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Physiological responses of grapevines to biodynamic management

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Abstract

A 3-year (2011–2013) field trial was carried out in a mature vineyard (*Vitis vinifera* L., cv. Sangiovese), planted in 2003, to assess physiological responses of grapevines to biodynamic management. Starting in 2007, the vineyard was managed with organic production protocols in accordance with EC Regulations (834/2007). In 2008, the vineyard (2 ha) was divided in two large plots, with each plot having similar soil physico-chemical properties. One of the plots was managed with organic protocols per EC Regulations and the other with biodynamic practices, consisting of spray application of preparations 500, 500 K, fladen and 501. During the 2011–2013 season, the biodynamic preparations were used at least twice per year, with the exception of 501 that was applied only once in 2013. Concentration of hormones and mineral elements in biodynamic preparations were determined. Biodynamically managed vines showed lower stomatal conductance in all years and lower leaf water potential in 2012. Leaf photosynthetic activity was not influenced by cultivation method. Biodynamic management led to an increase in leaf enzymatic activities of endochitinase (EC 3.2.1.14), exochitinase (β-*N*-acetylhexosaminidase, EC 3.2.1.52 and chitin 1,4-β-chitobiosidase) and β-1,3-glucanase (EC 3.2.1.39), which are typically correlated with plant biotic and abiotic stresses and associated with induced plant resistance. Year effects were observed with 1,3-β-glucanase, whose activity in 2012 was 4.1-fold higher than in 2013. Disease incidence and grape yields were not different between organic and biodynamic treatments. This study provided a strong indication of a stimulation of natural defense compounds in grapes grown under biodynamic cultivation, but subsequent effects on plant protection and productivity require further evaluation.

Key words: *Vitis vinifera*, organic and biodynamic viticulture, stomatal conductance, biodynamic preparations, induced resistance

Introduction

Increased consumer awareness of environmental pollution in agriculture and the importance of food quality in relation to human health have encouraged the practice of alternative agronomic strategies, such as organic and biodynamic farming (Ponzio et al., 2013). The countries with the largest organic food markets are the USA, followed by Germany and France (Willer and Lernoud, 2014). Italy ranks sixth in global organic food markets at $1.95 billion compared with $23 billion in the USA (Willer and Lernoud, 2014). In the viticultural sector, the organic trend is particularly pronounced, due to the cultural and social role played by wine-making and the attention paid to the whole production and bottling cycle.

According to FIBL and IFOAM surveys (Willer and Lernoud, 2014), over 280,000 ha of organic grapes were grown in 2014, which constituted 4% of the world's grape growing area (7 million ha in 2011 (Food and Agriculture Organization of the United Nations)). In Europe, 240,000 ha, representing 6% of the harvested grape area, is under organic management. Spain, France and Italy represent the countries with the largest organic grape area, which includes more than 55,000 ha of

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organic grapes (Willer and Lernoud, 2014). A considerable increase in the supply of organic grapes is expected from these countries (Willer and Lernoud, 2014).

Approximately 147,000 ha are managed worldwide according to Demeter biodynamic standards. There are 520 Demeter wineries in the world, with a total of 8000 ha of vineyards (Demeter International, 2012). Biodynamic viticulture is spreading the fastest in Argentina, Chile and France. Biodynamic viticulture techniques can vary, depending on the grower's beliefs and rates of adoption. However, biodynamic growers face similar restrictions as organic producers regarding the disallowance of synthetic pesticides and fertilizers. Overall, biodynamic farming constitutes a holistic approach relying mostly on natural resources (Lotter, 2003). This perspective is achieved through the compulsory use of a set of specific fermented preparations applied on crops or soil in very small amounts. These preparations, most of them proposed by Rudolf Steiner (Steiner, 1924), are claimed to stimulate the soil nutrient cycle, enhance photosynthesis, and assist in optimal evolution of compost, increasing both soil and crop quality (Koepf et al., 2001). Biodynamic management is considered to induce beneficial environmental effects on the energetic efficiency of sustainable agro-ecosystems (Turinek et al., 2009) by intercepting, storing and multiplying cosmic forces to confer vitality to soil and plants (Steiner, 1924).

Biodynamic preparations applied to soil, compost and crops have been reported to promote efficient cycling of nutrients, stimulate soil and plant vitality, and improve animal health and crop quality (Pfeiffer, 1983; Turinek et al., 2009). These substances are not expected to act as fertilizers, but rather to 'harmonize environmental processes that naturally occur', enabling the plant to grow in a balanced way (Koepf, 1989).

The biodynamic farm proposes to be a self-sufficient system with all fertilizers and animal feed produced on the farm. Growers following the Demeter Protocol adopt specific management strategies in the vineyards for soil (e.g., tillage without turning over soil) and canopy (no shoot trimming). In the winery, spontaneous fermentation is practiced instead of fermentation by the use of commercial yeasts. However, several growers adopt biodynamic practices without following any specific private certification protocols. Although biodynamic currently represents an alternative agricultural cultivation method, some factors (e.g., agronomic, economical, institutional, commercial and social) may discourage its implementation in vineyards. Currently, there is little evidence regarding the agronomic effects of biodynamic practices, necessitating scientific investigations that will play a crucial role in providing objective information on the agronomical and physiological behavior of crops, such as grapes.

