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Vibrational Signals for Mating Disruption Do Not Negatively Affect Grapevine Growth and Production

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ABSTRACT

Vibrational mating disruption (VMD) is a promising strategy to control *Scaphoideus titanus* populations in vineyards, and it is based on the prolonged application of a species-specific disturbance vibrational signal (DVS) on grapevines. Plants can react by different transcriptional, physiological, and morphological changes, according to the source and type of mechanical stimuli, but no information is available on possible side effects of DVS on grapevine plants. This study aimed to investigate grapevine response to DVS during VMD exposure under field and greenhouse conditions. No negative effects were observed on vine productivity, berry characteristics, and grape quality parameters in two consecutive seasons under field conditions. Chlorophyll, flavonol, and anthocyanin content, nitrogen balance index, stomatal conductance, electron transport rate, and leaf vapor pressure deficit were comparable in DVS-treated and control plants under field and greenhouse conditions. Moreover, no modulation of genes related to defense, growth, and secondary metabolism was found in the leaves of DVS-treated plants, indicating no negative impacts of VMD on grapevine physiology. The only observed difference associated with DVS treatment was an increased internodal length under field and greenhouse conditions with partial stimulation of shoot length. Although further studies are required to clarify the mechanism of internodal length stimulation, these results support the absence of negative effects of VMD on grapevines, encouraging its further application in commercial vineyards.

1 | Introduction

Vibrational mating disruption (VMD) is a novel behavioral manipulation technique for the control of pests [1] that is based on the propagation of a species-specific disturbance vibrational signal (DVS) through the plants to interfere with the mating communication of target insects (e.g., leafhoppers) to reduce their populations [2–4]. VMD was developed for the first time for *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae) since this species, as most Cicadellidae, relies on pherodones (i.e., species-specific vibrational signals) [5] to identify and localize mating partners [5, 6]. *Scaphoideus titanus* is a Nearctic leafhopper native to the deciduous forests of temperate North America, now largely distributed in Europe, where it is a major threat to grapevine production since it is the main vector of the flavescence dorée phytoplasma "16SrV-C and -D" [7]. This phytoplasma is a phloem-obligate pathogen that causes delayed or lack of bud break in spring, leaf curling downwards, and leaf yellowing (white grape variety) or reddening (red grape variety) in summer, drying of inflorescence and bunches, with scarce shoot lignification and negative impacts on grape yield and profitability of viticulture (removal of infected plants) [8]. The

control of *S. titanus* populations is the major strategy for limiting flavescence dorée phytoplasma infections, and it is based on mandatory insecticide treatments in several European countries as stated in EU Regulation 2022/1630 [8–10]. However, flavescence dorée phytoplasma is still spreading in several European regions and the tendency of the European policy to reduce the use of insecticides is fostering the development of alternative strategies, such as VMD, to decrease chemical treatments and increase the control efficacy against *S. titanus* [7, 8, 10]. The efficacy of VMD, with a significant reduction of *S. titanus* mating episodes, was demonstrated under controlled and field conditions [2, 3]. Nevertheless, the efficacy of VMD relies on the continuous transmission of a species-specific DVS to all the grapevine plants of the treated vineyard by stand-alone shakers (e.g., Tremos, CBC Europe) to mask the main frequency span of the natural insect pherodones (150–250 Hz) with an amplitude above the threshold of 1.5×10^{-2} mm/s [2, 3]. Similar to other mating disruption modalities, VMD should be applied in an area-wide concept in order to reduce the risk of reinfestation of the vineyards by insects who mated in untreated areas [11, 12]. However, no information is available on the possible side effects of DVS to grapevine plants.

Plants can react to mechanical stimuli (e.g., gravity, sound, substrate-borne vibrations, touch, and wind) [13–16], and morphological responses (called thigmomorphogenesis) can include changes in gene expression, flowering time, leaf folding, and stem elongation, according to the source and type of mechanical stimuli [13, 16, 17]. For example, genes related to defense response and signal transduction are known to be upregulated in response to touch stimuli in *Arabidopsis* plants [13]. Moreover, young roots of corn plants can grow toward the source of water-borne vibrations (frequencies of 200 and 300 Hz), indicating morphological changes in response to vibrational signals [15]. For example, plants respond to vibrational signals produced by insect chewing or flying, suggesting that vibrational signals contribute to induction of chemical defenses [18–23]. Thus, vibrational signals emitted by pests can provide a consistent set of cues, functioning as kairomones (i.e., vibrational signals that evoke a behavioral or physiological response in the receiver, i.e., beneficial to the receiver but not to the emitter) for activating plant response [5, 24]. Moreover, anthropogenic activities are changing the soundscapes and vibroscapes of our planet at an unprecedented rate and are considered to be a key threat to biodiversity [25]. For example, traffic noise can negatively affect growth and physiology of plants by inducing oxidative damage and interfering with hormonal balance in *Tagetes patula* and *Salvia splendens* [26].

Furthermore, the perception of herbivores in plants has been shown to lead to negative impacts in plant fitness, for example, in *Nicotiana attenuate*, where oral secretions from *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae) led to a decrease in photosynthetic carbon assimilation and stomatal conductance, suggesting that herbivore perception decreases photosynthetic carbon assimilation and reduces stomatal conductance [27]. Consequently, inquiring about possible impacts of DVS as a potential kairomone on plant physiology, but also measuring potential side effects on plant growth and fruit quality, is a fundamental step to understand the ecological impact of the VMD strategy and to further develop it. Therefore, since the utility of biomass, growth rate, and yield as an appropriate surrogate for fitness under many circumstances was highlighted [28], the aim of this study was to understand grapevine response to DVS by investigating shoot growth, internodal length, grape berry quality, physiological parameters, and gene expression levels in grapevine plants exposed to VMD compared to control plants under greenhouse and field conditions.

