKinney's Index of postharvest gray mold of table grapes cv. Italia that were stored at atmospheric pressure in airtight boxes containing flasks with water or essential oils of thyme, rosemary, or mint for 24 h and then stored at ambient temperature for 9 d.

Essential oils	Incidence (%)	Severity (1-5)	McKinney's Index (%)
Thyme	35.00±33.8 a	2.97±1.63 a	25.64±16.45 a
Rosemary	11.42±19.43 b	1.71±1.86 a	7.71±9.17 b
Mint	20.00±19.43 ab	2.65±1.68 a	13.00±5.29 ab
Water	31.07±31.54 a	2.73±1.96 a	25.07±21.8 a

When table grapes were exposed 24 h to vapors of essential oils at hypobaric pressure, and then stored at ambient temperature, both essential oils of mint and rosemary reduced incidence and the McKinney's Index of table grapes postharvest decay compared to the control (Table 3), while no difference between control and table grapes exposed to thyme essential oils were recorded. In particular, essential oil of rosemary reduced decay incidence and decay McKinney's Index by 60% and 66%, respectively, and essential oil of mint reduced decay incidence and decay McKinney's Index by 53% and 60%, respectively. The severity of table grapes decay was reduced only by treatments with rosemary essential oils.

Table 3. Incidence, severity and McKinney's Index of postharvest gray mold of table grapes cv. Italia that were stored at 50 kPa in airtight boxes containing flasks with water or essential oils of thyme, rosemary, or mint for 24 h and then stored at ambient temperature for 5 d.

Essential oils	Incidence (%)	Severity (1-5)	McKinney's Index (%)
Thyme	16.07±20.06 ab	1.89±1.95 ab	12.21±17.44 ab
Rosemary	7.85±12.57 b	1.16±1.71 b	4.92±8.37 b
Mint	9.28±10.51 b	1.82±1.83 ab	5.85±7.07 b
Control	19.64±14.26 a	3.04±1.66 a	14.64±10.98 a

Table 4, which summarizes the results obtained when the table grapes were exposed 24 h to vapors of essential oils at hypobaric pressure, stored 4 °C for 7 d, and then exposed to shelf life for 3 d, confirmed the previous data. Indeed, only treatments with vapors of rosemary essential oils reduced incidence and the McKinney's Index of table grapes postharvest decay compared to the control by 70% and 66% respectively, while essential oils of thyme and mint were not effective in reducing decay incidence and decay McKinney's Index. The severity of decay was the same between table grapes stored with essential oils and the control.

Table 4. Incidence, severity and McKinney's Index of postharvest gray mold of table grapes cv. Italia that were stored at 50 kPa in airtight boxes containing flasks with water or essential oils of thyme, rosemary, or mint for 24 h, stored at 4 °C for 7 d, and then exposed to shelf life for 3 d.

Essential oil	Incidence (%)	Severity (1-5)	McKinney's Index (%)
Thyme	14.64±15.02 a	2.39±1.82 a	9.57±9.93 ab
Rosemary	4.64±7.92 b	1.17±1.84 a	3.50±6.43 b
Mint	11.42±11.45 ab	1.88±1.63 a	6.50±6.76 ab
Control	15.35±17.73 a	2.11±1.80 a	10.28±13.22 a

2.3.2 Essential oils vaporization

Essential oils placed for 24 h in airtight boxes vaporized as proven by the weight differences of flasks at the beginning and at the end of the trials (Table 5). The vaporization at atmospheric pressure was lower than vaporization at hypobaric pressure. Indeed, at atmospheric pressure essential oils of thyme, rosemary, and mint had a vaporization of 0.4%, 2%, and 3% of their initial weight. At the same condition but at hypobaric pressure, the percentage of vaporization was 7 times, 3 times, and almost 2 times higher than vaporization at room pressure for thyme, rosemary, and mint essential oils, respectively.

Table 5. Vaporization of essential oils placed for 24 h at atmospheric pressure or at the hypobaric pressure of 50 kPa within airtight boxes.

	Initial weight (mg)		Final weight (mg)		Vaporization (%)	
Essential oils	100 kPa	50 kPa	100 kPa	50 kPa	100 kPa	50 kPa
Thyme	5.00	5.00	4.98	4.86	0.40	2.80
Rosemary	5.00	5.00	4.90	4.70	2.00	6.00
Mint	5.00	5.00	4.85	4.72	3.00	5.60

2.3.3 Sensory analysis

Essential oils of lavender, rosemary, or mint were analyzed in a sensory triangle discrimination test, while thyme essential oils was excluded because of its inefficacy in reducing postharvest fruit decay.

In the first sensory evaluation, table grapes were removed from airtight boxes and immediately tasted by 30 untrained panelists that could clearly perceive the flavor of essential oils (Table 6). Indeed, in all the comparisons water-essential oils, the number of panelists that correctly identified the different berry was higher than the number of panelists of the significance threshold. Similar results were obtained when 30 untrained panelists tasted table grapes that were exposed to essential oils and stored for 24 h before sensory evaluation. Again, panelists could perceive the differences on taste between berries exposed to essential oils vapors and control table grapes. On the contrary, when table grapes after exposure to essential oils were cold stored 48 h before sensory evaluation, panelists could not perceive the taste of essential oils. Indeed, in this case, the number of panelists that correctly found the “different berry” was not sufficient to reach the significant threshold, indicating that the taste differences between table grapes exposed to essential oil and the control were not perceivable. When table grapes were stored for 48 h at 25 °C before sensory evaluation, the panelists could perceive the difference in taste only after exposure to lavender, while the taste of rosemary and mint were not perceived by the 45 untrained panelists.

Table 6. Number of total and correct answers obtained from triangle discrimination tests using table grapes cv. Italia placed for 24 h at 50 kPa within airtight boxes containing flasks with water or essential oils of lavender, rosemary, or mint. After the treatment, the table grapes were immediately tasted by untrained panelists or stored at 24 h or 48 h at 4 °C or 25 °C before the sensory evaluation.

Discrimination tests	Time between treatments and analysis (h)	Total answers	Correct answers	Significance thresholds
Rosemary essential oil-Water	0 h	30	16*	15
Rosemary essential oil-Water	24 h at 25 °C	30	17*	15
Rosemary essential oil-Water	48 h at 4 °C	45	21	21
Rosemary essential oil-Water	48 h at 25 °C	45	21	21
Mint essential oil-Water	0 h	30	26*	15
Mint essential oil-Water	24 h at 25 °C	30	25*	15
Mint essential oil-Water	48 h at 4 °C	45	15	21
Mint essential oil-Water	48 h at 25 °C	45	20	21
Lavender essential oil-Water	0 h	30	18*	15
Lavender essential oil-Water	24 h at 25 °C	30	18*	15
Lavender essential oil-Water	48 h at 4 °C	45	20	21
Lavender essential oil-Water	48 h at 25 °C	45	22*	21

*significant recognition of the treatment (Lawless and Hildegarde, 2010)

2.4 DISCUSSION

Over the past years, essential oils have deserved attention as natural preservatives used to prolong postharvest life of fruit and vegetables (Sivakumar and Bautista-Baños, 2014). Essential oils could be applied as liquid solution (de Sousa et al., 2013), as additive in fruit coating such as co-formulant in chitosan coating (Romanazzi et al., 2017) or as biofumigant (Mehara et al., 2013). In general terms, fumigation was preferred over liquid application for the postharvest decay control since their use expects minimal handling of the food product and absence of the fruit wetting. However, fumigants are unable to penetrate into fruit surfaces, and therefore cannot control latent infections.

Exposure to *R. officinalis* essential oil vapors were effective in reducing postharvest decay of table grapes. Our results corroborate previous trials. When rosemary essential oils were applied alone or in combinations with oregano essential oils to grapes artificially contaminated with spores of *Aspergillus flavus* and *A. niger*, the rate of fungal infection, revealed by visible signs of fungal growth on fruits, was delayed throughout the assessed storage period at both room (12 d) and cold temperatures (24 d) as compared to the control (de Sousa et al., 2013). In addition, essential oils of *R. officinalis* were effective in reducing postharvest decay of stone and pome fruits previously inoculated with fungal pathogens (Lopez-Reyes et al., 2010; 2013). In previous research, sprays of table grapes with *T. vulgaris* essential oils, or exposure to thymol, which is among the thyme essential oil main components, were effective in reducing postharvest decay of table grapes (Guillén et al., 2007; Abdolahi et al., 2010; Shin et al., 2014). However, in our results, the exposure with *T. vulgaris* essential oils was not effective in reducing postharvest decay of table grapes. The reasons of this could be explained by variable compositions of essential oils or to different response of table grapes cultivars.

As demonstrated by weight loss of essential oils, at hypobaric condition the vaporization of essential oils was higher compared with room pressure. These results are the consequence of the Henry's law according to which the amount of dissolved gas into liquid solutions is proportional to its partial pressure in the gas phase. In our case, being the pressure into airtight containers lower than the atmospheric pressure, as a consequence, the amount of vaporization of essential oils was higher as compared to vaporization recorded with the same conditions at atmospheric pressure. At hypobaric condition the decay of table grapes was reduce by fumigation with *M. piperita* essential oil vapors of around 60%, while at atmospheric pressure, maintaining the same condition, table grapes decay was not significantly reduced by *M. piperita* essential oils. The higher effectiveness of *M. piperita* vapors in reducing postharvest decay of table grapes in hypobaric conditions could be due to the higher amount of essential oils vapors or to the direct consequences of the hypobaric treatments. Hypobaric environment was demonstrated to induce resistance in strawberry tissues that were treated for 4 h at 5 kPa. Activities of defense-related enzymes such as phenylalanine ammonia-lyase, chitinase and peroxidase increased in strawberry tissues exposed to hypobaric environment (Hashmi et al., 2013). In addition, during hypobaric treatment it is generated a lower partial pressure of oxygen that could contribute to slow the respiration rate of fruit tissues and retard senescence. The fruit remains fresh longer and ready to counteract fungal infection for longer time. On the other side, at hypobaric condition lower amount of oxygen is available for the development of pathogens that do not proliferate on fruit tissues. The combination of essential oil of *M. piperita* and hypobaric treatment decreased postharvest decay of table grapes, while *M. piperita* essential oil by itself did not. This responds to the approach of the multiple hurdles theory according to which the use of an individual alternative to synthetic fungicides might not be enough effective to obtain complete control of a disease, while the integration of many might replace the use of conventional fungicides, whereby each means of control provides a portion of the disease reduction. The optimal combination of the hurdles secures the microbial safety as well as the organoleptic quality, the nutritional value and the economic viability of food products.

Interactions of food matrix components with the essential oils should be investigated before their application is proposed for commercial practice. A challenge for essential oils application is their strong aroma even at low concentrations, which may adversely affect the organoleptic property of food items. Hence, the optimal concentration of essential oils or the ideal moment of their application before fruit consumption should be determined in front of essential oils recommendation as food preservative. In our sensory evaluation, untrained panelists could perceive, immediately or 24 h after the treatment, a different taste in the berries that were fumigated for 24 h with essential oils, but 48 h after the treatment, the panelists could not perceive any more this difference. Even in other sensory evaluations, essential oils did not negatively alter the taste of table grapes (Marandi et al., 2010; Sonker et al., 2015). However, in order to preserve the organoleptic property of fruit, the length exposure to essential oils could be decreased, while increased the period elapsing between fruit treatment and fruit consumption. In addition, the combination with hypobaric pressure could further reduce the amount of essential oils needed to control postharvest decay or it could improve the homogenous distribution of essential oils on the fruit surface. Too high concentrations of vapors could induce phytotoxic signs on the fruit or they could alter the sensory characteristics of the food produce, while reduced amounts of essential oils could be ineffective. Even the kind of essential oils gives different results in term of perception, indeed *Lavandula* × *hybrida* essential oil seemed more perceivable than *M. piperita* or *R. officinalis* essential oils.

In conclusion, the optimal combination of essential oil concentration, length of the treatment, time of application, kind of essential oil, arrangement with other technologies, are parameters to be determined in order to obtain the highest decay control by essential oils, without altering fruit taste. Further studies are needed to optimize the application of essential oil vapors with or without hypobaric treatment, and in order to find solutions to their introduction into commercial practices. Indeed, when essential oils are used as volatiles, their application could be challenging because of their possible dispersion.

2.5 REFERENCES

Abdolahi, A., Hassani, A., Ghosta, Y., Bernousi, I., Meshkatalsadat, M. H., 2010. Study on the potential use of essential oils for decay control and quality preservation of Tabarzeh table grapes. J. Plant Prot. Res. 50:45-52.

Apelbaum, A., Barkai-Golan, R., 1977. Spore germination and mycelial growth of postharvest pathogens under hypobaric pressure. Phytopathology 67:400–403.

Cappellini, R. A., Ceponis, M. J., Lightner, G. W., 1986. Disorders in table grape shipments to the New York market, 1972-1984. Plant Dis. 70:1075–1079.

Cindi, M.D., Soundy, P., Romanazzi, G., Sivakumar, D., 2016. Different defenseresponses and brown rot control in two *Prunus persica* cultivars to essential oil vapours after storage. Postharvest Biol. Technol. 119:9–17.

Deans, S. G., Ritchie, G., 1987. Antimicrobial properties of plant essential oils. Int. J. Food Microbiol. 5:165–180.

De Sousa, L. L., de Andrade, S. C. A., Athayde, A. J. A. A., de Oliveira, C. E. V., de Sales, C. V., Madruga, M. S., de Souza, E. L., 2013. Efficacy of *Origanum vulgare* L. and *Rosmarinus officinalis* L. essential oils in combination to control postharvest pathogenic *Aspergilli* and autochthonous mycoflora in *Vitis labrusca* L. (table grapes). Int. J. Food Microb. 165:312-318.

Feliziani, E., Romanazzi, G., Smilanick, L. J., 2014. Application of low concentrations of ozone during the cold storage of table grapes. Postharvest Biol. Technol. 93: 38-48.

Guillén, F., Zapata, P. J., Martínez-Romero, D., Castillo, S., Serrano, M., Valero, D., 2007. Improvement of the overall quality of table grapes stored under modified atmosphere packaging in combination with natural antimicrobial compounds. J. Food Sci. 72:185-190.

Hashmi, M. S., East, A. R., Palmer, J. S., Heyes, J. A., 2013. Hypobaric treatment stimulates defence-related enzymes in strawberry. Postharvest Biol. Technol. 85:77-82.

Janisiewicz, W. J., Korsten, L., 2002. Biological control of postharvest diseases of fruits. Annu. Rev. Phytopathol. 40:411–441.

Karabulut, O. A., Gabler, F. M., Mansour, M., Smilanick, J. L., 2004. Postharvest ethanol and hot water treatments of table grapes to control gray mold. Postharvest Biol. Technol. 34:169–177.