Several investigations have highlighted the phytoiatric effects (e.g., plant resistance-inducers) of microbial products of potential use in organic farming (Schmitt et al., 2004; Tamm et al., 2011). However, control of the main grape pathogens, *Plasmopara viticola*, *Uncinula necator* and *Botrytis cinerea*, is troublesome due to few protective treatments and because of the restriction on the amount of copper—limited to a maximum of 6 kg ha^{-1} yr^{-1}, regardless of cultivation method (EC Regulation 473/2002) (EC, 2002). In addition, the use of sulfur-containing compounds (Rauhut, 2009) may cause adverse effects on the sensorial properties of the wine (e.g., off-flavor). Finally, there are limitations on the amount of sulfites that can be added to the must during wine-making (EC, 2012) (e.g., 100 mg L^{-1} for organic red wine). Consequently, in organic and biodynamic vineyards, all field strategies, including canopy and soil management, strive to preserve and enhance plant health and resilience under biotic and abiotic stresses.

Research on the effect of biodynamic cultivation on grape physiology and productivity has been limited to one study of Reeve et al. (2005). On the other hand, the influence of biodynamic preparations on berry and wine composition and quality has been relatively extensively investigated (Reeve et al., 2005; Parpinello et al., 2015). Parpinello et al. (2015) demonstrated that the quality of 'Sangiovese' red wine was affected, to a great extent, by the application of biodynamic preparations. Moreover, by using 1H-NMR (Laghi et al., 2014), it was possible to discriminate between red wines from organic and biodynamic grapes. Such discrimination was also achieved by the droplet evaporation method (DEM) (Kokornaczyk et al., 2014).

The scientific literature on biodynamic grape cultivation almost exclusively focuses on berry and wine quality, without considering the important role played by leaves in plant physiology and health. Various explanations regarding the mode of action of biodynamic preparations on plants exist, including hormonal stimulation, and enhancement of plant growth, particularly at the root level (Stearn, 1976; Fritz and Köpke, 2005).

Plant hormones are organic molecules with the ability to regulate physiological, biochemical and metabolic processes in roots and shoots through the interaction with specific membrane receptors involving long-distance-signaling pathways, which in turn include specific secondary messengers of an organic or inorganic nature (Santner and Estelle, 2009). Major plant hormones are auxins (indole-3-acetic acid, IAA), abscisic acid (ABA) and cytokinins (CKs), although other classes of molecules are emerging as new plant hormones, such as salicylic acid and jasmonic acid (Shan et al., 2012). Auxins are involved in the regulation of plant developmental phases, such as phototropism, geotropism and hydrotropism. They also play a relevant role in the regulation of cell elongation and the development of lateral roots and absorbent hairs. The CKs are involved in the regulation of cell division and tissue differentiation, but also play a relevant role in the control of shoot:root balance, nutrient transport and root-to-shoot translocation as well as delaying leaf senescence. Main CKs derived from adenine and are

classified as aromatic CKs (topolin-types, benzyladenine) and isoprenoids (zeatin-types, isopentenyl-adenine-types) (Santner and Estelle, 2009; Shan et al., 2012). Abscissic acid is involved in the regulation of plant water homeostasis (leaf transpiration, stomatal closure) under osmotic stresses (Santner and Estelle, 2009; Shan et al., 2012).

Regulation of bacterial activity from biodynamic cultivation has also been reported; for example, lower values of the metabolic quotient for CO_2 were noted, suggesting higher complexity and diversity of soil microbial communities receiving long-term treatment of biodynamic preparation (Miller and Bassler, 2001). Chemical and molecular analysis conducted by Spaccini et al. (2012) on Horn Manure 500 preparation showed a complex composition of substances, including derivatives of lignin, and cyclic or linear lipids of plant and microbial origin. According to the authors, this particular molecular composition would make the 500 preparation more degradable than common compost, due to the high content of aromatic derivatives of lignin that are potentially more biologically active, promoting plant growth (Spaccini et al., 2012).

The hypothesis concerning the hormonal mode of action of biodynamic preparations proposed by Stearn (1976) was not supported by experiments and therefore remains questionable. It is well known, however, that plants exposed to a variety of biotic and abiotic stresses respond by inducing defense mechanisms (Kuć, 2001; Van Loon et al., 2006). Plants have the ability to acquire an enhanced level of resistance to pathogen attack or to different environmental changes after being exposed to several biotic or abiotic stimuli (Van Loon et al., 2006). Natural defense mechanisms of plants provide an alternative, non-conventional approach for plant protection through the use of new natural chemicals or bioagents (Dixon, 2001; Alabouvette et al., 2006). In grapes, stimulation of defense mechanisms was evidenced under various types of abiotic stresses or elicitor treatment in leaves, berries and inflorescences, as revealed by the increasing expression of genes encoding pathogenesis-related (PR) proteins or the stimulation of their corresponding activities (Trotel-Aziz et al., 2006; Petit et al., 2009). Both chitinase and glucanase activities have been detected in grapevine leaves following different stimulation (Derckel et al., 1996; Busam et al., 1997; Giannakis et al., 1998; Kikkert et al., 2000; Reuveni et al., 2001; Magnin-Robert et al., 2007). Several PR proteins have been characterized at the molecular level and have shown antifungal activity *in vitro* (Datta and Muthukrishnan, 1999). Some of them also have shown enzymatic activities, such as β-1,3-glucanase and chitinase, which are involved in the degradation of microbial cell wall structural polysaccharides (Kauffmann et al., 1987; Legrand et al., 1987). Salicylic acid, a well-known resistant inducer, was able to increase the basal leaf activity of chitinase by factor of 5.5 (Derckel et al., 1996). Bacteria applied by drenching cv. Chardonnay (*Vitis vinifera*) roots were also able to increase chitinase and ß-1,3-glucanase foliar activities from two- to fourfold (Magnin-Robert et al., 2007, 2013).