2 | Materials and Methods

2.1 | Application of VMD Under Field Conditions

The application of the DVS in the field followed previous described application of the VMD technique [3, 12]. In the experimental vineyard (*Vitis vinifera* cultivar Pinot Gris, clone: SMA-505, rootstock: SO4, "pergola doppia" as trellis with a planting distance of 4.5×0.5 m, GPS coordinates: 46°12′52″ N and 11°08′15″ E, area: 3100 m^2), the DVS was transmitted to the plants through the metal wires of the trellis using stand-alone shakers developed for *S. titanus* VMD and programmed to emit the species-specific DVS (Tremos; CBC Europe, Grassobbio, Italy) attached to the pole in the middle part of each row (25 m from both ends of the row). Based on the position of the shakers, three areas were identified in the vineyard: one DVS-treated area contained plants at a maximum 6 m of distance from the shaker, and two control areas contained plants at a distance greater than 15 m from the shakers (Figure S1). For each treatment, DVS-treated and control, 20 replicates (plants) were selected (±5% of the total number of plants in the vineyard). The vigor of the DVS-treated and control plants was comparable (i.e., stem diameter and number of buds after pruning). For each plant, vibrational amplitude and frequency profile were weekly measured with an accelerometer (model 352A24, sensitivity 100.2 mV g^{-1}; PCB Piezotronics, Depew, NY, USA) positioned on the second wire of the trellis next to the shoot and on the shoot in contact with that wire. The accelerometer was connected to a smartphone (iPhone 14 Pro, Apple Inc.) through a signal conditioner (DigiDAQ Model 485B39, PCB Piezotronics, Depew, NY, USA) and the recording was acquired using VibCloud (iTnnovate, Ohrid, Macedonia) with 6400 FFT lines and 50% overlap. The experiment was carried out in 2023 (season 1) and 2024 (season 2). Due to the physical nature of the treatment, which required the transmission of vibrations through the continuous trellis wires from a central shaker, randomized assignment of treated and control plants was not feasible. Season 1 trial was conducted with the aim of assessing growth and vine production differences in DVS-treated grapevine plants compared to control plants. Season 2 trial implemented the evaluation of physiological parameters to further understand the side-effects of DVS treatment on grapevine fitness.

2.2 | Application of VMD Under Greenhouse Conditions

Grafted grapevine plants (*Vitis vinifera* cultivar Pinot Noir, clone: ENTAV115, rootstock: SO4) were grown in 2.5 L pots containing a mixture of peat and pumice (3:1; GR Intensivo, Tercomposti, Calvisano, Italy) under greenhouse conditions with a 16 h light/8 h dark photoperiod at 25 ± 1°C and 70% ± 10% relative humidity (RH) on a simplified trellis (length: 70 cm, height: 120 cm) (Figure S2). A mini shaker (hand-made piezoelectric transducer, CBC Europe) was attached to the upper part of one pole, and the

DVS was continuously transmitted to the plant through each metal wire of the simplified trellis for 53 days [3]. As a control, plants of the same cultivar were grown in the same greenhouse without exposure to DVS. Vibrational amplitude and frequency profile were weekly assessed on apical and basal leaves of all plants in proximity to the wires using a laser Doppler vibrometer (VQ-500-D-V, Ometron, Coventry, UK) connected to a laptop through a LAN-XI data acquisition device (Brüel & Kjær Sound and Vibration A/S, Nærum, Denmark) with a 3.2 kHz sample size, and the recording was analyzed using the BK Connect software (Brüel & Kjær Sound and Vibration A/S, Nærum, Denmark). Spectral analysis (fast Fourier transform (FFT)) was conducted with 400 lines, 8 Hz frequency resolution, and 66.7% overlap. Five replicates (plants) were analyzed for each treatment, and the experiment was conducted in 2023 (experiment 1) and 2024 (experiment 2). Experiment 1 was conducted with the aim of assessing gene modulation in grapevine plants, while experiment 2 implemented the evaluation of plant growth and physiological parameters to confirm the results obtained during Season 1 of the field trial.

2.3 | Assessment of Plant Growth, Pigment Content, Stomatal Conductance, and Chlorophyll Fluorescence

Under field conditions, internodal length (at one time point, BBCH-69) and shoot length (at five time points before shoot trimming: BBCH-15, BBCH-19, BBCH-53, BBCH-65, and BBCH-69; 7 May, 17 May, 22 May, 5 June, and 10 June) were assessed with a ruler, respectively. Ten shoots were randomly selected for each plant, and the mean shoot length of each replicate (plant) was calculated. The mean internodal length of each replicate (plant) was assessed as the average of the first 10 internodes of two shoots randomly selected for each plant [29]. Under greenhouse conditions, internodal length and shoot length were assessed with a ruler at the end of the experiment, and the mean values of each replicate (plant) were calculated considering all shoots of each plant.

Chlorophyll content, flavonol content, anthocyanin content, and nitrogen balance index were assessed with a Dualex Scientific optical leaf clip sensor (Force-A, Orsay, France) [30]. Under field conditions, measurements were carried out on six leaves (two basal, two medial, and two apical leaves) of each plant at three time points (BBCH-19, BBCH-53, and BBCH-65). Under greenhouse conditions, measurements were carried out on three leaves (one basal, one medial, and one apical leaf) of each plant at seven time points (3, 7, 16, 21, 30, 42, and 53 days of treatment).

Stomatal conductance (g_s), leaf vapor pressure deficit, and light intensity were assessed using a porometer with pulse-amplitude modulation fluorometer LI-600 porometer/fluorometer instrument (LI-COR, Lincoln, NE, USA). The electron transport rate (ETR) was then calculated as $\mathrm{ETR} = \mathrm{PAR} \times F_q^{'}/F_m^{'} \times 0.5 \times 0.84$, where 0.5 and 0.84 are the electron partitioning between PSII and PSI and leaf absorbance, respectively, while PAR represents the photosynthetic active radiation at which the analysis was carried out and collected via a Li-600 PAR sensor. In vivo leaf measurements were carried out, with auto $g_s + F$ (stomatal conductance + chlorophyll fluorescence) configuration [31], on six leaves (two basal, two medial, and two apical leaves) of each plant at three time points (BBCH-19, BBCH-53, and BBCH-65) under field conditions. Under greenhouse conditions, in vivo leaf measurements were carried out with the LI-600 instrument on three leaves (one basal, one medial, and one apical leaf) of each plant at seven time points (1, 7, 16, 21, 30, 42, and 53 days of treatment).

All measurements were carried out in the mornings with same light intensity among the treatments.

2.4 | Assessment of Vine Productivity, Grape Quality, and Berry Characteristics

Vine productivity was assessed at harvest under field conditions (season 1: 05th September 2023 and season 2: 29th August 2024). Yield (kg/plant), number of bunches (bunches/plant), and mean bunch weight (g) were determined for each plant using a portable precision balance. Berry weight (g) and diameter (mm) were assessed for 10 berries of each plant using an analytical balance (KERN & SOHN GmbH, Germany) and a caliber.

Berry skin thickness (mm) of 10 berries for each plant was measured using a TAxT2i texture analyzer (Stable Micro Systems, Godalming, Surrey, UK), as previously described [32, 33]. Briefly, berries were frozen at −80°C, and slices of berry skin, including epidermis and a small amount of flesh tissue, were taken with a sharp razor blade after berry defrost. Slices of berry skin were analyzed with a compression test using a 2 mm cylinder probe (P/2, Stable Micro Systems, Godalming, Surrey, UK).