Latorre, B. A., Agosin, E., San Martin, R., Vasquez, G. S., 1997. Effectiveness of conidia of *Trichoderma harzianum* produced by liquid fermentation against *Botrytis* bunch rot of table grape in Chile. Crop Prot. 16:209–214.

Lawless, H. T., Hildegarde, H., 2010. Sensory evaluation of food: principles and practices, second ed. Springer.

Lichter, A., Romanazzi, G., 2017. Postharvest diseases of table grapes. In: Postharvest Pathology of Fruits and Vegetables. Prusky D., Adaskaveg J.E., Eds, APS Press, MN, US (in press).

Lopez-Reyes, J. G., Spadaro, D., Gullino, M. L., Garibaldi A., 2010. Efficacy of plant essential oils on postharvest control of rot caused by fungi on four cultivars of apples in vivo. Flav. Fragr. J. 25:171-177.

Lopez-Reyes, J. G., Spadaro, D., Prelle, A., Garibaldi, A., Gullino, M. L., 2013. Efficacy of plant essential oils on postharvest control of rots caused by fungi on different stone fruits in vivo. J. Food Prot. 76:631–639.

Marandi, R. J., Hassani, A., Ghosta, Y., Abdollahi, A., Pirzad, A., Sefidkon, F., 2010. *Thymus kotschyanus* and *Carum copticum* essential oils as botanical preservatives for table grape. J. Med. Plants Res. 4 :2424-2430.

Mari, M., Bautista-Baños, S., Sivakumar, D., 2016. Decay control in the postharvest system: Role of microbial and plant volatile organic compounds. Postharvest Biol. Technol. 122:70–81.

Meepagala, K. M., Sturtz, G., Wedge, D. E., 2002. Antifungal constituents of the essential oil fraction of *Artemisia dracunculus* L. Var. *dracunculus*. J. Agric. Food Chem. 50:6989-6992.

Mehra, L. K., MacLean, D. D., Shewfelt, R. L., Smith, K. C., Scherm, H., 2013. Effect of postharvest biofumigation on fungal decay, sensory quality, and antioxidant levels of blueberry fruit. Postharvest Biol. Technol. 85:109-115.

Meilgaard, M., Civille, G. V., Carr, B. T., 1999. Sensory Evaluation Techniques, third ed. CRC Press, Boca Raton, FL.

Mlikota, G. F., Smilanick, J. L., 2001. Postharvest control of table grape gray mold on detached berries with carbonate and bicarbonate salts and disinfectants. Am. J. Enol. Viticult. 52:12–20.

Montaño García, M. L., 1989. Adverse reactions induced by food additives: sulfites. Rev. Alerg. Mex. 36:107–109.

Prakash, B., Kedia, A., Mishra, P. K., Dubey, N. K., 2015. Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities - Potentials and challenges. Food Control 47:381-391.

Romanazzi, G., Nigro, F., Ippolito, A., 2000. Effectiveness of pre and postharvest chitosan treatments on storage decay of strawberries. Frutticoltura 62:71–75.

Romanazzi, G., Smilanick, J.L., Feliziani, E., Droby, S., 2016. Integrated management of postharvest gray mold on fruit crops. Postharvest Biol. Technol. 113, 69-76.

Romanazzi, G., Feliziani, E., Bautista Baños, S., Sivakumar, D., 2017. Shelf life extension of fresh fruit and vegetables by chitosan treatment. Crit. Rev. Food Sci. Nutr. 57:579-601.

Rosslenbroich, H. J., Stuebler, D., 2000. *Botrytis cinerea*—history of chemical control and novel fungicides for its management. Crop Protect. 19:557–561.

Shin M. H., Kim J. H., Choi H. W., Keum Y. S., Chun S. C., 2014. Effect of thymol and linalool fumigation on postharvest diseases of table grapes. Microbiology 42: 262-268.

Sivakumar D., Bautista-Baños S., 2014. A review on the use of essential oils for postharvest decay control and maintenance of fruit quality during storage. Crop Prot. 64:27-37.

Sonker, N., Pandey, A. K., Singh, P., 2015. Efficiency of *Artemisia nilagirica* (Clarke) Pamp. essential oil as a mycotoxicant against postharvest mycobiota of table grapes. J. Sci. Food Agric. 95:1932-1939.

Tripathi, P., Dubey, N. K., Shukla, A. K., 2008. Use of some essential oils as post-harvest botanical fungicides in the management of grey mould of grapes caused by Botrytis cinerea. World J. Microbiol. Biotechnol. 24:39–46.

Xu, W. T., Huang, K. L., Guo, F., Qu, W., Yang, J. J., Liang, Z. H., Luo, Y. B., 2007. Postharvest grapefruit seed extract and chitosa n treatments of table grapes to control *Botrytis cinerea*. Postharvest Biol. Technol. 46:86-94.

3 LOW RATE COPPER AND ALTERNATIVE PRODUCTS AGAINST *PLASMOPARA VITICOLA*

Abstract

Grapevine downy mildew (GDM) is one of the most serious diseases of grapevine. Limitations in the use of copper-based products in organic agriculture by the EU by Reg. EU/2002/473, promoted search for alternatives compounds. This two-year field trial aimed to evaluate the effectiveness against GDM of chitosan and of a mixture of laminarin and *Saccharomyces* spp. extracts (LSA), applied in different strategies with copper hydroxide. The effects of treatments on wine grape quantity and quality were also evaluated.

Under low disease pressure, all the treatments, except LSA alone, reduced GDM. With high disease pressure, Bordeaux mixture, copper hydroxide, chitosan, all the strategies including copper hydroxide with chitosan, and copper hydroxide with LSA, applying copper hydroxide for the first half of treatments, significantly reduced disease incidence on clusters by 43%, 47%, 36%, 40%, 48%, 37% and 37%, respectively, compared to the control, at the end of July 2014. The alternative compounds had no negative effects on the quantitative and qualitative parameters of wine grape berries.

Chitosan provided the best protection from GDM, applied alone or in strategy with copper hydroxide, so it can be proposed as a good alternative to copper formulations for GDM control in both organic and integrated disease management.

3.1 INTRODUCTION

Grapevine downy mildew (GDM) is caused by the biotrophic oomycete *Plasmopara viticola*, and it is one of the most serious diseases of grapevines worldwide (Gessler et al., 2011). It is potentially destructive especially in Europe, where grape-growing conditions are characterised by high humidity and abundant rainfall in the spring (Caffi et al., 2010). Over the last 20 years, the most widely used technique to control this pathogen was through the application of copper compounds, particularly in organic vineyards (Speiser et al., 2000). However, the high use of copper at rates of up to 8 kg/ha per year has led to accumulation of this heavy metal in the topsoil in many European countries (Rusjan et al., 2007). Copper, as a result of canopy foliar sprays, lasts in wood residues and accumulates in the soil where it cannot be metabolized by soil microorganisms, and is only eliminated from the vineyard through leaching (Duca et al., 2016). This excessive accumulation of copper in the topsoil has resulted in decreases in the carabid and earthworm populations, caused microbiological and enzymatic alterations, lowered the soil pH and reduced grapevine growth (Pontiroli et al., 2011).

As a consequence of these problems, the use of copper fungicides in organic agriculture was restricted by European Union Regulation 473/2002 (Commission Regulation EC; 2002). This Regulation limits the use of copper in organic farming to 6 kg/ha per year in the European countries. In response to these limitations, in recent years, research into alternative products, to reduce or eliminate copper-based formulations in organic agriculture, has been encouraged (Gessler et al., 2011). Some studies have identified natural compounds that have shown interesting qualities in terms of GDM control, including protein hydrolysates (Lachhab et al., 2014), chitosan (Maia et al., 2012; Rabea et al., 2005), laminarin (Aziz et al., 2003) and microorganisms (Alfonzo et al., 2012; Puopolo et al., 2013; Van Bruggen et al., 2016).

Chitosan is a deacetylated form of *N*-acetyl glucosamine, and it is a common polymer in shells of crustaceans, exoskeletons of insects, and cell walls of fungi. Among its several application area, chitosan has been shown to control preharvest and postharvest plant diseases (Romanazzi et al., 2017). Chitosan has eliciting activities that can lead to a variety of defence responses to microbial infections in host plants, which include the accumulation of phytoalexins, pathogen related proteins, and proteinase inhibitors, and the induction of lignin synthesis and callose formation (El Hadrami et al., 2010). Moreover, chitosan can also form physical barriers around the penetration sites of pathogens (Romanazzi et al., 2009) which can prevent them from spreading to healthy tissue, and fungicidal activities of chitosan have been well documented against various fungi (Feliziani et al., 2013).

Among the β-1,3-glucans, laminarin was derived from the brown alga *Laminaria digitata* and was shown to stimulate the natural defence responses in plants. Laminarin is involved in the induction of genes that encode various pathogenesis-related proteins with antimicrobial properties (Khan et al., 2009). Through its elicitation of plant defence reactions, laminarin can also reduce the development of many fungal pathogens, including *P. viticola* (Aziz et al., 2003).

The biological control of plant pathogens includes the use of microorganisms (Spadaro e Droby, 2016). Several biocontrol agents can promote vegetative growth, and induce resistance against pathogens in plants, including phytopathogenic fungi, bacteria and viruses, and in some cases, pest insects and nematodes (Harman et al., 2004; Liu et al., 2007). *Saccharomyces* spp. extract was reported to be active against *P. viticola*, reducing GDM symptoms (Romanazzi et al., 2016).

The present study evaluated the effectiveness of weekly foliar applications using natural compounds, individually applied or in strategies with copper formulations, aimed at controlling GDM. Compounds evaluated were Bordeaux mixture, copper hydroxide, laminarin, chitosan, and *Saccharomyces* spp. extracts. In addition, since these treatments can result in alterations to several physiological processes of the plants (Mandal et al., 2009) their influence on quantity and quality of some grape production parameters was evaluated.

3.2 MATERIALS AND METHODS

3.2.1 Vineyard treatments

The experimental field trials were conducted in 2014 and 2015 in a six-year-old commercial vineyard of *Vitis vinifera* cv. Montepulciano, grafted onto 420A rootstock, located at Montesicuro (AN), in central-eastern Italy (43°53’84.13”N; 13°48’15.85”E). The plants were spaced by 0.85 m in the rows, with 2.80 m between the rows, and they were grown according to the Guyot trellis system, leaving 10 buds per vine, with grass cover between the rows. The height of the fruiting cane was 60 cm from the ground. The vineyard was not irrigated, the fertilisers were distributed under vine in the winter, and additional hedging was applied in spring and summer, as common practices for the areas.
Over the two years, 10 treatments were tested, compared with an untreated control, and they were applied weekly until the end of July, with a total of 10 and 12 applications per year, respectively in 2014 and 2015. The active ingredients and the application rates are listed in Table 1. Throughout the experimental period, the distribution of the compounds occurred by adopting 4 different application strategies (Table 2).

Table 1. Copper and alternative formulation applied in the present study.

Commercial product	Active ingredient (%)	Supplier
Poltiglia Disperss	Bordeaux mixture (20)	Cerexagri Italia S.r.l. (IT)
Funguran	Copper hydroxide (19.2)	Certis Europe (IT)
Chito Plant	Chitosan (99.9), boron (0.05), zinc (0.05)	ChiPro GmbH (D)
Frontiere	Laminarin	BioAtlantis (IRL)
Oomisine	Microbial extract of *Saccharomyces* spp. (10), carbossilamine (10), manganese (3), boron (1.8), zinc (0.3), molybdenum (0.05)	Kalosgate (IT)

Table 2. Strategies adopted in the application of copper formulations and alternative products, and dates of applications.

Strategy	Treatments/Formulations Application rate	Applications 2014	Applications 2015
A	Bordeaux mixture (5 Kg/ha)	5/19, 5/26, 6/3, 6/9, 6/16, 6/24, 7/1, 7/8, 7/15, 7/22	5/14, 5/21, 5/27, 6/4, 6/12, 6/18, 6/25, 7/1, 7/8, 7/15, 7/21, 7/28
A	Copper hydroxide (2.8 L/ha)	5/19, 5/26, 6/3, 6/9, 6/16, 6/24, 7/1, 7/8, 7/15, 7/22	5/14, 5/21, 5/27, 6/4, 6/12, 6/18, 6/25, 7/1, 7/8, 7/15, 7/21, 7/28
A	Chitosan (5 Kg/ha)	5/19, 5/26, 6/3, 6/9, 6/16, 6/24, 7/1, 7/8, 7/15, 7/22	5/14, 5/21, 5/27, 6/4, 6/12, 6/18, 6/25, 7/1, 7/8, 7/15, 7/21, 7/28
A	Laminarin (1 L/ha) + microbial extract of *Saccharomyces* spp. (2 Kg/ha)	5/19, 5/26, 6/3, 6/9, 6/16, 6/24, 7/1, 7/8, 7/15, 7/22	5/14, 5/21, 5/27, 6/4, 6/12, 6/18, 6/25, 7/1, 7/8, 7/15, 7/21, 7/28
B	Copper hydroxide (2.8 L/ha)/	5/19, 6/3, 6/16, 7/1, 7/15	5/14, 5/27, 6/12, 6/25, 7/8, 7/21
	Chitosan (5 Kg /ha)	5/26, 6/9, 6/24, 7/8, 7/22	5/21, 6/4, 6/18, 7/1, 7/15, 7/28
C	Copper hydroxide (2.8 L/ha)/	5/19, 5/26, 6/3, 6/9, 6/16	5/14, 5/21, 5/27, 6/4, 6/12, 6/18
	Chitosan (5 Kg /ha)	6/24, 7/1, 7/8, 7/15, 7/22	6/25, 7/1, 7/8, 7/15, 7/21, 7/28
D	Chitosan (5 Kg /ha)/	5/19, 5/26, 6/3, 6/9, 6/16	5/14, 5/21, 5/27, 6/4, 6/12, 6/18
	Copper hydroxide (2.8 L/ha)	6/24, 7/1, 7/8, 7/15, 7/22	6/25, 7/1, 7/8, 7/15, 7/21, 7/28
B	Copper hydroxide (2.8 L/ha)/	5/19, 6/3, 6/16, 7/1, 7/15	5/14, 5/27, 6/12, 6/25, 7/8, 7/21
	Laminarin (1 L/ha) + microbial extract of *Saccharomyces* spp. (2 Kg /ha)	5/26, 6/9, 6/24, 7/8, 7/22	5/21, 6/4, 6/18, 7/1, 7/15, 7/28
C	Copper hydroxide (2.8 L/ha)/	5/19, 5/26, 6/3, 6/9, 6/16	5/14, 5/21, 5/27, 6/4, 6/12, 6/18
	Laminarin (1 L/ha) + microbial extract of *Saccharomyces* spp. (2 Kg /ha)	6/24, 7/1, 7/8, 7/15, 7/22	6/25, 7/1, 7/8, 7/15, 7/21, 7/28
D	Laminarin (1 L/ha) + microbial extract of *Saccharomyces* spp. (2 Kg /ha)/	5/19, 5/26, 6/3, 6/9, 6/16	5/14, 5/21, 5/27, 6/4, 6/12, 6/18
	Copper hydroxide (2.8 L/ha)	6/24, 7/1, 7/8, 7/15, 7/22	6/25, 7/1, 7/8, 7/15, 7/21, 7/28

Following the strategy A the plants were sprayed with the same compound(s) along the season, while in strategies B, C and D, the vines were treated with copper hydroxide and with a natural compound (chitosan or laminarin with *Saccharomyces* spp. Extract - LSA), according to different schedules. In particular, with the strategy B, copper hydroxide was applied alternately with the natural compound in the same plots; following the strategy C copper hydroxide was used for the first 5 or 6 applications (in 2014 and in 2015 respectively), while the natural compound for the following 5 or 6 applications (in 2014 and in 2015 respectively); with the strategy D the order of the products has been reversed as compared to strategy C. A randomised block design with four replicates was used, and the treatments were assigned to plots using a random-number generator (Excel; Microsoft Corp., Redmond, WA, USA). Each plot consisted of six/seven grapevines along a row, and the treated rows were separated each from the other by an untreated row. The treatments tested in both the years were applied to randomly selected vines between the different years.
Treatments started on 19 May, 2014, and on 14 May, 2015, when the environmental conditions became favorable to GDM infections, and plants were at the phenological stage of inflorescences swelling (BBCH 55), with the shoots about 20 cm long. The treatments were distributed by the spraying of a volume of 1.6 L/plot, equivalent to 1000 L/ha, using a motorised backpack sprayer (Honda GX 25, 25cc, 0.81 kW, Tokyo, Japan).