To our knowledge, there are no studies on the presence of hormones and potential resistance-inducing compounds in biodynamic preparations and their effects on biochemical defense mechanisms in grapes. In view of the above-mentioned considerations, this research aimed to characterize the composition of biodynamic preparations and evaluate their effects on leaf gas exchange, potential induced resistance and subsequent yields in 'Sangiovese' grapes in a Mediterranean climate.

Materials and Methods

Site description, experimental design and vineyard management

The long-term experiment was performed from 2008 to 2013 in a mature vineyard planted in 2003 with cv. Sangiovese grapes (clone FEDIT 30 ESAVE), *V. vinifera* L., grafted onto Kober 5BB, trained to spur pruned cordon (VSP). The vineyard was located in Tebano (Faenza, Emilia Romagna), Italy (44°17′7″N, 11°52′59″ E, 117 m a.s.l.), on a medium slope, with southeast/northwest and downhill-oriented rows. Vines were spaced at 2.8 m × 1.0 m (3571 plants ha^{-1}). Starting in 2007, this commercial vineyard was managed and certified as organic in accordance with EC Regulation 834, 2007 (EC, 2007). In 2008, the total surface (2 ha) was divided in two large uniform areas with similar soil chemical characteristics (Table 1). Each area was subjected to a specific cultivation method: (1) organic farming, managed according to the EC Regulation (EC, 2007) and (2) biodynamic farming, based on organic management, with the applications of biodynamic preparations according to Demeter standards. Each treatment covered seven plots, each with 12 monitored vines, for a total of 168 vines in the experiment. Randomization could have potentially caused problems in the management of this long-term experiment, due to possible effects of biodynamic preparations beyond the treatment area, including effects on soil microorganisms, mychorrizal fungi-mediated nutrient accumulation (Cheng and Baumgartner, 2004) and metabolite exchanges.

In 2011–2013, the number of buds (12–14) and bunches (11–15) was adjusted by winter pruning and cluster thinning, respectively. At the end of each vegetative season, herbaceous species were sown in alternate planting rows, such as fava bean (*Vicia faba*), barley (*Hordeum vulgare*), subterranean clover (*Trifolium subterraneum*) and mustard green (*Brassica juncea*) in both organic and biodynamic plots. Soil was managed by mowing the vegetation during late spring, which maintained biomass on the soil surface. Beginning in 2007, no irrigation water was applied, and the vineyard was not fertilized. A total of 14 soil samples (one sample from each experimental unit) were taken at 0–40 cm deep during the first year of the experiment (2008). The analysis showed no significant differences in the observed parameters (Table 1):

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Table 1. Soil chemical properties of organic and biodynamic experimental plots in 2008 at the beginning of the experiment.

Test type	Organic	Biodynamic	Significance
pH in H_2O	8.14	8.05	n.s.
Organic matter (OM)	1.9%	1.7%	n.s.
Total carbonates ($CaCO_3$)	16.6%	18.7%	n.s.
Active lime ($CaCO_3$)	8.11%	9.07%	n.s.
Total nitrogen (N)	1.42‰	1.31‰	n.s.
Assimilable phosphorus (P_2O_5)	33.43 μg g^{-1}	27.14 μg g^{-1}	n.s.
Exchangeable potassium (K_2O)	248 μg g^{-1}	289 μg g^{-1}	n.s.
Exchangeable sodium (Na)	46.57 μg g^{-1}	44.43 μg g^{-1}	n.s.
Exchangeable calcium (Ca)	3763 μg g^{-1}	3634 μg g^{-1}	n.s.
Exchangeable magnesium (Mg)	311 μg g^{-1}	277 μg g^{-1}	n.s.
Assimilable iron (Fe)	19.49 μg g^{-1}	18.35 μg g^{-1}	n.s.
Assimilable manganese (Mn)	7.55 μg g^{-1}	7.01 μg g^{-1}	n.s.
Assimilable zinc (Zn)	1.92 μg g^{-1}	2.08 μg g^{-1}	n.s.
Assimilable copper (Cu)	12.97 μg g^{-1}	16.27 μg g^{-1}	n.s.
Assimilable boron (B)	0.48 μg g^{-1}	0.45 μg g^{-1}	n.s.
Cation exchange capacity (CEC)	22.1 meq/100 g	21.2 meq/100 g	n.s.

n.s., no significance ($P \leq 0.05$).

these loamy clay alkaline soils contained medium–low organic matter (1.8%) and nitrogen (1.4‰) concentrations, high levels of carbonates (total carbonates 17.6%; active lime: 8.6%), medium–high content of assimilable phosphorus (P_2O_5: 30 μg g^{-1}) and potassium ions (K_2O: 268 μg g^{-1}), as well as assimilable iron (19 μg g^{-1}) and manganese (7 μg g^{-1}). Available copper in the soil was around 15 μg g^{-1}.