Grape must was obtained from all the bunches of each control and DVS-treated plant and analyzed in transmission mode using a Fourier transform infrared analyzer (FOSS WineScan, FOSS, Hamburg, Germany) following the OIV/OENO Resolution 390/2010 to assess sugar content (°Brix), pH, titratable acidity (g/L), tartaric acid (g/L), malic acid (g/L), and yeast-assimilable nitrogen (mg/L; only in season 2).

2.5 | Sample Collection, RNA Extraction, and Gene Expression Analysis

The gene expression analysis was performed on plants tested in greenhouse conditions. Leaf samples of DVS-treated and control plants were collected under greenhouse conditions at 1, 7, 17, and 27 days of treatment in experiment 1 and at 1, 27, and 50 days of treatment in experiment 2. Three and four replicates (plants) were collected for each treatment and time point of experiment 1 and experiment 2, respectively, and each replicate comprised two half leaves from the same plant. Samples were immediately frozen in liquid nitrogen and stored at −80°C. All samplings were carried out in the mornings with the same light intensity across the treatments.

Leaf samples were crushed to a fine powder using a mixer mill disruptor (MM200, Retsch. Haan, Germany) at 25 Hz for 30 s with refrigerated stainless steel jars and beads. Total RNA was extracted from 80 mg of leaf powder using the Spectrum Plant Total RNA kit (Sigma-Aldrich, Merck, St. Louis, MO, USA) with an on-column DNase treatment with the RNase-Free DNase Set (Qiagen, Hilden, Germany). RNA was then quantified by Qubit RNA Broad-Range Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the quality check was carried out with electrophoresis gel and NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA). The effectiveness of the DNase treatment was confirmed by running PCR with grapevine *actin*

primers (Table S1) in the absence of reverse transcription, and no amplification signals were detected.

The first strand cDNA was synthesized from 1.0 μg of total RNA using Superscript III (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and oligo-dT primer. Quantitative real-time PCR (qPCR) reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and specific primers (Table S1) using the LightCycler 480 (Roche, Branford, Connecticut, USA), as previously described [34]. In particular, genes encoding stilbene synthase (*STS*), phenylalanine ammonia-lyase (*PAL*), pathogenesis-related (PR) protein 1 (*PR-1*), PR protein 2 (*PR-2*), and PR protein 4 (*PR-4*) were used as markers of defense-related processes [34, 35]. For example, *PR-1* and *PR-2* are known as markers of the salicylic acid defense pathways [36] and *PR-4* is a marker of the jasmonic acid defense pathways [37], while *STS* and *PAL* are markers of the phenylpropanoid pathway [38]. Moreover, genes encoding gibberellic acid 3-oxidase 2 (*GA3OX2*) and gibberellic acid 20-oxidase 3 (*GA20OX3*) involved in the biosynthesis of gibberellic acid [39] and 3-epi-6-deoxocathasterone 23-monooxygenase (*CYP90D1*) involved in the biosynthesis of brassinosteroids [40] were used as markers of growth-related processes. Gene encoding 1-aminocyclopropane-1-carboxylic acid oxidase 2 (*ACO2*) was used as marker of the ethylene pathway [41], and UDP-glucose: flavonoid 3-O-glucosyltransferase (*UFGT*) involved in anthocyanins glycosylation [41] was used as marker of the anthocyanin pathway.

The LightCycler 480 SV 1.5.0 software (Roche, Branford, CT, USA) was used to extract Ct values based on the second derivative calculation and the reaction efficiency (Eff) was calculated with the LinRegPCR 11.1 software for each gene [42]. The expression level of each gene was calculated according to the Hellmann's equation [43], using *actin* and *VATP16* as housekeeping genes for normalization [44]. Briefly, relative quantities (RQ) were calculated according to the equation: $RQ = Eff^{(Ct-Ct')}$, where Ct is the threshold cycle and Ct′ is the average Ct of all the treatments analyzed. Normalized RQ (NRQ) were calculated by dividing the RQ by the normalization factor based on the RQ values of the two housekeeping genes [43].

2.6 | Statistical Analysis

2.6.1 | Statistical Analysis Was Carried Out Using R-Studio (Version 2024.09.1 + 394, PBC, Boston, MA, USA)

To evaluate the influence of DVS on internodal length, shoot length, physiological parameters, vine production, and quality under field conditions, generalized linear mixed models (GLMM) were fitted with treatment and season or time point as fixed effects and the biological replicate as random effect and normal distribution.

To evaluate the influence of DVS on physiological parameters under greenhouse conditions, GLMMs were fitted with treatment and time point as fixed effects and the biological replicate as random effect and normal distribution.

For each response variable, both the interaction and additive effects of the fixed factors were tested, and the Akaike's information criterion (AIC) was used to select the most parsimonious model, i.e., the one with the lowest AIC and significant coefficients [45] (Supporting Table 2). All GLMMs were fitted using the lme4 package. The residual distribution and fitness of the models were evaluated using "DHARMa" and "performance" packages [46, 47]. A Tukey post hoc test was performed to assess pairwise comparisons.

Normal distribution (Shapiro–Wilk normality test,$p > 0.05$) and homoscedasticity (F-test, $p > 0.05$) of data of gene modulation and grapevine growth parameters under greenhouse conditions and yeast-assimilable nitrogen under field conditions were checked, and significant differences between DVS-treated and control plants were assessed with two tailed and unpaired t-tests ($p \leq 0.05$). When normality (Shapiro–Wilk normality test, $p \leq 0.05$) or homoscedasticity (F-test, $p \leq 0.05$) assumptions were not respected, the Wilcoxon rank-sum exact test or the Welch's two-sample t-test were performed to assess significant differences between DVS-treated and control plants ($p \leq 0.05$), respectively.

3 | Results

3.1 | Growth and Physiological Parameters Under Field Conditions

At peak frequency, DVS-treated plants showed a higher vibrational amplitude than the background noise (mean ± SE: 37.61 ± 4.66 μm/s), while control plants had a vibrational amplitude comparable to the background noise in both seasons (1.02 ± 0.27 μm/s; Figure 1). As expected, the mean amplitude of DVS was slightly higher on the wires of the trellis compared to the plant shoot. The amplitude of the background noise was comparable on all DVS-treated and control plants, with most energy below 100 Hz. A waveform from a field recording is provided as a representative example of the DN temporal pattern, generated using the stand-alone shaker.