3.2.2 Climate data

The weather parameters (i.e., minimum and maximum temperatures; rainfall) and the phenological stages of budbreak, bloom, and veraison were obtained from the *Bollettino Agrometeorologico* published by Agrometeo, ASSAM, Marche region, relatively for the nearest weather station of the ASSAM to the experimental field.

3.2.3 Evaluation of grapevine downy mildew infections

Evaluation of GDM infections was carried out in all vines of each treatments on 100 leaves randomly selected per vine and all the clusters. During 2014, GDM symptoms were recorded for the grapevine leaves on 25 June and on 8, 15, 29 July, with the grape bunches scored on 8, 15, 29 July. During 2015, GDM symptoms were recorded for the grapevine leaves on 1, 7, 20 July, and on the grape bunches on 20 July, 4 August and 2 September. The disease incidence was expressed as the percentage of infected leaves or grape bunches. Moreover, the disease severity on the leaves was assigned to 11 levels, according to the percentage of surface covered by the GDM symptoms: from 0 (uninfected leaf) to 10 (over 90% of leaf surface showing symptoms). For the grape bunches, the disease severity was assigned to 8 levels, according to the number of infected berries and the percentage of the grape bunch that showed symptoms, as: 0, healthy grape bunch; 1, 1-5 infected berries; 2, 6-11 infected berries; 3, 12-25 infected berries; 4, 25% of grape bunch showing symptoms; 5, 26% to 50% of grape bunch showing symptoms; 6, 51% to 75% of grape bunch showing symptoms; and 7, >75% of grape bunch showing symptoms (Romanazzi et al., 2002). The infection index (or McKinney's index), which incorporates both the incidence and severity of the disease, was expressed as the weighted means of the disease as a percentage of the maximum possible level. In detail, this was calculated according to Equation:

$$I = [\Sigma(d \times f) / (N \times D)] \times 100$$

where d is the category of disease intensity scored for the grapevine leaves or the grape bunches, f is the disease frequency, N is the total number of organs examined (healthy and rotted), and D is the highest category of disease intensity that occurred on the empirical scale (McKinney, 1923).

3.2.4 Grape production

During 2015, at commercial harvest (12 October), a quantity of berries equal to 500 g per plot was collected from randomly selected clusters by clipping one or two terminal berries from the second lateral branch located at the top of the rachis of mature clusters from each plot. The berries were collected also by grapevines treated by Moncaro company, taking the samples from four different plots. The quality parameters of the musts, including the total soluble solids (°Brix), titratable acidity (g tartaric acid/L), and pH (Howell 2001; Mira de Orduña, 2010) were determined through laboratory analysis carried out by Moncaro winery. Moreover, on the day of harvest the grapes production per each treatment was recorded.

3.2.5 Statistical analysis

The data were submitted to analysis of variance according to a randomised block design, and the means were separated by Tukey's HSD tests, at $P \leq 0.05$ (Statsoft, Tulsa, OK, US). The statistical analysis to determine the homogeneity of the variance was tested using Levene's tests. Moreover, the data relating to grape yield and average weight of berries were analysed in the same way. Actual values are shown.

3.3 RESULTS

3.3.1 Climate data

In the first year (2014) the abundant rainfall in spring, coincident with an increase in temperature, was favourable for the onset of GDM infection. With rainfall even during the months of July and August, the climatic conditions were especially favourable for GDM development throughout the growing season, and this led to high disease incidence (Figure 1A).

Conversely, in the second year (2015) the climatic conditions in summer, including limited rainfall, were not favourable for the disease development. The first symptoms of GDM appeared on the vineyard in mid-June and at the end of the growing season the disease incidence was low (Figure 1B).

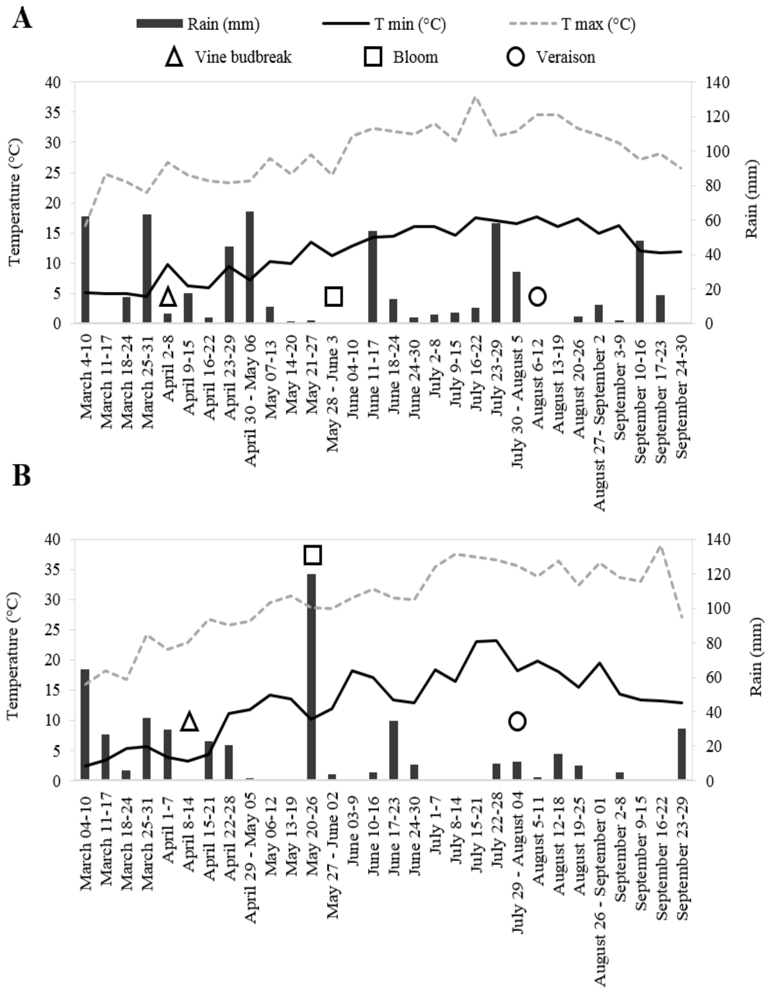

Figure 1. Climate data and main phenological stages of grapevines recorded through the study seasons, from March to September in 2014 (A) and 2015 (B) by the weather station located at Agugliano (AN).

3.3.2 Evaluation of grapevine downy mildew infections

3.3.2.1 First year (2014)

The first GDM infections appeared on the grapevine leaves on 20 June 2014. In the first survey on 25 June 2014, the disease incidence was lower than 6.4% among all of the treatments. The grapevines treated with Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C, D), and copper hydroxide + LSA (strategy C) showed the lowest results compared with the untreated control (data not shown). In the following assessments, an increase in the infection levels of GDM on the leaves was recorded, with the highest values on 29 July 2014 (Table 4).

On 8 July, the disease incidence on grapevine leaves was significantly reduced compared to the control only by treatment with copper hydroxide of 47% (Table 3). The disease severity according to table 3 was significantly reduced compared to the control by treatment with Bordeaux mixture, copper hydroxide, chitosan, chitosan + copper hydroxide (strategy D), and copper hydroxide + LSA (strategy C). The grapevines treated with copper hydroxide, copper hydroxide + chitosan (strategies B, D), and copper hydroxide + LSA (strategy C) showed significant reductions in GDM McKinney's index compared to the control, of 68%, 51%, 64%, and 54%, respectively.

In the assessment of GDM on grape bunches carried out on 8 July, the disease incidence was significantly reduced compared to the control by treatment with Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C, D) of 83%, 72%, 62%, 65%, 74%, and 67%, respectively. The disease severity and the McKinney's index of GDM on bunches was significantly reduced compared to the control by treatment with Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C, D), and copper hydroxide + LSA (strategy C); McKinney index was reduced by 92%, 87%, 83%, 81%, 89%, 87% and 67%, respectively (Table 3).

On 29 July, grapevines treated with Bordeaux mixture, copper hydroxide, copper hydroxide + chitosan (strategy C), and copper hydroxide + LSA (strategy C) showed on leaves significant reductions in GDM incidence, compared to the control, of 51%, 55%, 46% and 52%, respectively (Table 4). The disease severity was significantly reduced compared to the control by treatment with Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C), and copper hydroxide + LSA (strategy C) (Table 4). The McKinney's index of GDM was significantly reduced, compared to the control, by treatment with Bordeaux mixture, copper hydroxide, copper hydroxide + chitosan (strategies B, C), and copper hydroxide + LSA (strategy C), by 69%, 68%, 47%, 63% and 69%, respectively. In the assessment of GDM on grape bunches carried out on 29 July, there was a significant reduction in disease incidence, compared to the control, by treatment with Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C, D), and copper hydroxide + LSA (strategy C), of 43%, 47%, 36%, 40%, 48%, 37% and 37%, respectively, as also seen for the McKinney's index of 71%, 72%, 63%, 63%, 74%, 57% and 68%, respectively (Table 4). In the same survey, significant reductions of disease severity were reported on grape bunches treated with Bordeaux mixture, copper hydroxide, and copper hydroxide + chitosan (strategies B, C, D) (Table 4). Considering all of the disease assessments on 2014, the rank analysis for McKinney's index of GDM provided the following order of effectiveness of the treatments on the grapevine leaves and the grape bunches (Van Bruggen et al., 2016). On leaves: copper hydroxide = copper hydroxide + chitosan (strategy D) > copper hydroxide + LSA (strategy C) > copper hydroxide + chitosan (strategy B) > chitosan = copper hydroxide + chitosan (strategy C) > Bordeaux mixture > copper hydroxide + LSA (strategy B) > LSA > copper hydroxide LSA (strategy D) > control. On grape bunches: Bordeaux mixture > copper hydroxide + chitosan (strategy C) > copper hydroxide > copper hydroxide + chitosan (strategy D) > chitosan > copper hydroxide + chitosan (strategy B) = copper hydroxide + LSA (strategy C) > LSA > copper hydroxide + LSA (strategy D) > copper hydroxide + LSA (strategy B) > control.

Table 3. Disease incidence (DI), disease severity (Severity) and McKinney's index (MI) for the grape downy mildew infection recorded for the grapevine leaves and bunches on 8 July 2014, following the treatments with the different formulations during the season[a].

	8 July 2014					
	Leaves			Bunches		
Treatment (strategy)[b]	DI (%)	Severity (1-10)	MI (%)	DI (%)	Severity (1-10)	MI (%)
Bordeaux mixture (A)	13.4 ± 6.1 ab	2.3 ± 1.1 bc	3.4 ± 2.5 abc	10.7 ± 17.4 c	0.8 ± 1.3 d	3.0 ± 7.1 d
Copper hydroxide (A)	11.6 ± 5.6 b	1.6 ± 1.2 c	2.2 ± 2.2 c	17.6 ± 20.0 c	1.3 ± 1.6 bcd	4.6 ± 7.9 d
Chitosan (A)	12.7 ± 5.2 ab	2.3 ± 1.2 c	3.2 ± 2.5 abc	23.5 ± 23.4 bc	1.4 ± 1.3 bcd	6.0 ± 7.7 cd
LamE (A)	20.0 ± 5.9 ab	2.6 ± 1.3 ab	5.8 ± 3.8 ab	52.0 ± 35.4 ab	2.5 ± 1.9 abc	22.4 ± 21.7 abc
Cu + chitosan (B)	14.9 ± 9.0 ab	1.8 ± 0.9 abc	3.4 ± 3.2 bc	21.8 ± 24.9 c	1.2 ± 1.3 cd	6.9 ± 12.1 d
Cu + chitosan (C)	14.7 ± 7.3 ab	2.0 ± 1.0 abc	3.4 ± 2.6 abc	16.1 ± 20.8 c	0.9 ± 1.0 d	3.9 ± 5.7 d
Chitosan + Cu (D)	12.5 ± 4.8 ab	1.8 ± 1.0 c	2.5 ± 2.0 c	20.8 ± 25.5 c	0.8 ± 0.9 d	4.8 ± 10.6 d
Cu + LamE (B)	17.2 ± 6.6 ab	1.9 ± 0.7 abc	3.5 ± 2.3 abc	52.8 ± 32.8 ab	3.8 ± 2.2 a	35.7 ± 33.5 a
Cu + LamE (C)	12.3 ± 6.3 ab	2.2 ± 1.3 c	3.2 ± 3.0 bc	36.5 ± 31.6 abc	1.5 ± 1.3 bcd	11.7 ± 12.9 bcd
LamE + Cu (D)	20.3 ± 8.7 ab	2.7 ± 1.5 ab	6.2 ± 4.9 ab	55.3 ± 35.4 a	3.0 ± 2.1 ab	30.8 ± 27.5 ab
Control	21.8 ± 8.1 a	2.8 ± 1.4 a	6.9 ± 4.4 a	62.6 ± 36.9 a	3.5 ± 2.1 a	35.9 ± 28.1 a

[a] Data are means ± standard deviation. Values followed by different letters in the same column are significantly different (Tukey's honestly significant difference; $P \leq 0.05$).