Climatic conditions

Overall, the 2011 vegetative season was marked by average temperatures well above the seasonal normal, with temperature peaks of 30°C in August. From bud burst to harvest, the average relative humidity (RH) varied from 40 to 70%; highest values were observed during spring (92%) and the lowest (38%) during the latter part of August. The total rainfall (204 mm) from bud burst to harvest was sporadic during spring and almost absent during ripening. In 2012, the average temperature showed an increasing trend beginning in March (10°C) until August, and then stabilized around 30°C, at the end of the month. The average RH from bud burst to harvest varied from 35 to 70% showing the lowest value (37%) at the end of July, while higher peaks occurred during May (88%) and at the beginning of September (89%). The total rainfall (342 mm), registered from bud burst to harvest, mainly occurred in spring. In the 2013 vegetative season, the average temperature increased from March (10°C) until the beginning of August (32°C) when the maximum temperature was around 40°C. From bud burst to harvest, the average RH was in the range of 40–90%, with the lowest peak (41%) occurring at the beginning of July and the higher at the beginning of May (89%) and the end of September (92%). The total rainfall recorded from bud burst to harvest was 433 mm and mainly occurred in spring, at the end of August (55 mm) and during the second half of September (52 mm).

Pest management and harvest sampling

Both organic and biodynamic experimental plots were treated to control diseases and pests in the same manner, using organic products allowed by the EC Regulation (EC, 2002). Treatments consisted mainly of copper (an average of 6 kg ha^{-1} yr^{-1}) and sulfur (an average of 70 kg ha^{-1} yr^{-1}), enabling control of fungal pathogens (*P. viticola, U. necator* and *B. cinerea*). At harvest, the incidence (number of affected clusters per vine) and severity (number of affected berries per cluster) of bunch rot were recorded for each cluster of 12 vines per experimental plot (84 per treatment).

Also at harvest, the number of clusters per plant, productivity per vine (kg) and bunch weight (g) (Wunder Digital Dynamometer, Wunder SA-Bi S.r.l, Milan, Italy) were determined on 12 vines per plot. After leaf abscission, pruning wood weight (kg) was recorded and the Ravaz Index (Ravaz, 1903) (yield/pruning wood ratio) was calculated on the same plants.

Biodynamic preparation characterization and application

The biodynamic preparations consisting of cattle manure-horn (500), 500 K, 501 (silica powder) and fladen were purchased from a regional producer (Fondazione le Madri, Rolo, Italy) and characterized in 2012, in our laboratory, using chemical analyses (see methods below).

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The 500 preparation was manure from a healthy bovine placed in a bovine's horn, buried in the earth over winter for 6 months and unearthed in spring. To make fladen, a hole was dug, covered with sticks of birch and then filled with fresh and compact cow manure, without straw and inoculated with compost preparations. Eggshells and basalt were mixed together for 1 h with a spade, then placed in the ground, inside a box with its base open to the soil, for 8 weeks. The fladen preparation was used when dark brown, crumbly and free of manure smell. Prepared Horn Manure (500P or 500 K) was developed by Alex Podolinsky in Australia (Podolinsky, 1989). It is made from Horn Manure (500) into which the six compost preparations, normally used for making the compost pile, were added. Preparations were stored in glass jars with slightly loose lids and stored under cool conditions, in an untreated wooden box surrounded by peat. The box was placed in a location free of power lines. The peat was kept moist and the condition of the stored preparations was checked periodically. The horn cattle manure preparations were kept moist all the time. The Horn Silica (501) was composed of fine crushed quartz powder. The preparation was mixed with rainwater and transferred to cow horns and buried in the soil until late September or early October, when the horns were unearthed, contents were extracted and stored in a glass jar until the time of application.

The following preparations were used in the vineyard experiment from 2008 until 2013: 500, fladen 500 K 501. In 2010, trunk paste (130 kg ha^{-1}), a mixture of fresh cow manure, horsetail and stinging nettle infusion, sand, bentonite and water, was applied to the trunks to protect vines from biotic and abiotic stresses. In each application of cattle manure-horn (500; 100 g ha^{-1}), fladen (100 g ha^{-1}), 500 K (100 g ha^{-1}) were dissolved in 30 liters of warm water (32°C). Tap water was stored for a few days in a wooden barrel before the contents were dissolved. Through the process of 'dynamization' the water was energetically stirred until a deep crater was formed in the rotating liquid; then the direction of stirring was reversed in order to produce a 'chaotic turbulence,' before gradually creating a new crater in the other direction. After 1 h the liquid was allowed to settle and poured through a sieve into a copper backpack sprayer and the product was immediately sprayed as droplets onto the soil. The application was performed in the afternoon.

Horn Silica (501) was used in very small quantities (5 g ha^{-1}). This preparation was dissolved in warm water (32°C) and the stirring procedure was similar to the one described for 20 min. Horn silica was sprayed with a copper backpack sprayer as a fine mist over the plants during early morning close to sunrise. In 2011, 2012 and 2013, the biodynamic preparations were used as follows: two applications of cattle-manure horn were applied in the spring period; one application of 500 K after harvest in autumn; silica powder was sprayed in summer three times in 2011, twice in 2012 and once in 2013; fladen was applied twice in 2011 and 2012 and three times in 2013, according to the Maria Thun calendar, when possible. The application times were modulated according to environmental conditions and observations on plants.