DVS-treated plants showed higher internodal length compared to control plants with a mean (±SE) difference of 1.59 ± 0.09 cm and 0.78 cm ± 0.23 in seasons 1 and 2, respectively (Figure 2). The internodal length was significantly affected in a negative way by the interaction of treatment and season (estimate: −0.8110, $p = 0.02506$) (Supporting Table 3). Specifically, the DVS treatment had a positive effect (estimate: 1.5885, $p = 1.55e-08$) while the season had a negative effect (estimate: −1.7090, $p < 2e-16$) as showed in the fitted GLMM with the interaction effect (Supporting Table 3). Shoot length was not affected by DVS at the early and late stages of the season, but longer shoots were found in DVS-treated compared to control plants at the third and fourth time points; in particular, the fitted GLMM with the interaction effect showed a positive effect of both the DVS treatment and the time point (BBCH-53 and -65, estimate: 5.18, $p = 0.0491$, and estimate: 5.79, $p = 0.0282$, respectively; mean (±SE) difference of 5.18 cm ± 1.30; Figure 2) (Supporting Table 3). For all other parameters, we did not observe any differences between treatment and control plants; specifically, the fitted GLMMs demonstrated an effect only of the time point (positive effect on chlorophyll content $p < 2e-16$, negative effect on anthocyanin content $p = 9.092e-11$, positive effect on flavonoid content $p < 2e-16$, positive effect on nitrogen balance index $p < 2e-16$, and positive effect on stomatal conductance at the second time point $p = 5.923e-10$, while negative at third $p = 1.17e-08$, negative effect on electron transport rate

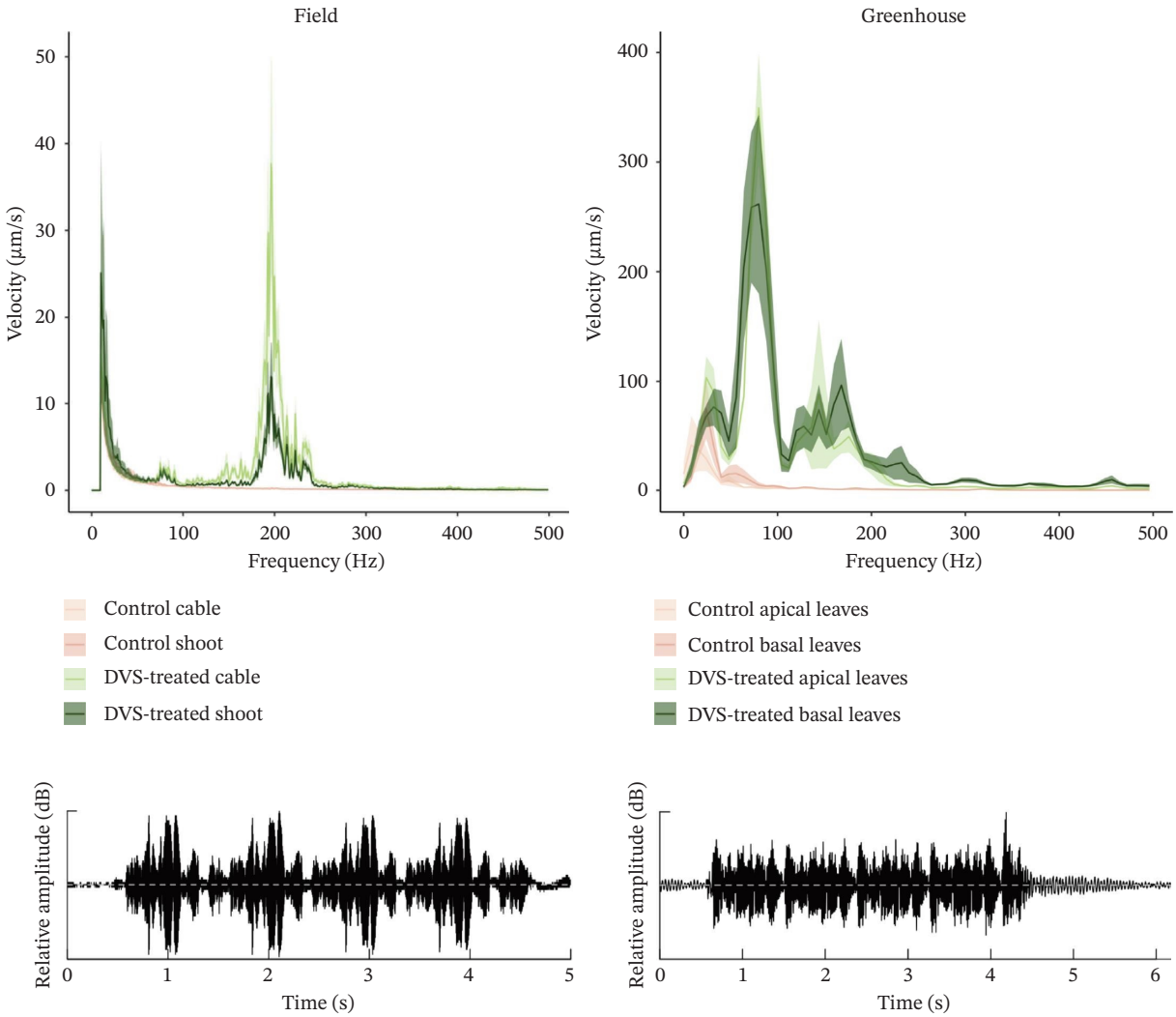

FIGURE 1 | Vibrational spectra above and waveform below of experimental field and greenhouse trials. The vibrational amplitude (as velocity of substrate displacement, µm/s) and frequency (Hz) of control plants (control) and plants treated with the disturbance vibrational signal (DVS-treated) were weekly assessed under field conditions and greenhouse conditions with an accelerometer and a laser doppler vibrometer, respectively. Under field conditions, the recording was done on the second wire of the trellis next to the shoot of each control plant (orange) and DVS-treated plant (light green) and the shoot in contact with the wire of each control plant (pink) and DVS-treated plant (dark green). Under greenhouse conditions, the recording of control and DVS-treated plants was done on apical leaves (orange and light green, respectively) and basal leaves (pink and dark green, respectively) in proximity to the wires.

$p = 5.132e - 13$ and negative effect on leaf vapor pressure at the second time point $p < 2e - 16$ (Figure 3) (Supporting Table 3).