[b] (A) = strategy A; LamE = laminarin applied with microbial extract of *Saccharomyces* spp.; Cu = copper hydroxide; (B) = strategy B; (C) = strategy C; (D) = strategy D.

Table 4. Disease incidence (DI), disease severity (Severity) and McKinney's index (MI) for the grape downy mildew infection recorded for the grapevine leaves and bunches on 29 July 2014, following the treatments with the different formulations during the season[a].

	29 July 2014					
	Leaves			Bunches		
Treatment (strategy)[b]	**DI (%)**	**Severity (1-10)**	**MI (%)**	**DI (%)**	**Severity (1-10)**	**MI (%)**
Bordeaux mixture (A)	29.8 ± 14.6 cd	2.9 ± 0.8 de	9.1 ± 5.2 d	47.4 ± 23.4 cd	1.6 ± 2.0 d	19.5 ± 21.0 c
Copper hydroxide (A)	27.6 ± 25.4 d	2.6 ± 1.1 e	9.2 ± 11.1 d	44.0 ± 30.5 d	2.1 ± 2.0 cd	18.5 ± 19.8 c
Chitosan (A)	50.4 ± 26.1 ab	3.5 ± 0.8 cd	19.1 ± 12.3 abc	53.3 ± 27.9 bcd	2.4 ± 1.6 abcd	24.6 ± 18.1 c
LamE (A)	58.9 ± 20.9 a	4.9 ± 0.8 a	28.7 ± 11.0 a	79.2 ± 25.1 ab	4.4 ± 2.1 ab	58.4 ± 28.6 a
Cu + chitosan (B)	44.2 ± 23.1 abcd	3.2 ± 1.0 cde	15.4 ± 10.2 bcd	50.5 ± 35.9 cd	2.1 ± 1.8 bcd	25.1 ± 23.8 c
Cu + chitosan (C)	33.1 ±19.9 bcd	2.8 ± 1.1 de	10.8 ± 8.6 cd	43.9 ± 31.9 d	1.2 ± 1.5 d	17.3 ± 19.5 c
Chitosan + Cu (D)	48.5 ± 23.8 abc	3.7 ± 0.8 bcd	18.5 ± 10.8 abc	53.1 ± 30.4 bcd	2.4 ± 2.2 bcd	29.2 ± 21.8 bc
Cu + LamE (B)	55.6 ± 24.1 a	4.0 ± 1.1 abc	23.7 ± 13.6 ab	73.0 ± 30.0 abc	4.5 ± 2.1 abc	53.5 ± 31.1 ab
Cu + LamE (C)	29.2 ± 15.1 cd	2.8 ± 1.0 de	8.9 ± 5.7 d	53.1 ± 33.6 bcd	2.3 ± 1.7 abcd	21.6 ± 14.2 c
LamE + Cu (D)	61.8 ± 21.1 a	4.5 ± 1.2 ab	28.3 ± 12.3 a	80.0 ± 26.2 ab	4.9 ± 2.2 a	65.5 ± 31.5 a
Control	61.3 ± 20.9 a	4.7 ± 1.1 ab	29.0 ± 10.7 a	83.8 ± 20.6 a	5.1 ± 2.2 a	67.3 ± 18.6 a

[a] Data are means ± standard deviation. Values followed by different letters in the same column are significantly different (Tukey's honestly significant difference; $P \leq 0.05$).
[b] (A) = strategy A; LamE = laminarin applied with microbial extract of *Saccharomyces* spp.; Cu = copper hydroxide; (B) = strategy B; (C) = strategy C; (D) = strategy D.

3.3.2.2 Second year (2015)

The first symptoms of GDM appeared on leaves in the middle of June 2015, about one month after the first treatment, and the disease assessments were carried out on the grapevine leaves on 1, 7 and 20 July 2015, while the surveys on grape bunches were carried out on 20 July, 4 August and 2 September 2015.

In the assessment of 7 July on leaves, disease incidence and McKinney's index were reduced significantly in all the plots in comparison to the untreated control (Table 5). Treatments with Bordeaux mixture, copper hydroxide, chitosan, LSA, copper hydroxide + chitosan (strategies B, C, D), and copper hydroxide + LSA (strategies B, C, D) reduced GDM incidence of 85%, 87%, 95%, 23%, 89%, 78%, 88%, 82%, 90%, and 46%, respectively and GDM McKinney's index of 90%, 91%, 96%, 31%, 92%, 84%, 92%, 85%, 93% and 56%, respectively. Disease severity was significantly reduced compared with the control by Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C, D), and copper hydroxide + LSA (strategies B, C) (Table 5).

In the assessment of GDM on grape bunches, carried out on 4 August, treatments with Bordeaux mixture, copper hydroxide, copper hydroxide + chitosan (strategies B, C), and copper hydroxide + LSA (strategies C, D) reduced significantly disease incidence of 52%, 73%, 62%, 59%, 49%, and 57%, respectively, compared to control (Table 5). In the same survey, considering the McKinney's index and the disease severity, significantly superior disease control compared with the control was observed after treatment with Bordeaux mixture, copper hydroxide, chitosan, copper hydroxide + chitosan (strategies B, C, D), and copper hydroxide + LSA (strategies B, C, D). Regarding McKinney's index, the disease reductions compared with the control were of 72%, 91%, 76%, 82%, 81%, 77%, 57%, 76% and 69% (Table 5).

Considering all of the disease surveys on 2015 here, the rank analysis for McKinney's index of GDM provided the following order of effectiveness of these treatments. On grapevine leaves: chitosan > copper hydroxide + LSA (strategy C) > copper hydroxide + chitosan (strategy B) > copper hydroxide + chitosan (strategy D) > Bordeaux mixture > copper hydroxide > copper hydroxide + LSA (strategy B) > copper hydroxide + chitosan (strategy C) > copper hydroxide + LSA (strategy D) > LSA > control. On the grape bunches, we recorded by order of effectiveness: copper hydroxide > copper hydroxide + chitosan (strategy B) > chitosan > copper hydroxide + chitosan (strategy C) > copper hydroxide + LSA (strategy C) > Bordeaux mixture > copper hydroxide + chitosan (strategy D) > copper hydroxide + LSA extracts (strategy B) = copper hydroxide + LSA (strategy D) > LSA > control.

Table 5. Disease incidence (DI), disease severity (Severity) and McKinney's index (MI) for the grape downy mildew infection recorded for the grapevine leaves on 7 July 2015, and on the grape bunches on 4 August 2015, following the treatments with the different formulations during the season[a].

	Leaves, 7 July 2015			Grape bunches, 4 August 2015		
Treatment (strategy)[b]	**DI (%)**	**Severity (1-10)**	**MI (%)**	**DI (%)**	**Severity (1-10)**	**MI (%)**
Bordeaux mixture (A)	2.3 ± 2.0 d	0.9 ± 0.5 cd	0.3 ± 0.2 d	17.5 ± 16.8 bc	1.0 ± 1.0 bc	4.1 ± 5.4 c
Copper hydroxide (A)	1.9 ± 2.2 d	0.7 ± 0.5 de	0.2 ± 0.3 d	10.1 ± 11.6 c	0.5 ± 0.6 c	1.3 ± 1.6 c
Chitosan (A)	0.7 ± 1.4 d	0.4 ± 0.6 e	0.1 ± 0.2 d	21.6 ± 23.1 abc	0.8 ± 0.7 bc	3.5 ± 3.6 c
LamE (A)	11.8 ± 5.3 b	1.4 ± 0.4 ab	1.7 ± 0.9 b	33.1 ± 24.7 ab	1.8 ± 1.5 ab	11.1 ± 11.6 ab
Cu + chitosan (B)	1.7 ± 1.8 d	0.8 ± 0.5 de	0.2 ± 0.2 d	14.1 ± 17.7 bc	0.7 ± 0.7 c	2.7 ± 4.4 c
Cu + chitosan (C)	3.4 ± 2.1 d	1.1 ± 0.4 bcd	0.4 ± 0.3 d	14.9 ± 27.4 bc	0.6 ± 0.6 c	2.8 ± 6.2 c
Chitosan + Cu (D)	1.8 ± 1.4 d	0.8 ± 0.5 de	0.2 ± 0.2 d	21.3 ± 23.0 abc	0.8 ± 0.6 c	3.3 ± 3.6 c
Cu + LamE (B)	2.7 ± 3.0 d	0.9 ± 0.6 cd	0.4 ± 0.5 d	24.6 ± 28.4 abc	1.2 ± 1.0 bc	6.3 ± 11.6 bc
Cu + LamE (C)	1.6 ± 1.7 d	0.8 ± 0.5 cd	0.2 ± 0.2 d	18.6 ± 17.6 bc	0.9 ± 0.7 bc	3.6 ± 3.8 c
LamE + Cu (D)	8.3 ± 4.2 c	1.3 ± 0.3 abc	1.1 ± 0.6 c	15.8 ± 14.8 bc	1.3 ± 1.3 bc	4.6 ± 6.6 bc
Control	15.3 ± 7.0 a	1.5 ± 0.4 a	2.4 ± 1.4 a	36.7 ± 24.6 a	2.6 ± 1.9 a	14.6 ± 12.0 a

[a] Data are means ± standard deviation. Values followed by different letters in the same column are significantly different (Tukey's honestly significant difference; $P \leq 0.05$).

[b] (A) = strategy A; LamE = laminarin applied with microbial extract of *Saccharomyces* spp.; Cu = copper hydroxide; (B) = strategy B; (C) = strategy C; (D) = strategy D.

3.3.3 Grape production

Grape production was not affected by the treatments in terms of either quantity or quality (Table 6). The production per plant and the quality parameters of the berry juice musts, such as the titratable acidity, sugar concentration and pH, were not significantly different among the treatments and the untreated control.

Table 6. Quantity and quality of the grape production parameters from the grapevines treated with the different formulations during the 2015 season[a].

Treatment[b]	Production /vine (Kg)	Sugar content (%Brix)	Total acidity (g/L)	pH
Bordeaux mixture (A)	6.45 ± 1.65	21.50 ± 0.76	3.96 ± 0.45	3.31 ± 0.04
Copper hydroxide (A)	7.65 ± 2.36	22.61 ± 0.91	4.32 ± 0.59	3.25 ± 0.04
Chitosan (A)	4.89 ± 0.66	22.11 ± 1.22	3.85 ± 0.10	3.26 ± 0.04
LamE (A)	6.53 ± 1.17	21.70 ± 0.53	3.66 ± 0.29	3.33 ± 0.05
Cu + chitosan (B)	8.15 ± 3.12	22.44 ± 1.78	4.23 ± 0.43	3.30 ± 0.03
Cu + chitosan (C)	7.20 ± 0.93	22.67 ± 0.55	3.85 ± 0.28	3.28 ± 0.03
Chitosan + Cu (D)	6.45 ± 0.74	22.47 ± 1.24	4.11 ± 0.32	3.31 ± 0.08
Cu + LamE (B)	7.79 ± 1.50	22.08 ± 0.85	4.35 ± 0.70	3.29 ± 0.06
Cu + LamE (C)	6.64 ± 1.94	21.49 ± 1.10	4.09 ± 0.39	3.27 ± 0.03
LamE + Cu (D)	6.99 ± 1.09	22.20 ± 0.30	4.16 ± 0.35	3.28 ± 0.07
Farm application	7.95 ± 2.82	23.22 ± 0.63	4.01 ± 1.00	3.41 ± 0.12
Control	6.17 ± 1.83	22.31 ± 0.38	3.80 ± 0.57	3.38 ± 0.10

[a] Data are means ± standard deviation.
[b] (A) = strategy A; LamE = laminarin applied with microbial extract of *Saccharomyces* spp.; Cu = copper hydroxide; (B) = strategy B; (C) = strategy C; (D) = strategy D.

3.4 DISCUSSION AND CONCLUSIONS

The two years over which this trial was conducted were characterized by different climatic conditions and, consequently, the data obtained relating to the parameters examined were very different in each year. The surveys allowed to find differences in the effectiveness of the treatments and of the strategies with the copper-based products and the natural compounds applied to the vegetation.

On the basis of our field trials, in condition of low disease pressure, all the formulations applied, except LSA, provided good crop protection against GDM.

In conditions of high disease pressure, good effectiveness was ensured by both the copper-based products, by chitosan applied alone or in strategy with copper and by the application of copper hydroxide in strategy with LSA, in this last case using copper in the first half of treatments and the natural product in the second half of the applications. In particular, the formulations that provided the best grapevine protection were Bordeaux mixture, copper hydroxide and this last product in strategy with chitosan, applying copper in the first half of treatments and chitosan in the second one.

Both Bordeaux mixture and copper hydroxide provided the best protection against GDM under both the higher (2014) and lower (2015) disease pressure. The presence of abundant rainfall in the growing season of 2014 has not altered the persistence of both the products, although they are contact fungicides. However, the amount of copper for the treatment with Bordeaux mixture using this strategy were twice that allowed in organic agriculture in the European Union, while with copper hydroxide they were close to the limits imposed by European Union Regulation 473/2002, recently extended to IPM, that may be further reduced in the near future for organic agriculture.

Among the natural formulations individually applied, chitosan provided a good crop protection applied alone or together with copper compounds under both low and high disease pressure. The effectiveness of chitosan for the reduction of downy mildew incidence and severity has also been reported for different plant species, through enhanced expression of genes related to defense proteins (Sharathchandra et al., 2004; Farouk et al., 2008; Manjunatha et al., 2008; Nandeeshkumar et al., 2008). Some studies on GDM have shown that foliar application of chitosan can significantly reduce infections caused by *P. viticola* on leaves and bunches (Aziz et al., 2006; Dagostin et al., 2011; Maia et al., 2012; Romanazzi et al., 2016).

LSA under high disease pressure was not effective against GDM, and also in condition of low pressure it did not reduce symptoms compared to the untreated control, except for disease incidence on leaves. The lack of effectiveness observed in the grapevines treated with LSA partially disagrees with the results obtained in other field trials, where grapevines treated with this mixture reported less downy mildew symptoms on leaves and grapes in condition of low disease pressure (Romanazzi et al., 2016).

When copper hydroxide was used in strategy with the alternative compounds, a reduced GDM incidence, severity ad infection index were observed when the copper was applied in the first half of the season. The first half of treatments was carried out when grapevine was in the phenological phases of floral bud differentiation, bloom and fruit set, then results very susceptible to GDM (Ash, 2000). Copper hydroxide protected more appropriately the more susceptible phenological stages of grapevine than the other two natural formulations, and in particular the LSA.