Plant hormone analysis

The following plant hormones were studied in this experiment: zeatin (Z), dihydrozeatin (DHZ), trans- and cis-zeatin riboside (t-ZR and c-ZR), dihydrozeatin riboside, isopentenyladenine, isopentenyladenosine, IAA and ABA. The extraction and purification of plant regulators were carried out using the method described by Dobrev and Kaminek (2002) and Aguirre et al. (2009). Liquid Chromatography–Mass Spectrometry quantification of cytokines (CKs): the CKs were quantified by HPLC linked to a 3200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using a reverse-phase column (Tracer Excel 120 ODSA 3 mm, 100 × 4.6 mm^2, Teknokroma, Barcelona, Spain). A linear gradient of methanol (A) and 0.05% formic acid in water (B) was used: 35–95% A in 11 min, 95% A for 3 min and 95–35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.25 ml min^{-1}, the injection volume was 50 ml, and the column and sample temperatures were 30 and 20°C, respectively.

Liquid Chromatography–Mass Spectrometry quantification of IAA and ABA: these hormones were quantified by HPLC linked to a 3200 Q TRAP LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada), equipped with an electrospray interface, using a reverse-phase column (Synergi 4 m Hydro-RP 80 A, 150 × 2 mm^2, Phenomenex, Torrance, CA). A linear gradient of methanol (A) and 0.5% acetic acid in water (B) was used: 35% A for 1 min, 35–95% A in 9 min, 95% A for 4 min and 95–35% A in 1 min, followed by a stabilization time of 5 min. The flow rate was 0.20 ml min^{-1}, the injection volume was 50 ml, and the column and sample temperatures were 30 and 20 °C, respectively. Detection and quantification were performed by multiple reaction monitoring in the positive-ion mode, employing a multilevel calibration graph with deuterated CKs, IAA and ABA as internal standards. Compound-dependent parameters are described in Aguirre et al. (2009). The source parameters included the following: curtain gas: 25.0 psi; GS1: 50.0 psi; GS2: 60.0 psi; ion spray voltage: 5000 V for CKs and 4000 V for ABA, IAA; CAD gas: medium and temperature: 600°C. The source parameters included the following: curtain gas: 25.0 psi; GS1: 50.0 psi; GS2: 60.0 psi; ion spray voltage: 4000 V; CAD gas: medium and temperature: 600°C.

Elemental analysis

Concentrations of C (total and humic acid-C) and N in dried samples of biodynamic preparations were

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determined by elemental analysis using a LECO CHN 900 analyzer (LECO Corporation, St Joseph, MI, USA).

In order to determine mineral nutrients (K, P, meso- and micro-nutrients) in biodynamic preparations, biodynamic preparation samples were dried at 100°C for 24–48 h in an air-oven, and sample digestions (250 mg of sample) were carried out with 3 ml of HNO_3 (65% Sigma-Aldrich Trace Metal grade) and 2 ml of hydrogen peroxide (33%, 'ultrapure' quality Merck, Darmstadt) at 100°C in a termostatized bath for 4 h. Each sample was diluted to 10 ml with 18.2 MΩ type 1 water. When fluorhydric acid was used, 1 ml of fluorhydric acid (40%, 'ultrapure' quality Merck, Darmstadt) was also used along with nitric acid and hydrogen peroxide as described previously.

Subsequently, samples were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) on a Thermo Scientific iCAP 6500 (Thermo Fischer, Waltham, MA).

Leaf gas exchange and water potential measurements

Measurement of leaf gas exchange in 2011, 2012 and 2013 was determined on three plants per experimental plot using an infrared gas analyzer (LI-COR 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, Li-Cor, Inc., Lincoln, NE). Measurements were performed in the morning between 9:00 and 11:00 AM on a leaf from the middle part of the shoot. Leaves were illuminated by the LI-COR 6400 LED light source providing a photosynthetic photon flux density ca. 1000 µmol $m^{-2} \cdot s^{-1}$. The level of CO_2 was fixed at 380 ppm within the leaf chamber. Net photosynthesis was measured when foliar CO_2 uptake was steady. Leaf water potential (MPa) was measured at midday during berry ripening on two plants per experimental plot using the Scholander pressure chamber method (Scholander et al., 1965). From each vine, one mature, completely expanded, sun-exposed, healthy leaf from the eighth node was selected. Leaf gas exchange and water potential were measured at least once annually.

Leaf enzymatic activities

Samples of ten leaves from each experimental plot were collected in June and July, 2012 and 2013. We sampled fully expanded young leaves, from the 8th node in June and the 12th node in July. Each leaf sample was weighed, quickly frozen in liquid nitrogen, and immediately ground to fine powder using a pre-chilled mortar and pestle. Proteins were extracted with a chilled sodium acetate buffer, 20 mM, pH 5.5 (1 ml g^{-1} fresh weight), containing 1% (w/v) polyvinylpolypyrrolidone (Sigma Chemical Co, St. Louis, MO). Extractions were carried out at 4°C for 90 min with continuous gentle stirring. Each crude extract was centrifuged twice at 12,000 g for 20 min at 4°C. The supernatant was filtered using a GV Millex® Syringe Filter Unit (Millipore Corporation, Billerica, MA) to remove solid particles. Protein concentrations were determined by the protein–dye-binding method (Bradford, 1976), using BSA (BioRad Laboratories, Inc., Hercules, CA) as a standard. Each enzyme activity assay was performed twice in triplicate.