3.2 | Vine Productivity and Quality Under Field Conditions

At the harvest of season 1 and season 2, yield, bunch number, sugar content (season 1: control = 21.15 ± 0.10°Brix, DVS-treated = 21.11 ± 0.11°Brix; season 2: control = 20.49 ± 0.11°Brix, DVS-treated = 20.31 ± 0.15°Brix), pH (season 1: control = 3.26 ± 0.01, DVS-treated = 3.27 ± 0.01; season 2: control = 3.11 ± 0.01, DVS-treated = 3.11 ± 0.01), titratable acidity (season 1: control = 8.44 ± 0.06, DVS-treated = 8.56 ± 0.08; season 2: control = 8.15 ± 0.14, DVS-treated = 8.03 ± 0.16 g/L (tartaric acid)), tartaric acid, and malic acid were comparable in DVS-treated and control plants. The seasonality did not affect the yield and the number of bunches (GLMM estimate = −0.6645; estimate = −3.3204, $p > 0.05$) (Supporting Table 3). The variables related to the grape quality were significantly affected only by the seasonality in a negative way as showed in the fitted GLMMs (Supporting Table 3). The bunch weight was significantly affected in a negative way only by the DVS treatment as showed in the fitted GLMMs (estimate: −13.656, $p = 0.0336$; Table 1) (Supporting Table 3).

Moreover, mean berry weight was not affected by the DVS treatment, while it was positively affected by the season as showed in the fitted GLMM (season 1: DVS-treated 1.39 ± 0.04 g, control 1.47 ± 0.02 g, and season 2: DVS-treated 1.75 ± 0.03 g, control 1.73 ± 0.04 g; estimate: 0.30042, $p = 6.55e - 12$) (Supporting Table 3). Likewise, yeast-assimilable nitrogen (Table 1, *t*-test: $N = 40$, df = 38, $p > 0.05$), mean berry diameter (DVS-treated 13.18 ± 0.11 mm, control 12.92 ± 0.13 mm; *t*-test: $N = 40$, df = 38, $p > 0.05$) and berry skin thickness (DVS-treated 0.16 ± 0.001 mm, control 0.16 ± 0.001 mm; *t*-test: $N = 40$, df = 38, $p > 0.05$) were comparable in DVS-treated and control plants in season 2.

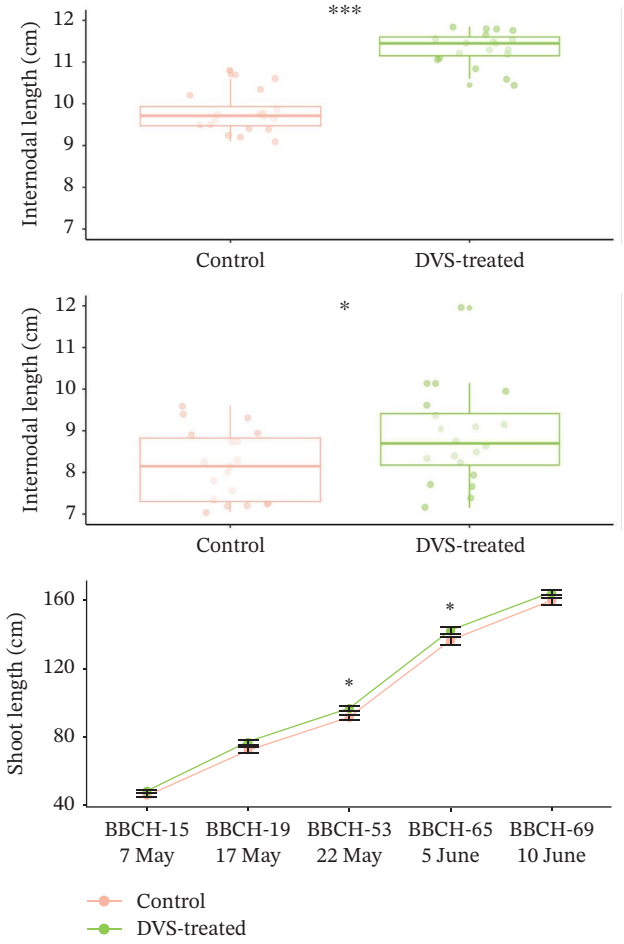

FIGURE 2 | Shoot growth under field conditions. Internodal length and shoot length of control plants (pink) and plants treated with the disturbance vibrational signal (DVS-treated; green) were assessed under field conditions in season 1 and season 2. Internodal length was assessed at BBCH-69 in season 1 and season 2 (14 July 2023 and 05 June 2024, respectively). Mean and standard error values of 20 replicates (plants) are reported for each treatment. Asterisks indicate significant differences between DVS-treated and control plants according to the fitted GLMM (Tukey pairwise comparison $N = 80$, df = 76; $^{*}p$ value < 0.05; $^{**}p$ value = 0.0027; $^{***}p$ value < 0.0001).

3.3 | Grapevine Growth, Physiological Parameters, and Gene Expression Under Greenhouse Conditions

On DVS-treated plants, the amplitude measured at 180–210 Hz was between 24 and 300 μm/s, while control plants lacked the peak at 180–210 Hz and the corresponding amplitude was comparable to the background noise under greenhouse conditions (Figure 1). A peak was detected on DVS-treated plants at 70–100 Hz that had a higher amplitude compared to the peak at 180–210 Hz. Apical and basal leaves showed comparable mean amplitude, and background noise was found on DVS-treated and control plants with most of the energy concentrated at frequencies below 50 Hz.

Shoot internodes were significantly longer in DVS-treated plants compared to control plants with a difference of 1.15 ± 0.32 cm (Figure 4; *t*-test: $N = 14$, df = 12, $p = 0.05$), while no significant difference in shoot length was found between DVS-treated and control plants at the end of the experiment under greenhouse conditions (*t*-test: $N = 14$, df = 12, $p > 0.05$; Figure 4). All the results of the fitted GLMMs are reported in Supporting Table 3. Specifically, the chlorophyll content, the anthocyanin content, the nitrogen balance index, the stomatal conductance and the electron transport rate were significantly affected only by the time point. Specifically, starting from the fifth time point, a negative effect was recorded on chlorophyll content; from the fourth time point, a negative effect was recorded on anthocyanin content; only the third time point affected flavonoid content; from the sixth time point, a negative effect was recorded on nitrogen balance index; all the time points negatively affected stomatal conductance; and only the fourth and sixth time points positively affected electron transport rate (Figure 5). Leaf vapor pressure deficit was significantly affected by the treatment in a negative way and even by the time point in a negative way at the second and third time points, while in a positive way, starting from the fifth time point as showed in the fitted GLMM (Supporting Table 3). Moreover, the expression of marker genes was comparable in DVS-treated and control plants, such as *STS*, *PAL*, *ACO2*, *UFGT*, *PR-4*, *PR-1*, and *PR-2* genes at 1, 7, 17, and 27 days of treatment in experiment 1, and *STS*, *PAL*, *ACO2*, *UFGT*, *CYP90D1*, *GA3OX2*, and *GA20OX3* genes at 1, 27, and 50 days of treatment in experiment 2 (*t*-test, Welch's two-sample *t*-test or Wilcoxon rank-sum test: $N = 10$, df = 8, $p > 0.05$; Table 2).