In other studies that have been carried out on grapevines to determine the effectiveness against GDM of treatments with low-rate copper formulations, applied individually or in combination with chitosan, both the strategies guaranteed good crop protection, especially under conditions of low disease pressure (La Torre et al., 2010; Romanazzi et al., 2010). The treatments with the combination in different strategies of low copper concentrations and chitosan or LSA appeared to be appropriate to control GDM under low disease pressure (e.g., in 2015) and, using chitosan as second compound, also in condition of high disease pressure (e.g., in 2014). In the treatments where LSA or chitosan were applied in strategy with low copper rates, the quantity of copper was only 2.8 and 3.2 kg/ha/year, respectively during 2014 and 2015. This combination represents a good solution instead of treatment that use full doses of copper and represent a 50% reduction in copper kg/ha/year. Moreover, it has been shown that low copper formulations allow to reduce the quantity of copper in pruning wood and in the soil even in the spring after applications, compared to full doses formulations (Duca et al., 2016). On the other hand, the treatment with chitosan, which uses no copper at all, gave good levels of protection, although these were lower than those obtained with the application of copper formulations under high disease pressure in 2014.

Chitosan can be considered as an alternative product to copper, applied individually or in strategy with low copper formulations, especially in organic farming, in light of the recent approval of chitosan hydrochloride for its use in agriculture as a plant protection product by European Commission Regulation (EU) number 563/2014 of 23 May 2014. The use of copper in the first treatments followed by the applications of chitosan might be useful to limit GDM symptoms, in condition of both high and low disease pressure, and the negative impact of copper accumulation in the topsoil, consequently to its excessive use. However, it remains necessary to carry out large-scale vineyard trials to verify the effectiveness of these GDM management strategies, and to further define their effects on the quality of the wines that are produced.

3.5 REFERENCES

Alfonzo, A., Piccolo, S. L., Conigliaro, G., Ventorino, V., Burruano, S., Moschetti, G., 2012. Antifungal peptides produces by *Bacillus amyloliquefaciens* AG1 active against grapevine fungal pathogens. Ann. Microbiol. 62:1593–1599.

Ash, G., 2000. Downy mildew of grape. The Plant Health Instructor DOI: 10.1094/PHI-I-2000-1112-01.

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bézier, A., Lambert, B., Joubert, J.M., and Pugin, A., 2003. Laminarin elicits defense responses in grapevine and induces protection against *Botrytis cinerea* and *Plasmopara viticola.* Mol. Plant-Microbe Interact. 16:1118–1128.

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M., Vernet, G., 2006. Chitosan oligomers and copper sulfate induce grapevine defense reactions and resistance to gray mold and downy mildew. Phytopathology 96:1188–1194.

Caffi, T., Rossi, V., and Bugiani, R., 2010. Evaluation of a warning system for controlling primary infections of grapevine downy mildew. Plant Dis. 94:709–716.

Commission Regulation (EC) No 473/2002 of 15 March 2002 amending Annexes I, II and VI to Council Regulation (EEC) No 2092/91 on organic production of agricultural products and indications referring thereto on agricultural products and food-stuffs, and laying down detailed rules as regards the transmission of information on the use of copper compounds. Official Journal L. 075:21–24.

Dagostin, S., Schärer, H. J., Pertot, I., Tamm, L., 2011. Are there alternatives to copper for controlling grapevine downy mildew in organic viticulture. Crop. Prot. 30:776–788.

Duca, D., Toscano, G., Pizzi, A., Rossini, G., Fabrizi, S., Lucesoli, G., Servili, A., Mancini, V., Romanazzi, G., Mengarelli, C., 2016. Evaluation of the characteristics of vineyard pruning residues for energy applications: effect of different copper-based treatments. J. Agric. Eng. 497:22–27.

El Hadrami, A., Adam, L. R., El Hadrami, I., Daayf, F., 2010. Chitosan in plant protection. Mar Drugs. 8:968–987.

Farouk, S., Ghoneem, K. M., Abeer, A. A., 2008. Induction and expression of systemic resistance to downy mildew disease in cucumber by elicitors. Egypt J. Phytopathol. 36:95–111.

Feliziani, E., Smilanick, J. L., Margosan, D. A., Mansour, M. F., Romanazzi, G., Gu, S., Gohil, H. L., Rubio Ames, Z., 2013. Preharvest fungicide, potassium sorbate, or chitosan use on quality and storage decay of table grapes. Plant Dis. 97:307–314.

Gessler, C., Pertot, I., and Perazzoli, M., 2011. *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 50:3–44.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., Lorito, M., 2004. *Trichoderma* species – opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2:43–56.

Howell, G. S., 2001. Sustainable grape productivity and the growth-yield relationship: a review. Am. J. Enol. Vitic. 52:165−174.

Khan, W., Rayirath, UP., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., Critchley, A. T., Craigie, J. S., Norrie, J. Prithiviraj, B., 2009. Seaweed extracts as biostimulants of plant growth and development. J. Plant Growth Regul. 28:386–399.

La Torre, A., Talocci, S., Miele, M., 2010. Evaluation of anti-downy mildew effectiveness and economic sustainability of substances of natural origin. Petria 20:46–48.

Lachhab, N., Sanzani, S., Adrian, M., Chiltz, A., Balacey, S., Boselli, M., Ippolito, A., Poinssot, B., 2014. Soybean and casein hydrolysates induce grapevine immune responses and resistance against *Plasmopara viticola*. Front Plant Sci. 5 716:1–10.

Liu, X. G., Gao, K. X., Kang, Z. S., He B. L., 2007. Systemic resistance induced by biocontrol agents in plants and its biochemical and cytological mechanisms. Chin. J. Appl. Ecol. 18:1861–1868.

Maia, A. J., Leite, C. D., Botelho, R. V., Faria, C. M. D. R., Machado, D., 2012. Chitosan as an option to control mildew in the sustainable vinegrowing. Semina: Ciênc Agrár (Londrina) 33:2519–2530.

Mandal, K., Saravanan, R., Maiti, S., Kothari, I. L., 2009. Effect of downy mildew disease on photosynthesis and chlorophyll fluorescence in *Plantago ovata* Forsk. J. Plant Dis. Prot. 116:164–168.

Manjunatha, G., Roopa, K. S., Prashanth, G. N., Shetty, H. S., 2008. Chitosan enhances disease resistance in pearl millet against downy mildew caused by *Sclerospora graminicola* and defence-related enzyme activation. Pest Manage. Sci. 64:1250–1257.

McKinney, H. H., 1923. Influence of soil, temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. J. Agric. Res. 26:195–217.

Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality and production. Food Res. Int. 43:1844–1855.

Nandeeshkumar, P., Sudisha, J., Ramachandra, K. K., Prakash, H. S., Niranjana, S. R., Shekar, S. H., 2008. Chitosan induced resistance to downy mildew in sunflower caused by *Plasmopara halstedii*. Physiol. Mol. Plant. Pathol. 72:188–194.

Pontiroli, R., Rizzotti. R., Zerbetto F., 2001. Low rate copper formulations in control of grapevine downy mildew in Oltrepò Pavese. Inf. Fitopatol. 10:62–66.

Puopolo, G., Giovannini, O., Pertot, I., 2013. *Lysobacter capsici* AZ78 can be combined with copper to effectively control *Plasmopara viticola* on grapevine. Microbiol. Res. 169:633−642.

Rabea, E. I., El Badawy, M., Rogge, T. M., Stevens, C. V., Höfte, M., Steurbaut, W., Smagghe, G., 2005. Insecticidal and fungicidal activity of new synthesized chitosan derivatives. Pest. Manage. Sci. 61:951–960.

Romanazzi, G., Feliziani, E., Bautista Baños, S., Sivakumar, D., 2017. Shelf life extension of fresh fruit and vegetables by chitosan treatment. Crit. Rev. Food Sci. Nutr. 57:579-601.

Romanazzi, G., Mancini, V., Feliziani, E., Servili, A., Endeshaw, S., Neri, D., 2016. Impact of alternative fungicides on grape downy mildew control and vine growth and development. Plant Dis. 100:1–10.

Romanazzi, G., Mlikota Gabler, F., Margosan, D., Mackey, B. E., Smilanick, J. L., 2009. Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. Phytopathology. 99:1028–1036.

Romanazzi, G., Santini, M., Murolo, S., Masciulli, A., D'Ercole, G., Patrizio, F., 2010. Evaluation of the effectiveness of copper formulations used at reduced rates and of alternative compounds in the control of *Plasmopara viticola*. Petria 20:9–12.

Romanazzi G., Nigro F., Ippolito A., Di Venere D., Salerno M., 2002. Effects of pre- and postharvest chitosan treatments to control storage grey mold of table grapes. J. Food Sci. 67:1862−1866.

Rusjan, D., Strlic, M., Pucko, D., Korosec-Koruza, Z., 2007. Copper accumulation regarding the soil characteristics in sub-Mediterranean vineyards of Slovenia. Geoderma 141:111–118.

Sharathchandra, R. G., Niranjan, Raj. S., Shetty, N. P., Amruthesh, K. N., Shekar Shetty H., 2004. A chitosan formulation Elexa induces downy mildew disease resistance and growth promotion in pearl millet. Crop Prot. 23:881–888.

Spadaro, D., Droby, S., 2016. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. Trends Food Sci. Technol. 47:39−49.

Speiser, B., Berner, A., Häseli, A., Tamm, L., 2000. Control of downy mildew of grapevine with potassium phosphonate: effectivity and phosphonate residues in wine. Biol. Agric. Hortic. 17:305–312.

Van Bruggen, A. H. C., Gamlielb, A., Finckhc, M. R., 2016. Plant disease management in organic farming systems. Pest Manage. Sci. 72:30–44.

4 EVALUATION OF LOW COPPER RATE TREATMENTS FOR THE CONTROL OF GRAPEVINE DOWNY MILDEW

Abstract

Grapevine downy mildew (GDM) is one of the most serious diseases of grapevines. With limitations in the use of copper-based products imposed for organic agriculture by the European Union, research for compounds at reduced dose of copper is encouraged. The aim of this research was to follow a two-year trial to evaluate the control of GDM using some compounds at reduced dose of copper on different cultivars and in different localities. In the first year, under low disease pressure, Bordoflow resulted in the lowest GDM incidence on leaves, while on grape bunches Coprantol Hi Bio the lowest GDM incidence. In the second year with high disease pressure Bordoflow again gave the best protection against GDM on leaves, while on grape bunches Heliocuivre provided the best GDM protection. The data obtained by this experiment confirmed that the cv. Pecorino was the less susceptible variety to GDM compared to cv. Montepulciano and cv. Passerina.

4.1 INTRODUCTION

Grapevine downy mildew (GDM) is caused by the biotrophic oomycete *Plasmopara viticola*, and it is one of the most serious diseases of grapevines worldwide (Gessler et al., 2011). It is potentially destructive especially in Europe, where grape-growing conditions are characterised by high humidity and abundant rainfall in the spring (Caffi et al., 2010). Over the last 20 years, the most widely used technique to control this pathogen was through the application of copper compounds, particularly in organic vineyards (Speiser et al., 2000). However, the massive use of copper at rates of up to 8 kg/ha per year has led to accumulation of this heavy metal in the topsoil in many European countries (Rusjan et al., 2007). Copper, as a result of canopy foliar sprays, lasts in wood residues and accumulates in the soil (Duca et al., 2016), where it cannot be metabolized by soil microorganisms, and is only eliminated from the vineyard through leaching. This excessive accumulation of copper in the topsoil has resulted in decreases in the carabid and earthworm populations, caused microbiological and enzymatic alterations, lowered the soil pH and reduced grapevine growth (Pontiroli et al., 2001).

As a consequence of these problems, the use of copper fungicides in organic agriculture was restricted by European Union Regulation 473/2002 (European Commission 2002). This Regulation limits the use of copper in organic farming to 6 kg/ha per year in the European countries.

The present study evaluated the effectiveness of foliar applications using compounds at reduced dose of copper, for the control of GDM on three wine grape cultivar (Montepulciano, Pecorino, Passerina) and in three different localities (Valtesino, AP; Forola, AP; San Giovanni, AP) whitin the Marche region. Compounds evaluated were Bordoflow, Heliocuivre and Coprantol Hi Bio used at the lowest dose of label.

4.2 MATERIALS AND METHODS

4.2.1 Vineyard treatments. The experimental field trials were conducted in 2013 and 2014 in three commercials vineyards of *Vitis vinifera* cv. Montepulciano, Pecorino and Passerina, situated in the localities of Valtesino (AP), Forola (AP) and San Giovanni (AP), in central-Italy. The plants were spaced by 1.0 m in the rows, with 2.2 m between the rows, and they were grown according to the Guyot trellis system, leaving 10 buds per vine, with grass cover between the rows. The height of the fruiting cane was 60 cm from the ground. Over the two years, four treatments were tested, compared with an untreated control; the active ingredients and the application rates are listed in Table 1. A randomised block design with four replicates was used, and the treatments were assigned to plots using a random-number generator (Excel; Microsoft Corp., Redmond, WA, USA). Each plot consisted of eight or ten grapevines along a row, and the treated rows were separated each from the other by an untreated row. The treatments tested in both the years were applied to randomly selected vines between the different years. Treatments began on 20 May, 2013, and on 22 May, 2014, when the vines become sensitive to GDM infection. At the time of first product application, plants were at the phenological stage of inflorescences swelling (BBCH 55) and the shoots were about 20 cm long. Treatments were repeated until August, with a total of 8 applications per year. The treatments were distributed by the spraying of a volume equivalent to 1,000 L/ha, using a motorised backpack sprayer (Honda GX 25, 25cc, 0.81 kW, Tokyo, Japan).

Table 1. Treatment formulations, application rates, and frequencies used in the present study.

Active ingredient (%)	Commercial product	Supplier	Application rate	Number of applications	Trial years
Copper hydroxide (26.2)	Heliocuivre	Biogard (IT)	300 ml/ha	8	2013; 2014
Copper hydroxide (25)	Coprantol Hi Bio	Syngenta (CH)	160 g/hl	8	2013; 2014
Copper sulphate (10)	Bordoflow	Manica spa (IT)	400 ml/ha	8	2013; 2014

4.2.2 Climate data. The weather parameters (i.e., minimum and maximum temperatures; rainfall) and the phenological stages of budbreak, bloom, and veraison were obtained from the data provided by the weather station of Offida (AP) and from the *Bollettino Agrometeoreologico* published by Agrometeo, ASSAM, Marche region.