Detection of 1,3-β-glucanase activity (EC 3.2.1.39)

1,3-β-glucanase activity in leaves was analyzed by measuring the rate of production of reducing sugars employing laminarin (Sigma Chemical St. Louis, MO), as the substrate and following a modified version of the procedure of Kauffmann et al. (1987). The reaction mixture was composed of a 0.4 ml sodium acetate buffer, 0.1 M, pH 5.2 containing 1 mg ml^{-1} laminarin and 100 µl enzymatic solution. After 3 h incubation at 37°C, 0.3 ml of alkaline copper reagent was added (Ashwell, 1957) and the mixture was heated at 100°C for 20 min. After cooling, 0.2 ml of Nelson's chromogenic reagent was added and the absorbance was measured at 660 nm (Ashwell, 1957). Glucose and enzyme standards and substrate blanks were included. One unit of 1,3-β-glucanase activity was defined as the amount of enzyme that released 1 mg glucose min^{-1}.

Detection of chitinase activity

The activities of endochitinase (EC 3.2.1.14), β-N-acetylhexosaminidase (EC 3.2.1.52) and chitin 1,4-β-chitobiosidase (Lorito, 1998) were assayed following a modified procedure by Tronsmo (Tronsmo and Harman, 1993). The three chitinase assays were based on colorimetric determinations of p-nitrophenyl cleaved from the chitin-analogous substrates, p-nitrophenyl-β-D-N,N′,N″-triacetylchitotriose, p-nitrophenyl-N-acetyl-β-D-glucosaminide and p-nitrophenyl-β-D-N,N′-diacetylchitobiose (all from Sigma, respectively). Fifty µl of each substrate stock solution (2 mg ml^{-1} in 50 mM acetate buffer, pH 5.0) were added to 30 µl of the crude protein extract from each sample. After incubation for 2 h in a water bath at 37° C, the reaction was stopped by adding 0.5 ml of 0.2 M Na_2Ca_3 and the absorbance was measured at 405 nm. Chitinase activities were calculated using an absorption coefficient for the p-nitrophenyl of 18.5 mM^{-1} cm^{-1}.

Statistical analysis

Comparison of means and analysis of variance between treatments (ANOVA) of vine physiological parameters (net photosynthesis, stomatal conductance and water potential), plant productivity and number of clusters per vine were performed using SAS 6.04 software (SAS Institute, Cary, NC). Means were compared by the Student–Newman–Keuls test ($P \leq 0.05$). Bunch weight,

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Table 2. Plant productivity, bunch number per plant, bunch weight at harvest from organically and biodynamically grown cv. Sangiovese vines in 2011–2013.

Year	Treatment	Productivity (kg plant^{-1})	Bunch (No. plant^{-1})	Bunch weight (kg)
2011	Organic	2.0	14	0.150
	Biodynamic	2.1	14	0.150
	Significance	n.s.	n.s.	n.s.
2012	Organic	2.6	15	0.161
	Biodynamic	2.6	15	0.160
	Significance	n.s.	n.s.	n.s.
2013	Organic	3.9	12	0.329
	Biodynamic	3.8	12	0.308
	Significance	n.s.	n.s.	n.s.

n.s., not significant.
Means followed by the same letter are not significantly different as per the Student–Newman–Keuls test, $P \leq 0.05$, (bunch number, plant productivity) and Kruskal–Wallis test, performed by Dunn's comparison test (bunch weight), $P < 0.05$.

bunch rot incidence and severity, and the Ravaz-index were analyzed using the Kruskal–Wallis non-parametric test, followed by Dunn's comparison test. Data on the activity of each enzyme were statistically analyzed by three-way ANOVA with treatment, year and month as main factors using SAS 6.04 software.

Results and Discussion

Plant parameters

Cultivation method (organic versus biodynamic) did not influence grape vegetative (pruning weight) and productive (yield) parameters (Table 2). Estimated yield was 7.64 t ha^{-1} for the organic system and 7.14 t ha^{-1} for the biodynamic system in 2011; 9.43 (organic) and 9.31 (biodynamic) in 2012; 13.99 (organic) and 13.39 (biodynamic) in 2013. In 2011 and 2012, the incidence of fungal diseases was negligible and was not influenced by treatments. However, in 2013, a minor incidence of bunch rot was observed during the latter part of September, but no differences in the incidence (organic: 4.3%; biodynamic 3.2%) and severity (organic: 0.8%; biodynamic: 0.7%) of *B. cinerea* were observed between cultivation methods. It is important point out that the 2011–2012 growing seasons were characterized by low rainfall, high temperatures and a general decline in plant productivity (Table 2).

Characterization of biodynamic preparations

The mineral profile of the biodynamic preparations is presented in Table 3. Both macro- and micro-elements were detected in all preparations. As expected, a high concentration of Si was found in 501, particularly when fluorhydric acid was used as extracting agent. Concerning plant regulators, the CK isopenthyl adenosine was found in the biodynamic preparation 500 (0.087 pM g^{-1}), 500 K (0.323 pM g^{-1}) and fladen (0.238 pM g^{-1}), whereas it was below detection limits in 501. In all the investigated preparations, the concentrations of isopenthyl adenine (CK), IAA and ABA were below the detection limits.