4 | Discussion

VMD is a promising pest control strategy that aims to be environmental friendly since it is based on the continuous transmission of a species-specific vibrational signal to grapevine plants to interfere with the target species communication and reduce population density in an area-wide approach [3, 12]. However, plants can react to vibrational stimuli with hormonal, metabolic, morphological, and transcriptional changes [13, 15, 18–23], raising the question on possible side effects of DVS exposure on grapevine health (e.g., growth and production). Observed results suggest that VMD does not negatively affect grapevine plants and is compatible with sustainable strategies for pest management. The only parameter negatively influenced by the DVS treatment was the average bunch weight, despite that, no negative effects were observed on vine productivity (bunch number and yield), berry characteristics (weight, diameter, and skin thickness), and grape quality parameters (sugar content, pH, titratable acidity, tartaric acid, malic acid, and yeast-assimilable nitrogen) in two consecutive seasons under field conditions. Likewise, physiological parameters assessed on grapevine leaves (chlorophyll content, flavonol content, anthocyanin content, nitrogen balance index, stomatal conductance, electron transport rate, and leaf vapor pressure deficit) were comparable in DVS-treated and control plants under field and greenhouse conditions, indicating no negative impacts of VMD on grapevine physiology. The only exception was on leaf vapor pressure deficit, which was slightly decreased under greenhouse conditions by the treatment, suggesting a more favorable growing environment and a potential positive effect of VMD on improving the hydric stress resistance on grapevine plants. However, this effect was not confirmed in the field trial, probably because leaf vapor pressure deficit is extremely dependent on microclimatic conditions. Moreover, no modulations of genes related to salicylic acid (*PR-1* and *PR-2*) [36], jasmonic acid (*PR-4*) [37], and ethylene (*ACO2*) [41]

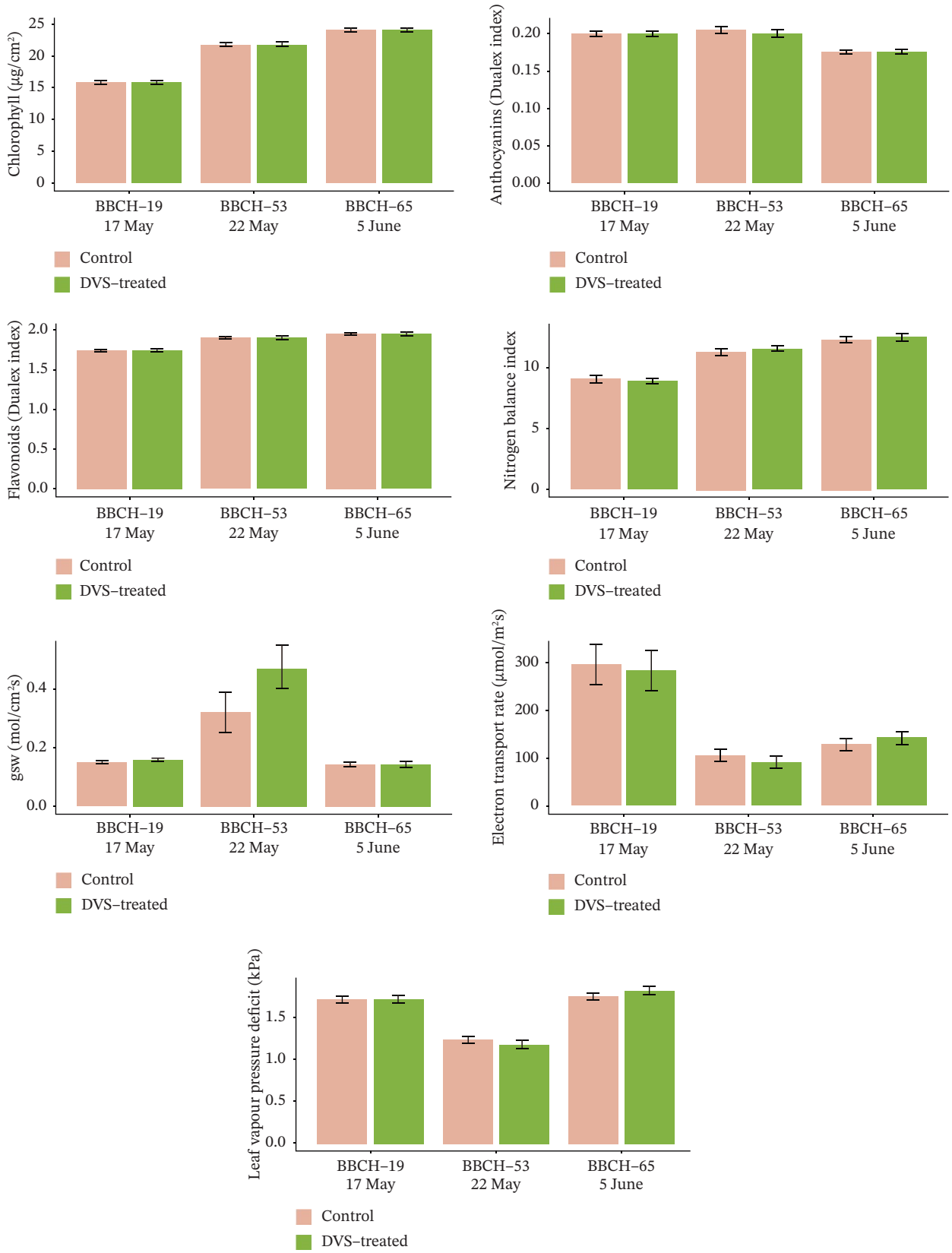

FIGURE 3 | Physiological parameters under field conditions. Chlorophyll content, anthocyanin content, flavonoid content, and nitrogen balance index of control plants (pink) and plants treated with the disturbance vibrational signal (DVS-treated; green) were assessed using a Dualex instrument at three time points (BBCH-19, BBCH-53, and BBCH-65; 14 May, 24 May, and 05 June 2024, respectively). At the three time points, stomatal conductance (g_s), electron transport rate, and leaf vapor pressure deficit were assessed using a LI-COR LI-600 instrument. Mean and standard error values of 20 replicates (plants) are reported for each treatment and time point. No significant differences between DVS-treated and control plants were found for each parameter and time point, according to the fitted GLMM (p value > 0.05).

TABLE 1 | Vine productivity and quality of control plants and plants treated with the disturbance vibrational signal (DVS) under field conditions.