4.2.3 Evaluation of grapevine downy mildew infections. Evaluation of GDM infections was carried out in all vines of each treatments on 100 leaves randomly selected per vine and all the clusters. During 2013, GDM symptoms were recorded for the grapevine leaves on 30 May, 6 June, 1 July and for the grape bunches scored on 2 September. During 2014, GDM symptoms were recorded for the grapevine leaves on 30 May, 1 July, and 9 August and on the grape bunches just on 9 August. The disease incidence was expressed as the percentage of infected leaves or grape bunches. Moreover, the disease severity on the leaves was assigned to 11 levels, according to the percentage of surface covered by the GDM symptoms: from 0 (uninfected leaf) to 10 (over 90% of leaf surface showing symptoms). For the grape bunches, the disease severity was assigned to eight levels, according to the number of infected berries and the percentage of the grape bunch that showed symptoms, as: 0, healthy grape bunch; 1, 1-5 infected berries; 2, 6-11 infected berries; 3, 12-25 infected berries; 4, 25% of grape bunch showing symptoms; 5, 26% to 50% of grape bunch showing symptoms; 6, 51% to 75% of grape bunch showing symptoms; and 7, >75% of grape bunch showing symptoms. The infection index (or McKinney's index), which incorporates both the incidence and severity of the disease, was expressed as the weighted means of the disease as a percentage of the maximum possible level. In detail, this was calculated according to Equation:

$$I = [\Sigma(d \times f) / (N \times D)] \times 100$$

where d is the category of disease intensity scored for the grapevine leaves or the grape bunches, f is the disease frequency, N is the total number of organs examined (healthy and rotted), and D is the highest category of disease intensity that occurred on the empirical scale (McKinney 1923).

4.2.5 Statistical analysis. The data were submitted to analysis of variance according to a randomised block design, and the means were separated by Tukey's HSD tests, at $P \leq 0.05$ (Statsoft, Tulsa, OK, US). The statistical analysis to determine the homogeneity of the variance was tested using Levene's tests.

4.3 RESULTS

4.3.1 Climate data. In the first year the unfavorable climatic conditions for disease development led to a low incidence of GDM (Figure 1A). In 2014 the abundant rainfall in spring, coincident with an increase in temperature, were favorable for the onset of GDM infection. With rainfall even during the months of July and August, the climatic conditions were favorable for GDM development throughout the growing season, and this led to high disease incidence (Table 2).

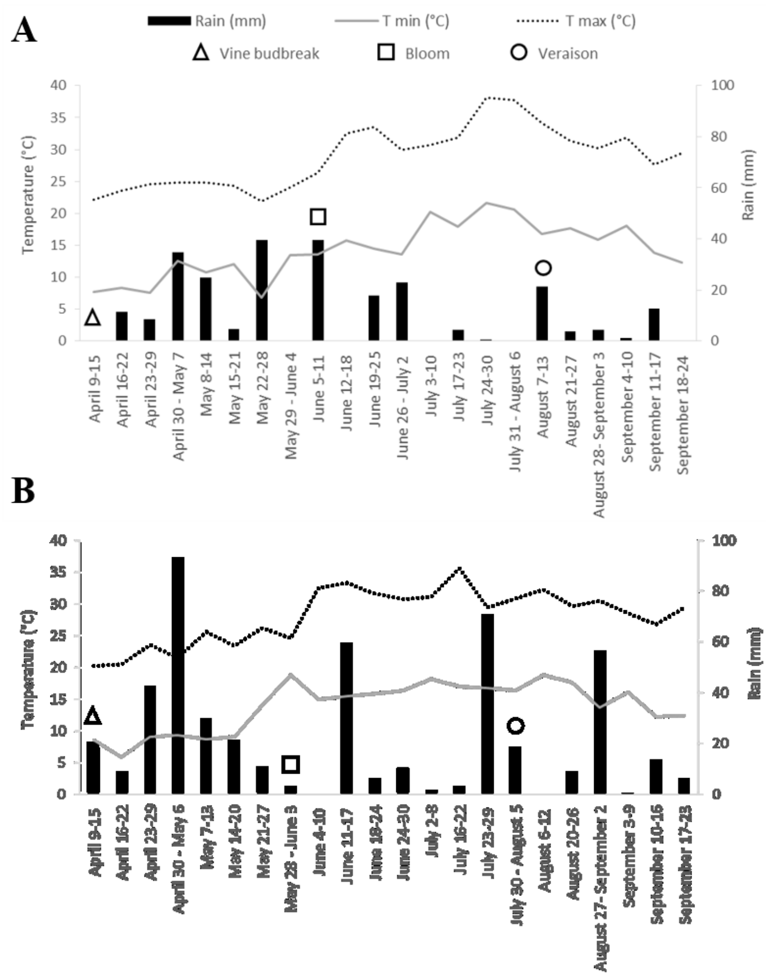

Figure 1. Climate data and main phenological stages of grapevines recorded through the study season, from April to September in 2013 (A), and 2014 (B) by the weather station located at Offida (AP).

4.3.2 Evaluation of grapevine downy mildew infection first year (2013). The first GDM infections appeared on the grapevine leaves on 30 May, 2013. In the survey the disease incidence was very low among all of the treatments, at <1%. In the following assessments, an increase in the infection levels of GDM on the leaves was recorded, with the highest values on 1 July, 2013, and soon after the harvest, on 2 September, 2013.

On 1 July, the grapevines cv. Montepulciano in locality Valtesino, treated with Bordoflow, Heliocuivre and Coprantol Hi Bio, did not show significant reductions for all the disease parameters on leaves compared to the control. The grapevines cv. Montepulciano in locality Forola showed significant reductions in GDM incidence compared to the control only when treated with Coprantol Hi Bio, while for the McKinney's index both Coprantol Hi Bio and Bordoflow guaranteed a significant reduction compared to the control. The grapevines cv. Montepulciano in locality San Giovanni showed significant reductions in GDM incidence, McKinney's index and disease severity compared to the control when treated with all the three products (Table 2). In the assessment of GDM on grape bunches carried out on 2 September on cv. Montepulciano in locality Forola, the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 75%, 75% and 86%, respectively and the McKinney's index by 79%, 84% and 93% respectively. The survey carried out on 2 September on grape bunches on cv. Montepulciano in locality San Giovanni showed significant reductions in GDM incidence and McKinney's index compared to the control on plots treated with all the three products (Table 2).

On 1 July, the grapevines cv. Pecorino in locality Valtesino, treated with Bordoflow, Heliocuivre and Coprantol Hi Bio showed significant reductions in GDM symptoms on leaves compared to the control, reducing disease incidence by 45%, 34% and 43% respectively and McKinney's index by 75%, 61%, and 70%. The grapevines cv. Pecorino in locality Forola showed significant reductions in GDM incidence and McKinney's index on leaves compared to the control by treatments with Bordoflow and Heliocuivre. The grapevine leaves cv. Pecorino in locality San Giovanni showed significant reductions in GDM incidence and McKinney's index compared to the control only treatment with Coprantol-Hi Bio and Heliocuivre, disease severity only treatment Coprantol-Hi Bio reduced disease severity (Table 3). In the assessment of GDM on grape bunches carried out on 2 September on cv. Pecorino in locality Valtesino only the product Coprantol-Hi Bio showed significant reductions in McKinney's index and in disease severity, but did not show significant reduction in GDM incidence; on grape bunches surveys carried out on 2 September on cv. Pecorino in locality Forola only the treatment with Heliocuivre showed a significant reduction in GDM incidence, while for McKinney's index all the treatments showed significant reduction compared to the control. In locality San Giovanni no treatments showed any significant reduction in GDM compared to the control, due to the low disease level (Table 3).

On 1 July, the grapevines cv. Passerina in locality Valtesino, treated with Bordoflow, Heliocuivre and Coprantol Hi Bio showed significant reductions in GDM symptoms compared to the control, reducing disease incidence by 46%, 46% and 44% respectively and the McKinney’s index by 70%, 67%, 64%; on 1 July, the grapevines cv. Passerina in locality Forola treated with Bordoflow, Heliocuivre and Coprantol Hi Bio showed significant reductions in GDM symptoms compared to the control, reducing disease incidence by 52%, 50% and 46% respectively, and the McKinney’s index by 76%, 66%, 61%. No significant reductions were recorded for disease severity on plots treated with Coprantol Hi Bio. In locality San Giovanni the grapevines cv. Passerina treated with Bordoflow, Heliocuivre and Coprantol Hi Bio showed significant reduction in GDM symptoms compared to the control, reducing disease incidence by 76%, 64% and 82% respectively, and the McKinney’s index by 81%, 76%, and 89% (Table 4).

In the assessment of GDM on grape bunches carried out on 2 September on cv. Passerina in locality Valtesino, the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 50%, 70% and 41%, respectively; the McKinney’s index by treatments with Bordoflow and Heliocuivre of 63% and 74% and disease severity was significantly reduced by all the treatments. On 2 September on cv. Passerina in locality Forola the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 79%, 52% and 55%, respectively, the McKinney’s index was reduced by 85%, 57% and 63% and disease severity was reduced in all the treated plots; on 2 September on cv. Passerina in locality San Giovanni the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 62%, 59% and 70%, respectively, the McKinney’s index was reduced by 35%, 44% and 52% and disease severity was reduced only by plots treated with Coprantol Hi Bio (Table 4).

Considering all of the disease assessments, the rank analysis in 2013 (Romanazzi et al., 2009) provided the following order of effectiveness of the treatments on the grapevine leaves and the grape bunches. On leaves: Bordoflow > Heliocuivre > Coprantol Hi Bio > control. On grape bunches: Coprantol Hi Bio > Bordoflow > Heliocuivre > control.

4.3.3 Evaluation of grapevine downy mildew infection second year (2014). The first symptoms of GDM appeared on the leaves at the end of May 2014, and the disease assessments were carried out on 30 May, on 1 July and on 9 August 2014. The last survey on the grapevine leaves on 9 August, 2014, revealed a further increase in the disease pressure. On leaves of cv. Montepulciano in locality Valtesino, the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 20%, 20% and 22%, respectively, the McKinney's index was reduced by 30%, 36% and 35% and disease severity significantly reduced by all three treatments. On cv. Montepulciano in locality Forola, the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi-Bio by 31%, 32% and 40%, respectively and the McKinney's index was reduced by 33%, 32% and 46%, while for disease severity only the treatment with Coprantol Hi Bio showed significant disease reduction. In locality San Giovanni the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 16%, 30% and 45%, respectively and the McKinney's index by 41%, 41% and 48%; the disease severity was significantly reduced by all three treatments (Table 2).

In the assessment of GDM on grape bunches carried out on 9 August on cv. Montepulciano in locality Valtesino, the disease incidence was significantly reduced only by the treatment with Bordoflow, while for McKinney's index and disease severity not significant reductions compared to the control were recorded. In locality Forola all the three treatments showed significant reductions in GDM incidence compared to the control, while no treatment showed significant reductions compared to the control regarding McKinney's index and disease severity. In locality San Giovanni the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 32%, 41% and 36%, respectively, the McKinney's index by 45%, 52% and 48% and disease severity was significantly reduced by all three treatments (Table 2).

In the assessment of GDM on leaves carried out on 9 August on cv. Pecorino in locality Valtesino, the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 39%, 49% and 37%, respectively and McKinney's index by 41%, 52% and 38%, while disease severity was reduced only by Heliocuivre. In locality Forola all the disease parameters were significantly reduced compared to the control on leaves only by treatments with Coprantol Hi Bio, reducing incidence by 43% and McKinney's index of 48%. In locality San Giovanni the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 32%, 33% and 32%, respectively and McKinney's index was reduced by 54%, 37% and 37%, while disease severity only by treatments with Bordoflow and Coprantol Hi Bio (Table 3). In the assessment of GDM on grape bunches carried out on 9 August on cv. Pecorino in locality Valtesino, the disease incidence was significantly reduced compared to the control only by treatments with Bordoflow, while no significant differences compared to the control were recorded regarding McKinney's index and disease severity. In locality Forola the disease incidence and McKinney's index were significantly reduced compared to the control only by treatments with Bordoflow by 44% and 54% respectively; no significant differences were recorded compared to the control regarding disease severity. In locality San Giovanni the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 68%, 68% and 69%, respectively, the McKinney's index by 80%, 80% and 80% and disease severity was significantly reduced by all three treatments (Table 3).

In the assessment of GDM on leaves carried out on 9 August on cv. Passerina in all the localities all the disease parameters considered were significantly reduced by all the treatments compared to the untreated control. In locality Valtesino, the disease incidence was significantly reduced by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 21%, 31% and 37%, and the McKinney's index by 34%, 44% and 52% respectively, in locality Forola the disease incidence was significantly reduced by 36%, 32% and 35% and the McKinney's index by 42%, 40% and 44% respectively and in locality San Giovanni the disease incidence was significantly reduced by 21%, 22% and 50% and the McKinney's index by 47%, 53% and 73% respectively (Table 4). In the assessment of GDM on bunches carried out on 9 August on cv. Montepulciano in locality Valtesino the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 34%, 38% and 49%, respectively and the McKinney's index by 43%, 48% and 57%; the disease severity was significantly reduced by all three treatments. In locality Forola the disease incidence was significantly reduced compared to the control by treatments with Bordoflow, Heliocuivre and Coprantol Hi Bio by 21%, 22% and 50%, respectively and the McKinney's index was reduced by 47%, 53% and 73%, while no significant reductions were recorded regarding disease severity. In locality San Giovanni the disease incidence was significantly reduced compared to the control by treatments with

Bordoflow, Heliocuivre and Coprantol Hi Bio by 17%, 26% and 40%, respectively and the McKinney's index was reduced by 49%, 61% and 73%; the disease severity was significantly reduced by all three treatments (Table 4).

Considering all of the disease assessments, the rank analysis in 2014 (Romanazzi et al. 2009) provided the following order of effectiveness of the treatments on the grapevine leaves and bunches. On leaves: Bordoflow > Heliocuivre = Coprantol Hi Bio >control. On grape bunches: Heliocuivre > Bordoflow >Coprantol Hi Bio >control.

Table 2. Disease incidence and severity, and McKinney's index for the GDM infection recorded for the grapevine leaves and bunches, cv. Montepulciano, on 1 July and 2 September, 2013, and on 9 August, 2014, following the treatments with the different formulations during the season.