The concentration of isopenthyl adenosine found in the biodynamic preparations, 500, 500 K and fladen, was very low when compared with the concentration of CKs used in commercial products, which typically ranges between 0.1 and 1 mg l^{-1}. Considering that application rates of CK-based foliar products are between 1 and 3 liters ha^{-1} (0.1–3 mg CK ha^{-1}), the concentration of active CKs incorporated in soil with the biodynamic preparations (around 1.114×10^{-5} mg of isopenthyl adenosine per ha) was negligible.

We cannot ascribe observed physiological effects to a specific component of the biodynamic preparations. The extremely low amounts of plant regulators supplied by the biodynamic preparations suggest that the hormonal mode of action proposed by Stearn (1976) is unlikely. The silicon supplied by the biodynamic preparation 501 (quartz powder) may have induced some physiological effects detected in leaves, as silicon can stimulate plant resistance to biotic and abiotic stress (Ma and Takahashi, 2002; Van Bockhaven et al., 2013), but again, no direct evidence exists from this experiment for this theory. With regard to bunch rot infection in 2011–2013, no differences were observed between treatments. However, in 2010 vegetative season, characterized by frequent rainfall and elevated yields, significantly higher values were observed in organically managed experimental plots (34%) compared to the biodynamic ones (12%).

Leaf gas exchange and water potential

The application of biodynamic preparations reduced stomatal conductance in all years (reduction of 33.3, 54.7 and 90.6% in 2011, 2012 and 2013, respectively) and leaf water potential in 2012 (Table 4). Leaf photosynthetic

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Table 3. Concentration of mineral elements in the biodynamic preparations.

Mineral Elements										
(a) TOC, HC, TOC/HC, N, P, K, Ca, Mg, S										
Preparation	TOC[1] (%)	HC[2] (%)	TOC/HC (%)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)	S (%)	
500	19.60	9.90	50.00	1.93	0.43	0.64	3.10	0.66	0.35	
500 K	19.10	14.60	76.00	1.60	0.54	0.73	2.88	0.75	0.30	
Fladen	17.40	10.10	58.00	1.60	0.59	0.92	5.03	1.15	0.28	
501	0.11	n.d.	–	1.38	0.00	0.01	0.04	0.01	0.00	
501—FH[3]	n.d.[4]	n.d.	–	0.03	0.00	0.00	0.06	0.01	0.01	
(b) Na, Fe, B, Cu, Mn, Mo, Zn, Si										
Preparation	Na (%)	Fe (%)	B (μg g^{-1})	Cu (μg g^{-1})	Mn (μg g^{-1})	Mo (μg g^{-1})	Zn (μg g^{-1})	Si (μg g^{-1})		
500	0.06	1.22	29.70	45.80	576.60	1.10	108.00	37.00		
500 K	0.10	1.15	33.50	60.10	542.10	1.90	169.00	94.00		
Fladen	0.29	1.40	38.30	44.90	478.50	2.00	99.00	40.00		
501	0.01	0.01	0.00	0.30	0.90	0.00	1.00	586.00		
501—FH[3]	0.03	0.01	158.80	0.40	2.50	0.00	9.00	58,944.00		

[1] Total carbon.
[2] Humic acid carbon.
[3] Digested using fluorhydric acid.
[4] n.d. under limit of detection.

activity was not influenced by treatments (Table 3). One could compute water use efficiency (WUE) from these results using either gas exchange or carbon isotope ratios in leaf dry matter. In the shorter term, it is common to use leaf gas exchange measurements by relating net CO_2 assimilation rate (A_N) with stomatal conductance (g_s)—i.e., the so-called intrinsic water use efficiency ($WUE_i = A_N/g_s$) (Tomás et al., 2014). The parameters A_N and g_s are involved in carbon and water economy, respectively, and in grapes, a non-linear relationship between A_N and g_s has been reported, suggesting that the diverse responses by WUE_i are mostly related to variations in g_s (Medrano et al., 2012). In our study, grapevines treated with biodynamic preparations exhibited lower g_s conductance. Since photosynthetic activity was not influenced by treatments (Table 4), these results indicated a higher WUE (Chaves et al., 2010) in biodynamically managed vines, a relevant physiological and agronomic feature in grape cultivation considering that irrigation is not practiced and in some cases not allowed (e.g., some Protected Designation of Origin, PDO areas). Furthermore, reduction in stomatal conductance has been associated with enhanced plant tolerance toward biotic (Zeng et al., 2010) and abiotic stresses (Salazar-Parra et al., 2012, 2014), which is considered to be one of the key aspects of biodynamic viticulture by its practitioners (Turinek et al., 2009).

Leaf enzymatic activities

Cultivation method had a differential influence on leaf enzyme activities, including chitinases (endochitinase, β-N-acetylhexoaminidase and chitin 1,4-β-chitobiosidase) and 1,3-β-glucanase (Table 5). No significant interactions related to treatment × year and treatment × month were detected. In 2012 and 2013 years, leaf enzymatic activities were higher in plants under biodynamic management. Means from 2012 and 2013 data showed increases of 82.5, 52.3, 54.5 and 74.0%, respectively, for endochitinase, β-N-acetylhexoaminidase, chitin 1,4-β-chitobiosidase and 1,3-β-glucanase, compared with organic management. All enzymes displayed higher activity in 2012 than in 2013. Year effects were observed with 1,3-β-glucanase, whose activity in 2012 was 4.1-fold higher than in 2013.