Grape parameter	Season 1		Season 2	
	Control	DVS-treated	Control	DVS-treated
Yield (kg/plant)	6.37 ± 0.51	5.61 ± 0.396	5.69 ± 0.44	5.00 ± 0.31
Bunch number (bunches/plant)	41 ± 3	40 ± 2	38 ± 3	35 ± 2
Bunch weight (g)	152.65 ± 3.24[b]	138.63 ± 5.48[a]	150.21 ± 4.59[b]	145.48 ± 4.35[b]
Sugar content (°Brix)	21.15 ± 0.10	21.11 ± 0.11	20.49 ± 0.11	20.31 ± 0.15
pH	3.26 ± 0.01	3.27 ± 0.01	3.11 ± 0.01	3.11 ± 0.01
Titratable acidity (g/L)	8.44 ± 0.06	8.56 ± 0.08	8.15 ± 0.14	8.03 ± 0.16
Tartaric acid (g/L)	9.44 ± 0.08	9.47 ± 0.06	8.27 ± 0.12	8.19 ± 0.09
Malic acid (g/L)	3.51 ± 0.16	3.52 ± 0.13	3.25 ± 0.14	3.06 ± 0.17
Yeast-assimilable nitrogen (mg/L)			410.62 ± 7.72	397.09 ± 11.3

Note: Vine productivity parameters were assessed at grape harvest of experimental field trials of season 1 and season 2. The must obtained from all bunches of each plant was analyzed using the FT-IR technique. For each parameter, mean and standard error values of 20 replicates (plants) are reported for each treatment. The fitted GLMMs revealed a negative effect of the DVS treatment only for the bunch weight (estimate = −13.656, $p = 0.0336$). Different letters indicate significant differences ($p < 0.05$) among treatments after Tukey's post hoc test. Yeast-assimilable nitrogen was not assessed in samples of season 1.

pathways were found in DVS-treated plants, as well as genes involved in secondary metabolism (e.g., *PAL*, *STS*, and UFGT) [38, 41], suggesting that VMD did not affect the expression of defense-related processes in grapevine in our experimental conditions. Vibrational measurements confirmed that, in both experimental settings (field and greenhouse conditions), the DVS-treated plants vibrated with higher amplitude compared to control plants in correspondence with the frequency range used for VMD of *S. titanus* (150–210 Hz) [12]. This confirms that observed effects are due to the vibrational stimulation. Some low-frequency background noise was detected in both settings, probably because they were noninsulated environments and the noise likely derived from greenhouse fan, wind, or other environmental factors [48, 49]. However, the noise was comparable between DVS-treated and control plants, indicating that the only difference between the two groups was the higher amplitude of vibrations used for VMD. The 80 Hz dominant peak detected in greenhouse conditions was probably caused by resonance effects of the small metal trellis structure, since the shaker applied to the pole was built to propagate the vibration in field conditions up to 25 m [3]. However, the distance between poles was 70 cm under greenhouse conditions, and poles were connected on the bottom with a metallic platform on which the potted plants were placed. Thus, potted plants were vibrated by touching the wires and also by standing on the platform, whereas the canopy is vibrated by touching the wires under field conditions.

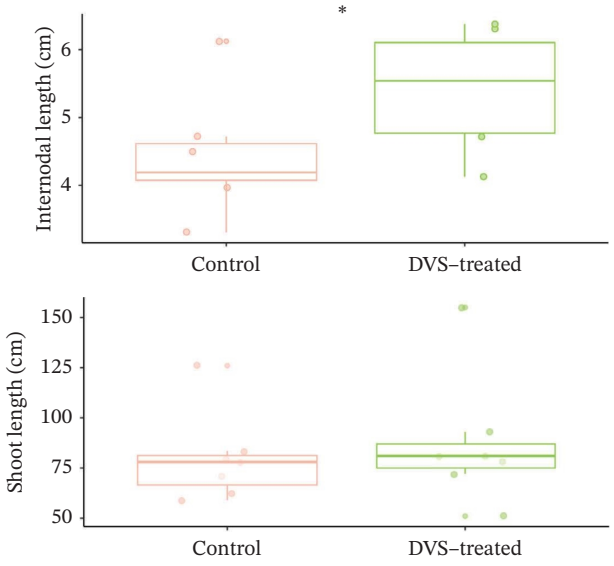

FIGURE 4 | Shoot growth under greenhouse conditions. Internodal length and shoot length of control plants (pink) and plants treated with the disturbance vibrational signal (DVS-treated; green) were assessed at the end of the experiments under greenhouse conditions. Mean and standard error values of seven replicates (plants) are reported for each treatment. Asterisks indicate significant differences after a *t*-test ($N = 14$, df = 12, p value = 0.05).

Previous studies indicate that herbivore-induced vibrations, such as caterpillar feeding vibrations, can affect the levels of defense-related compounds (e.g., glucosinolate, anthocyanins, and alkaloids), phytohormones (e.g., auxins, cytokinins, gibberellins, abscisic acid, and salicylic acid), and volatile organic compounds (e.g., β-ionone and methyl salicylate), suggesting that the sole mechanical stimulus caused by the herbivore activity on the plant can induce defense mechanisms [18–20, 22, 23]. Furthermore, the impact of sound vibration was found on the anthocyanin content of grapevine cell cultures [41]. The absence of DVS effects on parameters tested in our study is in contrast with these previous studies, and it could be related to the plant species (grapevine instead of model plants such as *Arabidopsis thaliana*), the tested system (whole plants instead of cultured cells), or the type of vibrational stimulus applied (disturbance noise instead of sound vibration). Regarding the latter, DVS is a *S. titanus* intraspecific signal emitted by rival males to interrupt mating duets, and it is not directly linked to plant damage, which is the case of caterpillar and leaf miner chewing tested in previous studies [18–20, 22, 23]. Moreover, the time of exposure to vibrations was different when comparing *S. titanus* rival signals and chewing vibrations. Rival signals are usually emitted for a few seconds, and when the ongoing mating duet stops, the rival male begins emitting other intraspecific signals to establish a mating duet with the female [50]. The application of the *S. titanus* rival signal continuously to the plants is due to the need to suppress mating, but it is not the natural condition. On the other hand, chewing vibrations have been tested for exposure times that are