Treatment and locality[b]	1 July, 2013[a]			2 September, 2013[a]			9 August, 2014[a]					
	Leaves			Grape bunches			Leaves			Grape bunches		
	Disease incidence (%)	Disease severity (1-10)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-7)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-10)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-7)	McKinney's index (%)
Valtesino												
Bordoflow	26.5±20,5 a	2.3±2.0 a	7.9±6.0 a				33.5±4.5 b	3.9±0.2 b	13.3±2.2 b	5.9±1.3 b	4.0±0.7 a	2.3±0.6 a
Heliocuivre	25.1±19.0 a	1.8±1.3 a	6.5±5.2 a				33.4±5.3 b	3.6±0.3 b	12.3±3.0 b	7.1±1.7 ab	3.6±0.6 a	2.6±0.8 a
Coprantol Hi Bio	28.7±21.5 a	1.8±1.3 a	7.6±5.9 a				32.6±6.7 b	3.8±0.7 b	12.5±3.2 b	8.0±1.8 a	3.6±0.7 a	2.9±0.8 a
Control	20.0±14.8 a	1.9±1.4 a	5.6±4.4 a				41.2±4.3 a	4.6±0.4 a	19.1±3.2 a	6.8±1.8 ab	3.8±1.1 a	2.6±0.9 a
Forola												
Bordoflow	5.7±4.0 ab	1.2±0.8 a	0.8±0.7 b	1.8±1.5 b	3.1±2.8 b	0.9±0.8 b	21.4±2.6 b	3.1±0.1 a	6.7±0.9 b	4.0±0.5 b	2.5±0.6 a	1.0±0.3 a
Heliocuivre	6.6±4.3 ab	1.1±0.6 a	0.9±0.7 ab	1.8±1.6 b	2.2±2.4 b	0.7±0.8 b	21.1±2.9 b	3.2±0.2 a	6.8±1.1 b	4.4±0.9 b	2.7±0.5 a	1.2±0.3 a
Coprantol Hi Bio	4.8±4.9 b	1.0±0.6 a	0.6±0.4 b	1.0±1.1 b	2.0±2.3 b	0.3±2.4 b	18.9±2.2 c	2.8±0.1 b	5.4±0.7 c	4.0±0.8 b	2.4±0.6 a	1.0±0.2 a
Control	8.0±5.0 a	1.3±0.8 a	1.4±1.0 a	7.1±4.1 a	4.9±2.7 a	4.3±2.5 a	31.4±2.9 a	3.1±0.1 a	10.0±1.1 a	5.4±0.7 a	2.6±0.3 a	1.4±0.3 a
San Giovanni												
Bordoflow	2.2±1.6 b	1.6±0.7 b	0.4±0.4 b	0.7±0.8 b	2.7±3.0 ab	0.38±0.4 b	16.8±2.8 b	1.6±0.2 b	2.8±0.8 b	12.84±3.2 b	1.6±0.3 b	2.1±0.8 b
Heliocuivre	2.8±2.7 b	1.4±1.0 b	0.6±0.5 b	0.4±0.5 b	2.2±2.9b	0.27±0.3 b	14.1±3.1 bc	1.6±0.3 b	2.3±0.7 bc	11.19±2.6 b	1.4±0.4 b	1.7±0.9 b
Coprantol Hi Bio	1.7±2.1 b	1.1±0.9 b	0.3±0.4 b	0.9±1.1 b	3.1±3.0 ab	0.47±0.6 b	10.9±2.9 c	1.4±0.2 b	1.6±0.7 c	12.19±3.6 b	1.5±0.3 b	1.9±0.7 b
Control	6.0±3.3 a	1.9±0.7a	1.3±1.0 a	2.0±1.4 a	4.3±1.7 a	1.08±0.8 a	20.2±4.0 a	2.7±0.4 a	5.5±1.5 a	18.91±3.8 a	2.9±0.5 a	5.5±1.6 a

[a] Data are means ± SD.

[b] Values followed by different letter(s) in the same column are significantly different (Tukey's HSD; $P \leq 0.05$).

Table 3. Disease incidence and severity, and McKinney's index for the GDM infection recorded for the grapevine leaves and bunches, cv. Pecorino, on 1 July and 2 September, 2013, and on 9 August, 2014, following the treatments with the different formulations during the season.[a]

Treatment and locality[b]	1 July, 2013[a]			2 September, 2013[a]			9 August, 2014[a]					
	Leaves			Grape bunches			Leaves			Grape bunches		
	Disease incidence (%)	Disease severity (1-10)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-7)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-10)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-7)	McKinney's index (%)
Valtesino												
Bordoflow	14.7±4.6 b	1.4±0.3 b	2.2±1.1 b	3.0±1.8 a	1.9±0.7 ab	0.7±0.4 ab	24.0±2.8 b	3.5±0.1 ab	8.6±1.1 b	5.9±1.3 b	4.0±0.7 a	2.3±0.6 a
Heliocuivre	17.7±4.7 b	1.8±0.5 b	3.4±1.1 b	2.9±1.8 a	2.2±1.3 ab	0.7±0.6 ab	20.1±2.4 c	3.4±0.3 b	7.0±1.0 c	7.1±1.7 ab	3.6±0.6 a	2.6±0.8 a
Coprantol Hi Bio	15.4±8.0 b	1.5±0.5 b	2.6±1.6 b	2.7±2.4 a	1.6±1.3 b	0.6±0.7 b	24.8±4.3 b	3.6±0.2 ab	9.0±1.4 b	8.0±1.8 a	3.6±0.7 a	2.9±0.8 a
Control	26.8±7.4 a	2.9±0.8 a	8.6±3.3 a	3.5±2.4 a	2.5±1.6 a	1.1±0.9 a	39.6±4.4 a	3.6±0.2 a	14.6±2.2 a	6.8±1.8 ab	3.8±1.1 a	2.6±0.9 a
Forola												
Bordoflow	3.4±2.4 b	1.2±0.9 a	0.5±0.4 b	1.5±1.5 ab	1.4±1.5 a	0.3±0.4 b	24.3±4.8 ab	3.3±0.2 ab	8.0±2.0 ab	4.7±1.0 b	2.6±0.4 a	1.3±0.3 b
Heliocuivre	2.8±2.4 b	1.0±0.9 a	0.4±0.5 b	0.9±1.2 b	1.0±1.5 a	0.2±0.3 b	25.9±4.2 ab	3.3±0.3 ab	8.7±1.6 ab	8.9±1.2 a	2.7±0.4 a	2.8±0.4 a
Coprantol Hi Bio	4.3±2.4 ab	1.4±0.9 a	0.7±0.5 ab	1.4±1.3 ab	1.6±1.7 a	0.3±0.4 b	18.6±4.5 b	2.6±0.4 b	6.1±2.2 b	8.6±2.1 a	2.2±0.4 a	2.1±0.8 a
Control	5.9±3.6 a	1.5±0.9 a	1.1±0.7 a	2.3±2.1 a	1.9±1.5 a	0.7±0.9 a	32.5±5.0 a	3.5±0.3 a	11.8±2.2 a	8.4±1.1 a	3.0±0.4 a	2.8±0.4 a
San Giovanni												
Bordoflow	0.4±0.6 ab	0.5±0.7 ab	0.0±0.1 ab	1.1±0.9 a	1.2±1.0 a	0.2±0.2 a	24.9±4.0 b	3.1±0.2 c	6.2±0.9 b	4.2±3.0 b	1.2±0.8 b	0.2±0.2 b
Heliocuivre	0.3±0.5 b	0.4±0.6 ab	0.0±0.0 b	1.1±1.1 a	1.2±1.4 a	0.2±0.2 a	24.4±3.2 b	3.4±0.2 ab	8.5±0.9 b	4.2±2.8 b	1.4±0.9 b	0.2±0.2 b
Coprantol Hi Bio	0.2±0.6 b	0.3±0.6 b	0.0±0.0 b	0.9±1.9 a	1.6±1.4 a	0.2±0.2 a	25.0±3.6 b	3.3±0.3 b	8.4±0.8 b	4.1±2.7 b	1.3±0.8 b	0.2±0.1 b
Control	0.9±0.7 a	0.8±0.5 a	0.1±0.1 a	1.8±1.3 a	1.8±1.2 a	0.4±0.3 a	36.6±5.3 a	3.6±0.3 a	13.4±2.3 a	13.2±1.9 a	2.2±0.4 a	1.0±0.2 a

[a] Data are means ±SD.

[b] Values followed by different letter(s) in the same column are significantly different (Tukey's HSD; $P \leq 0.05$)

Table 4. Disease incidence and severity, and McKinney's index for the GDM infection recorded for the grapevine leaves and grape bunches, cv. Passerina, on 1 July and 2 September, 2013, and on 9 August, 2014, following the treatments with the different formulations during the season.[a]

Treatment and locality[b]	1 July, 2013[a]			2 September, 2013[a]			9 August, 2014[a]					
	Leaves			Grape bunches			Leaves			Grape bunches		
	Disease incidence (%)	Disease severity (1-10)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-7)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-10)	McKinney's index (%)	Disease incidence (%)	Disease severity (1-7)	McKinney's index (%)
Valtesino												
Bordoflow	13.4±4.5 b	1.7±0.3 b	2.4±0.8 b	4.7±1.9 b	4.3±1.0 b	2.3±1.1 b	30.7±6.0 b	3.4±0.5 b	10.6±3.1 b	9.9±2.2 b	3.3±0.6 b	3.4±1.0 b
Heliocuivre	13.5±4.6 b	1.8±0.5 b	2.6±0.1 b	2.8±1.3 c	3.3±1.4 b	1.6±0.8 c	27.0±5.7 bc	3.2±0.5 b	9.0±2.8 bc	9.5±2.1 b	3.2±0.5 bc	3.1±0.9 b
Coprantol Hi Bio	14.0±4.5 b	2.0±0.4 b	2.9±1.2 b	5.5±2.1 b	4.0±1.2 b	2.6±1.3 ab	24.6±6.8 c	3.1±0.4 b	7.8±3.5 c	7.7±3.8 b	2.6±0.7 c	2.6±1.4 b
Control	24.7±7.1 a	3.4±0.9 a	8.1±3.1 a	9.4±1.3 a	6.7±0.1 a	6.3±0.9 a	39.2±5.4 a	4.0±0.3 a	16.1±3.1 a	15.2±2.5 a	4.0±0.4 a	6.0±1.1 a
Forola												
Bordoflow	6.1±4.0 b	1.5±0.9 b	1.2±0.9 b	1.4±1.4 c	3.6±2.9 c	0.7±0.8 c	22.5±3.9 b	3.4±0.2 b	7.8±1.9 b	3.6±0.5 b	2.4±0.4 a	0.9±0.2 b
Heliocuivre	6.4±3.9 b	1.5±0.9 b	1.3±0.9 b	3.2±1.8 b	5.1±2.2 b	2.0±1.3 b	23.9±3.2 b	3.3±0.1 b	8.1±1.2 b	3.9±0.6 b	2.4±0.4 a	0.9±0.2 b
Coprantol Hi Bio	6.8±4.7 b	1.6±1.0 ab	1.5±1.3 b	3.0±2.8 b	5.1±1.9 b	1.7±1.4 b	22.7±2.9 b	3.3±0.2 b	7.5±1.1 b	3.8±0.6 b	2.5±0.4 a	0.9±0.2 b
Control	12.8±8.6 a	2.3±1.4 a	3.9±3.0 a	6.8±1.7 a	6.8±0.2 a	4.7±1.1 a	35.4±3.6 a	3.7±0.2 a	13.5±1.8 a	4.8±0.5 a	2.6±0.5 a	1.2±0.2 a
San Giovanni												
Bordoflow	5.1±3.0 b	1.9±0.8 b	1.1±0.8 b	1.4±1.2 b	2.2±1.9 ab	0.4±0.5 b	17.4±3.7 b	1.7±0.3 b	3.2±0.9 b	14.2±4.0 b	1.7±0.4 b	2.6±1.1 b
Heliocuivre	7.4±2.7 b	1.7±0.5 b	1.4±0.5 b	1.5±1.2 b	1.9±1.6 ab	0.4±0.5 b	17.2±3.8 b	1.6±0.3 b	2.8±0.9 bc	12.7±3.7 bc	1.5±0.3 b	2.0±0.8 bc
Coprantol Hi Bio	3.7±2.9 b	1.1±0.6 b	0.6±0.5 b	1.1±1.1 b	1.6±1.8 b	0.3±0.5 b	11.0±3.7 c	1.1±0.2 b	1.6±0.8 c	10.4±2.6 c	1.3±0.3 b	1.4±1.5 c
Control	21.1±8.0 a	2.5±0.5 a	5.9±2.9 a	3.7±2.2 a	3.4±1.9 a	1.5±1.1 a	22.0±5.6 a	2.7±0.6 a	6.0±2.3 a	17.2±3.6 a	2.7±0.6 a	5.1±1.6 a

[a] Data are means ±SD.

[b] Values followed by different letter(s) in the same column are significantly different (Tukey's HSD; $P \leq 0.05$)

4.4 DISCUSSION

Based on the results obtained from the two-year experimental field trial, all the copper formulations tested have proved to be effective in the control of grapevine from *P. viticola*, also during 2014, a year with a growing season characterized by climatic conditions favorable to the development of GDM. In most of the surveys, the values for the three disease parameters, recorded in plots treated with all the copper-based products, were significantly lower than those in the untreated control. The data obtained by this experimental test also confirm the difference in susceptibility to GDM by the different with grapevine cultivars, with Montepulciano that showed higher disease levels than Passerina and Pecorino, resulting these latter varieties less susceptible to GDM. Considering the different locations where the test was conducted, in the site of San Giovanni the lowest incidence values were recorded, probably for higher ventilation in the vineyard, which reduced the relative humidity present; while in the site placed in location Valtesino the highest infection values were recorded in the first year. In the second year, no significant differences in disease development were recorded among the three localities. Among the different low-rate copper formulations in the first year, Coprantol Hi Bio has proved to be the most effective product in GDM control especially on bunches, compared to the other two formulations, while in the second year Heliocuivre ensured the best crop protection on bunches. In the protection of vegetation, the surprising result was given by Bordoflow which showed the highest effectiveness compared to the other formulations, despite having provided a lower copper intake (2 kg / ha of copper). Also in other studies low copper formulations, applied alone or together with alternative product, were effective in the protection of grapevine against downy mildew, especially in condition of low disease pressure (Romanazzi et al., 2010; Romanazzi et al., 2016).

In conclusion at the end of this two-year experimental study we can say that all the low-rate copper formulations, used to reduced copper intake, ensured good grapevine protection in all the vineyards and for all the tested varieties, inculding a year characterized by favorable conditions for the development of *P. viticola*.

4.5 REFERENCES

Caffi, T., Rossi, V., and Bugiani, R., 2010. Evaluation of a warning system for controlling primary infections of grapevine downy mildew. Plant Dis. 94:709–716.

Duca, D., Toscano, G., Pizzi, A., Rossini, G., Fabrizi, S., Lucesoli, G., Servili, A., Mancini, V., Romanazzi, G., Mengarelli, C., 2016. Evaluation of the characteristics of vineyard pruning residues for energy applications: effect of different copper-based treatments. J. Agric. Eng. 497:22–27.

Gessler, C., Pertot, I., and Perazzoli, M., 2011. *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathol. Mediterr. 50:3–44.

McKinney. H. H., 1923. Influence of soil, temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. J. Agric. Res. 26:195–217.

Pontiroli, R., Rizzotti, R., and Zerbetto, F., 2001. Low rate copper formulations in control of grapevine downy mildew in Oltrepò Pavese. Informatore Fitopatologico 10:62–66.

Romanazzi, G., Mancini, V., Feliziani, E., Servili, A., Endeshaw, S., and Neri, D., 2016. Impact of alternative fungicides on grape downy mildew control and vine growth and development. Plant Dis. 100:1-10.