In our experiment, the above-mentioned physiological data collected under field conditions are consistent with the enzymatic activities endochitinase, β-N-acetylhexosaminidase, chitin 1,4-β-chitobiosidase and 1,3-β-glucanase measured on leaves in the laboratory (Table 5). Differences in these enzymes were also detected between resistant and susceptible *Vitis* cultivars, including interspecific hybrids, on the order of ninefold for chitinase (from 0.18 to 1.98 cpm × 10^{-6}/h.g FWt) and sevenfold for β-1,3 glucanase (from 6.4 to 46.9 μmol h.g FWt^{-1}) (Giannakis et al., 1998). These enzymatic concentrations were differentially expressed in leaves of the two management systems, with leaves from the biodynamic system

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Table 4. Net photosynthesis, stomatal conductance and water potential measured on grapevines cultivated with the organic and biodynamic methods during the vegetative growth period in the 2011–2013.

Physiological parameters			
Net photosynthethis (μmol CO_2 m^{-2} s^{-1})			
Treatment	2011	2012	2013
Organic	6.91	5.98	11.47
Biodynamic	6.10	4.69	10.96
Significance	n.s.	n.s.	n.s.
Stomatal conductance (mol H_2O m^{-2} s^{-1})			
Organic	0.12 a	0.14 a	0.32 a
Biodynamic	0.08 b	0.06 b	0.03 b
Significance	*	*	*
Leaf water potential (−MPa)			
Organic	1.20	1.74 b	0.81
Biodynamic	1.32	1.96 a	0.90
Significance	n.s.	*	n.s.

n.s.;*, not significant; significant at $P = 0.05$, respectively.
Means followed by the same letter do not differ by the Student–Newman–Keuls test ($P \leq 0.05$).

Table 5. Activities of endochitinase, β-N-acetylhexosaminidase, chitin 1,4-β-chitobiosidase and 1,3-β-glucanase enzymes of grape leaves under organic and biodynamic management in 2012 and 2013.

Enzyme activity[1]				
Treatment	**2012**	**2013**	**Mean**	**Significance**
Endochitinase (U g $protein^{-1}$)				
Organic	5.38	1.57	3.48 b	*
Biodynamic	9.92	2.78	6.35 a	
Mean	7.65 a	2.18 b		
Significance	***			
β-N-acetylhexosaminidase (U g $protein^{-1}$)				
Organic	6.84	1.87	4.36 b	*
Biodynamic	10.26	3.01	6.64 a	
Mean	8.55 a	2.44 b		
Significance	***			
chitin 1,4-β-chitobiosidase (U g $protein^{-1}$)				
Organic	7.53	2.41	4.97 b	*
Biodynamic	12.07	3.29	7.68 a	
Mean	9.80 b	2.85 a		
Significance	***			
1,3-β-glucanase (U mg $protein^{-1}$)				
Organic	52.17	14.96	33.57 b	*
Biodynamic	95.91	20.95	58.43 a	
Mean	74.04 a	17.96 b		
Significance	**			

[1] One unit (U) of chitinase activity (endochitinase, β-N-acetylhexosaminidase and chitin 1,4-β-chitobiosidase) is defined as the amount of enzyme that releases 1 μM p-nitrophenyl min^{-1}; one unit of 1,3-β-glucanase activity is defined as the amount of enzyme that releases 1 mg glucose min^{-1}.
Within columns or lines, means followed by different letters differ significantly at different P levels: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

1.52- to 1.82-fold greater for chitinases and 1.74-fold greater for 1,3-β-glucanase, compared with the organic system (Table 5).

There have been several reports of a correlation between an increase of these enzymes and reduction of symptoms caused by *B. cinerea* (Magnin-Robert et al., 2007, 2013). Moreover, the effect of induced resistance by β-aminobutyric acid against downy mildew (*P. viticola*) (Reuveni et al., 2001) in grapes, as well as fosethyl aluminum against *Phaemoniella chlamydospora* and

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Phaeoacremonium aleophilum, the main pathogens linked to esca disease, has been documented (Di Marco et al., 2011). Also polyphenols have been cited to be involved in plant resistance mechanisms against biotic stress factors (Daayf and Lattanzio, 2008). It is noteworthy that their level was higher in biodynamically grown berries compared with organically grown vines (Reeve et al., 2005). Despite these reported effects in the literature, we did not observe any direct effect of increased enzymatic activities on improved plant defenses.

Another hypothesis is that silicon, which is contained in the biodynamic quartz powder, led to a decrease in stomatal conductance, as previously shown (Gao et al., 2006), and to increase chitinase and 1,3-β-glucanase enzyme activities (Chérif et al., 1994; Dann and Muir, 2002).

Concluding remarks

Since in our experiment different biodynamic preparations were used, we are not able to distinguish among effects arising from the distinct preparations. Moreover, the observed effects on vine physiology cannot be ascribed to a specific component of the biodynamic preparation. However, it is possible that silicon supplied by the biodynamic preparation 501 (quartz powder) may have played an important role. Biodynamic theories do not claim any particular substance effect, but rather, attribute the possible influence to the entire complex of biodynamic preparations (Steiner, 1924). Additional research is needed to further ascribe causal relationships between biodynamic preparations and plant physiological effects.

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