Romanazzi, G., Mlikota Gabler, F., Margosan, D., Mackey, B. E., and Smilanick, J. L., 2009. Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. Phytopathology 99:1028–1036.

Romanazzi, G., Santini, M., Murolo, S., Masciulli, A., D'Ercole, G., and Patrizio, F., 2010. Evaluation of the effectiveness of copper formulations used at reduced rates and of alternative compounds in the control of *Plasmopara viticola*. Petria 20:9–12.

Rusjan, D., Strlic, M., Pucko, D., and Korosec-Koruza, Z., 2007. Copper accumulation regarding the soil characteristics in sub-Mediterranean vineyards of Slovenia. Geoderma 141:111–118.

Speiser, B., Berner, A., Häseli, A., and Tamm, L., 2000. Control of downy mildew of grapevine with potassium phosphonate: effectivity and phosphonate residues in wine. Biol. Agric. Hortic. 17:305–312.

5 ISOLATION OF *BOTRYTIS* SPP. FROM OVERWINTERED TREE AND GROUND TISSUES SAMPLED FROM CHERRY ORCHARDS IN CENTRAL OTAGO, NEW ZEALAND

5.1 INTRODUCTION

Botrytis cinerea is a necrotrophic pathogen able to infect a broad range of host crops and in cherries is responsible for *Botrytis* rot or postharvest gray mold.

The aim of this project was to survey cherry orchards from Central Otago region of New Zealand for resistance to the most commonly used fungicides, iprodione, carbendazim and the mixture boscalid + pyraclostrobin.

5.2 MATERIALS AND METHODS

5.2.1 Botrytis taxonomy

Recent research by Peter Johnson (Landcare Research, New Zealand) demonstrated that there are at least two closely related species of *Botrytis* in New Zealand vineyards (*B. cinerea* and *B. pseudocinerea*).

This project has not looked at the different *Botrytis* spp. in New Zealand cherry orchards, and hereafter we refer to all the isolates collected as *Botrytis* spp., until the genetic population structures are defined (Johnston et al., 2013).

5.2.2 Orchards

There were twenty-two cherry orchards representing different cherry growing regions in Central Otago were sampled. Four of these orchards were part of the original SummerGreen Futures project (2012–2015). Eleven orchards consisted of cv. Sweetheart, ten were cv. Lapins and one was cv. Stella.

5.2.3 Sampling procedure

There were 15 sample sites per orchard block and these were sampled using an M-sampling pattern. Each sample site was one or two trees. At each sample site, up to 35 cherry mummies were sampled from the ground under the tree(s) in the herbicide strip and then up to 15 cherry mummies or old fruit stalks were sampled from the tree(s), placed into separate paper bags and couriered to Plant and Food Research in Ruakura Research Centre.

The overall aim was to obtain 15–25 different *Botrytis* spp. isolates from each orchard (consisting of at least one isolate from each sample site, and 22 orchard blocks = 440 isolations total required for the survey of fungicide resistance to the most commonly used fungicides.

5.2.4 Tissue processing and induction of *Botrytis* spp.

In order to induce *Botrytis* spp. to produce fresh conidiophores, samples were placed onto one folded sterile paper towel in a sushi tray. One half of the paper towel was for ground samples and the other half for the tree sample. The area between the two samples was marked with a permanent black marker to ensure easy separation of samples. Each paper towel received 20 ml sterile distilled water (SDW) to ensure samples became re-imbibed and high relative humidity (HRH) was maintained during incubation. All tissues were spaced out to avoid clumping and ‘nesting’, and the number recorded. Each HRH chamber was closed and placed into 40L plastic bins.

In order to suppress secondary spread caused by food mites, shallow white plastic trays were lightly smeared with Odena oil and a black plastic grid placed on top of this to prevent oil coming into direct contact with the HRH chambers. Tap water was added to the base of the large plastic bins, sealed with a lid and incubated on the lab bench with natural and fluorescent light at 20°C for three to four days. The number of tissue samples with typical *Botrytis* spp. conidia from the ground and tree samples were counted and data expressed as the proportion (%) of tissues infected with *Botrytis* spp.

Botrytis spp. conidia and mycelium were carefully removed from infected samples with the aid of sterile wooden toothpicks and transferred to potato dextrose agar (PDA) plates that were amended with chloramphenicol (PDA+), then sealed with Parafilm. All plates were incubated in the lab at 20°C for three to four days and inspected for typical *Botrytis* spp. cultures. Pure cultures were obtained by removing a small section of PDA and mycelium from just behind the margin of rapidly growing cultures and placed mycelial surface down onto PDA+ plates. These cultures were sealed with Parafilm and incubated in the dark in a growth room held at 18°C. If any contaminants were present, the process of subculturing was repeated until a pure culture was obtained. After three or four days’ growth, six (5 mm diameter) mycelial plugs were removed and placed into a sterile micro tube with 2 ml steril distilled water for storage in a household refrigerator until required for resistance testing. Each *Botrytis* spp. isolate was sent to Plant Diagnostics Limited in Christchurch for subculturing and resistance testing.

5.3 RESULTS

Overall, the total number of tissue samples tested was 10258; of these 6581 were taken from the ground and 3667 were taken from the trees. The total number of *Botrytis* spp. infected samples from the ground and tree was 1170 (Incidence = 18%) and 439 (Incidence = 12%), respectively.

Botrytis spp. incidence (%) on the tree and ground samples combined ranged from 1 to 57%. The factors responsible for such a diverse range of *Botrytis* infection in cherry tissues are not known but are important from an epidemiological point of view and will be investigated by the PFR research team during the next growing season. Overall, and using the methods described earlier, at least 20 *Botrytis* spp. isolates per orchard were successfully obtained and all purified isolates were sent to Plant Diagnostics Limited in Christchurch for resistance testing. A smaller survey of *Botrytis* spp. sampled from four cherry blocks at pre flowering in 2013 found that 90% were resistant to carbendazim and 75% were resistant to iprodione. This study clearly showed that mummified cherry fruits from the ground and tree were acting as reservoirs of isolates of *Botrytis* spp. that were resistant to commonly used fungicides and were capable of successfully overwintering in cherry orchards under Central Otago conditions.

5.4 CONCLUSIONS AND RECOMMENDATIONS

This part of the SummerGreen Futures II project has confirmed the findings from a smaller survey carried out in 2013, during the SummerGreen Futures I project, that mummified cherry fruit (ground and tree) and cherry pedicels left in the tree from the previous season are an important source of overwintering *Botrytis* spp., thereby providing an inoculum bridge from the end of the previous season to potentially infect new blossom in the following spring. Research must now address the need to destroy these tissues in a cost-effective way and prevent *Botrytis* spp. from overwintering from one season to the next in this region. Analysis of spray diaries, including the products and active ingredients used from each CO block, will assist with identifying why some blocks had a high incidence of *Botrytis* spp. compared to blocks with a low incidence. Soil organic matter and other cultural operations management practices will also be investigated.

Experiments will be established in 2017 in selected orchards (with high *Botrytis* spp.) to evaluate the suppression of overwintering

Botrytis spp. by strip picking, using cost-effective mulches and tissue removal to ensure the trees remain free of any cherry tissues left behind after harvest (late summer 2017). Implementing these new postharvest practices will also assist with reducing the risk of fungicide resistant isolates successfully overwintering from one season to the next.

5.6 REFERENCES

Johnston, P. R., Hoksbergen, K., Park, D., Beever, R. 2014. Genetic diversity of *Botrytis* in New Zealand vineyards and the significance of its seasonal and regional variation. Plant Pathol. 63:888-898.

6 CONCLUDING REMARKS

Essential oils have deserved attention as natural preservatives used to prolong postharvest life of fruit. Essential oils could be applied as liquid solution (de Sousa et al., 2013), as additive in fruit coating such as co-formulant in chitosan coating (Romanazzi et al., 2017) or as biofumigant (Mehra et al., 2013). In general term, over liquid application, fumigation is to be preferred for the postharvest decay control since its use expects minimal handling of the food product and absence of the fruit wetting. However, fumigants being enable to penetrate into fruit surface, cannot control latent infections. Exposure to rosemary essential oil vapors was effective in reducing postharvest decay of table grapes. Our results corroborate previous trials. When rosemary essential oils were applied alone or in combinations with oregano essential oils to grapes artificially contaminated with spores of *Aspergillus flavus* and *A. niger*, the rate of fungal infection, revealed by visible signs of fungal growth on fruits, was delayed throughout the assessed storage period at both room (12 d) and cold temperatures (24 d) as compared to the control (de Sousa et al., 2013). In addition, essential oils of rosemary were effective in reducing postharvest decay of stone and pome fruits previously inoculated with fungal pathogens (Lopez-Reyes et al., 2010, 2013).

As demonstrated by weight loss of essential oils, under hypobaric condition the vaporization of essential oils was higher. This results are the consequence of Henry's law according to which the amount of dissolved gas into liquid solutions is proportional to its partial pressure in the gas phase. In our case, being the pressure into airtight containers lower than the atmospheric pressure, as a consequence, the amount of vaporization of essential oils was higher as compared to vaporization recorded with the same conditions at atmospheric pressure. Under hypobaric conditions, the decay of table grapes was reduce by exposure to mint essential oil vapors of around 60%, while at atmospheric pressure, maintaining the same condition, table grapes decay was not significantly reduced by mint essential oils. The higher effectiveness of mint vapors in reducing postharvest decay of table grapes under hypobaric conditions could be due to the higher amount of essential oils vapors or to the direct consequences of the hypobaric treatments. Essential oil fumigation treatment can be considered as a good alternative treatment due to the low volume used in table grapes decay control. It can be easily implemented at the packinghouses and does not cause deleterious effect on the skin and additionally it can be used in both organic and conventional agriculture. Furthermore, application of essential oil vapours as a biofumigant will be a good candidate for postharvest treatment since its application requires minimum handling in the packing line (Mercier and Jiménez, 2004; Cindi et al., 2016).

Grapevine downy mildew (GDM) is one of the most serious diseases of grapevine and until now, for limiting the disease spread, treatments were carried out mainly with copper compounds, producing several environmental damages caused by the accumulation of this heavy metal in the soil. Since the copper-based compounds are the only fungicides that can be used in organic farming, the research of alternative products is particularly important in this context. The field trials to evaluate alternative or low copper rate compounds against GDM highlighted that the use of copper may be reduced by applying it in mixture or alternated with other natural compounds, such as *Saccharomyces* spp. extracts or laminarin, or at low rate. The most promising alternative compound in limiting the disease was the biopolymer chitosan, which ensured an excellent grapevine protection under both high and low disease pressure. It was effective when applied alternately with copper-based products but also when applied as a single product for the whole duration of the growing season, proving to be able to completely replace copper-based compounds. Another positive aspect provided by chitosan is the limitation of plant vigour, with the reduction of growth parameters of grapevines (Romanazzi et al., 2016). The search for techniques to better manage excess plant vigour is essential in modern viticulture, in order to produce high quality grapes (Dry and Loveys, 1998; Chaves et al., 2007). All these positive features make chitosan an excellent compound to use in grapevine protection. Furthermore, in 2014 the use of chitosan has been regulated by the EU (Reg. 563/2014), which approved chitosan hydrochloride as the first basic compound that can be used as plant protection product. In the other field trial all the low copper rate formulations tested against GDM ensured a good crop protection, also under high disease pressure, especially Bordoflow on leaves and Heliocuivre on grape bunches.

Given the limitations in the use of conventional chemical products through the implementation of IPM, that is mandatory in the EU (Directive 2009/128/EC) by January 2014, the use of low environmental impact fungicides and alternative products will be increasingly frequent and the integration of different control measures will provide an adequate limitation of plant pathogens.

REFERENCES

Chaves, M. M., Santos, T. P., Souza, C. R., Ortuno, M. F., Rodrigues, M. L., Lopes, C. M., Maroco, J. P., Pereira, J. S., 2007. Deficit irrigation in grapevine improves water-use efficiency while controlling vigour and production quality. Ann. Appl. Biol. 150:237–252.

Cindi, M.D., Soundy, P., Romanazzi, G., Sivakumar, D., 2016. Different defenseresponses and brown rot control in two Prunus persica cultivars to essential oilvapours after storage. Postharvest Biol. Technol. 119, 9–17.

De Sousa, L. L., de Andrade, S. C. A., Athayde, A. J. A. A., de Oliveira, C. E. V., de Sales, C. V., Madruga, M. S., de Souza, E. L., 2013. Efficacy of *Origanum vulgare* L. and *Rosmarinus officinalis* L. essential oils in combination to control postharvest pathogenic *Aspergilli* and autochthonous mycoflora in *Vitis labrusca* L. (table grapes). Int. J. Food Microbiol. 165:312-318.

Dry, P. R., Loveys, B. R., 1998. Factors influencing grapevine vigour and the potential for control with partial rootzone drying. Aust. J. Grape Wine Res. 4:140–148.

Lopez-Reyes, J. G., Spadaro, D., Gullino, M. L., Garibaldi A., 2010. Efficacy of plant essential oils on postharvest control of rot caused by fungi on four cultivars of apples in vivo. Flav. Fragr. J. 25:171-177.

Lopez-Reyes, J. G., Spadaro, D., Prelle, A., Garibaldi, A., Gullino, M. L., 2013. Efficacy of plant essential oils on postharvest control of rots caused by fungi on different stone fruits in vivo. J. Food Prot. 76:631–639.

Mehra, L. K., MacLean, D. D., Shewfelt, R. L., Smith, K. C., Scherm, H., 2013. Effect of postharvest biofumigation on fungal decay, sensory quality, and antioxidant levels of blueberry fruit. Postharvest Biol. Technol. 85:109–115.

Mercier, J., Jiménez, J. I., 2004. Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus*. Postharvest Biol. Technol. 31, 1–8.

Romanazzi, G., Mancini, V., Feliziani, E., Servili, A., Endeshaw, S., and Neri, D. 2016. Impact of alternative fungicides on grape downy mildew control and vine growth and development. Plant Dis. 100:1-10.

Romanazzi, G., Feliziani, E., Bautista Baños, S., Sivakumar, D., 2017. Shelf life extension of fresh fruit and vegetables by chitosan treatment. Crit. Rev. Food Sci. Nutr. 57:579-601.

7 ACKNOWLEDGMENTS

Thanks go to Professor Gianfranco Romanazzi, tutor of my PhD project, Dr. Sergio Murolo and Dr. Lucia Landi for their availability in helping me during my PhD.

I would like also to thank Dr. Valeria Mancini, Dr. Erica Feliziani, Dr. Renzo Foglia for their support during the experimental trials.

Part of the PhD project research was carried out at the New Zealand Plant and Food Research, located at Ruakura, Hamilton. I am very thankful to Dr. Philip Elmer for the opportunity given to me and for his availability.