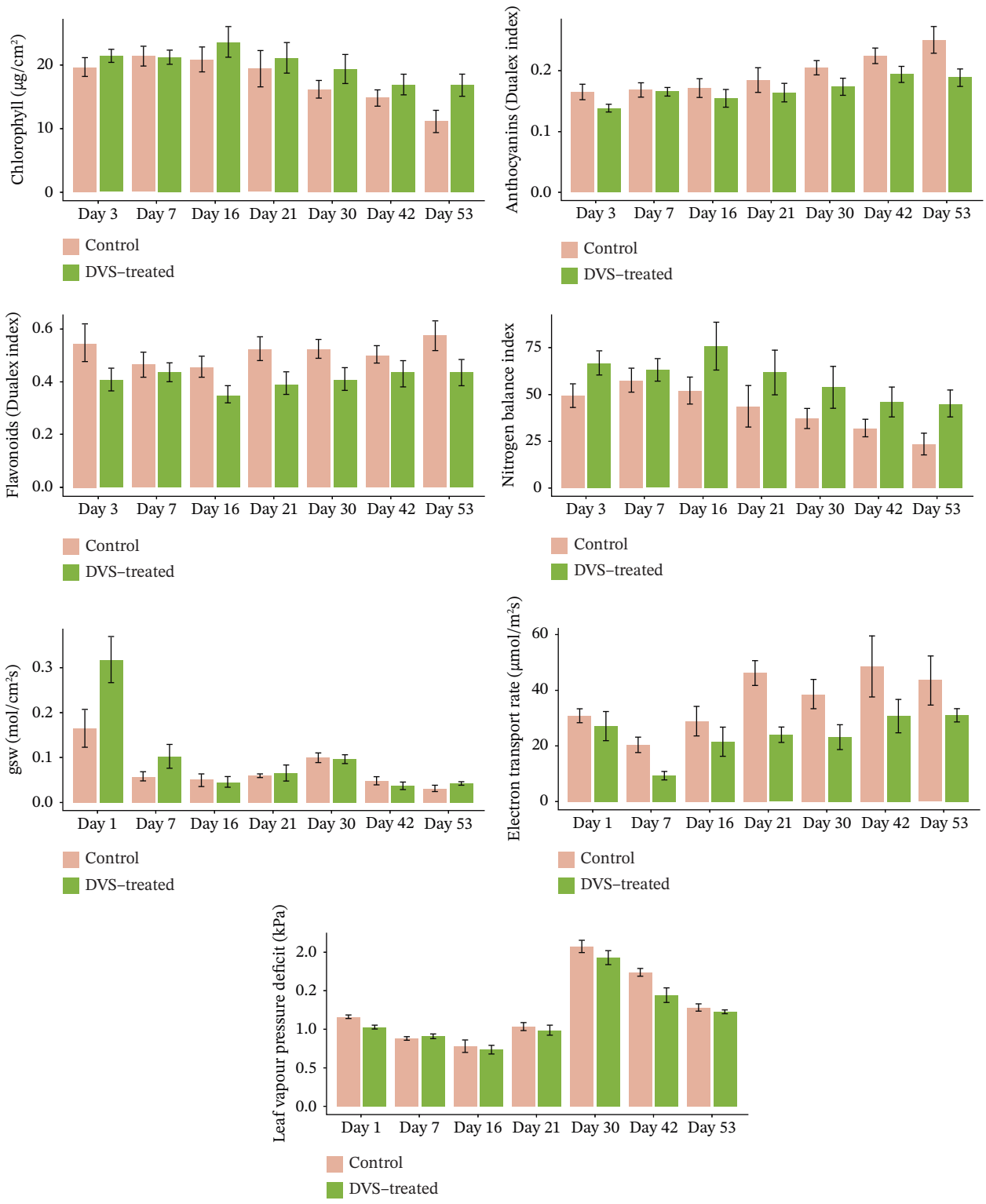

FIGURE 5 | Physiological parameters under greenhouse conditions. Chlorophyll content, anthocyanin content, flavonoid content, and nitrogen balance index of control plants (pink) and plants treated with the disturbance vibrational signal (DVS-treated; green) were assessed using a Dualex instrument at seven time points. Stomatal conductance (g_s), electron transport rate, and leaf vapor pressure deficit were assessed using a LI-COR LI-600 instrument at seven time points. Mean and standard error values of five replicates (plants) are reported for each treatment and time point. Only leaf vapor pressure deficit was significantly affected by the treatment in a negative way as showed in the fitted GLMM (Supporting Table 3). No significant differences between DVS-treated and control plants were found for the other parameters and time points, according to the fitted GLMM (p value > 0.05).

coherent with the natural context [18–20, 22, 23]. Moreover, the amplitude of the playback in previous studies (mm/s) [18–20, 22, 23] is higher than the one used in this study (μm/s), suggesting that an amplitude threshold may exist for plant recognition. The different results obtained varying the type of signal, the time of exposure, and the amplitude suggest that the

TABLE 2 | Gene expression analysis of control plants and plants treated with the disturbance vibrational signal (DVS) under greenhouse conditions.

A

Gene	Day 1		Day 7		Day 17		Day 27	
	Control	DVS-treated	Control	DVS-treated	Control	DVS-treated	Control	DVS-treated
STS	1.76 ± 1.27	0.47 ± 0.34	0.15 ± 0.03	0.44 ± 0.20	0.34 ± 0.02	0.61 ± 0.38	0.64 ± 0.32	3.39 ± 2.35
PAL	1.60 ± 1.04	1.01 ± 0.58	0.77 ± 0.17	1.34 ± 0.63	1.22 ± 0.28	2.10 ± 0.85	3.43 ± 1.49	5.35 ± 2.00
ACO2	1.12 ± 0.39	0.63 ± 0.21	0.18 ± 0.02	0.29 ± 0.05	0.42 ± 0.19	0.69 ± 0.46	0.77 ± 0.24	1.71 ± 0.97
UFGT	1.08 ± 0.28	2.53 ± 0.96	5.93 ± 1.95	2.35 ± 1.31	1.36 ± 0.39	2.39 ± 1.40	2.72 ± 0.90	3.47 ± 2.28
PR-4	1.35 ± 0.62	0.25 ± 0.08	0.50 ± 0.10	0.51 ± 0.18	0.43 ± 0.09	0.46 ± 0.12	0.45 ± 0.18	1.73 ± 0.91
PR-1	0.72 ± 0.40	0.15 ± 0.08	0.19 ± 0.07	0.17 ± 0.11	0.69 ± 0.51	0.28 ± 0.12	0.25 ± 0.10	0.40 ± 0.22
PR-2	0.55 ± 0.20	0.21 ± 0.03	0.55 ± 0.06	0.50 ± 0.13	0.56 ± 0.07	0.49 ± 0.04	0.48 ± 0.16	0.64 ± 0.23

B

Gene	Day 1		Day 27		Day 50	
	Control	DVS-treated	Control	DVS-treated	Control	DVS-treated
STS	1.85 ± 0.79	1.74 ± 0.91	0.51 ± 0.15	0.59 ± 0.23	0.47 ± 0.12	0.60 ± 0.36
PAL	1.50 ± 0.71	1.68 ± 1.03	0.56 ± 0.07	1.61 ± 1.09	1.68 ± 0.82	1.13 ± 0.42
ACO2	1.16 ± 0.27	0.92 ± 0.37	0.60 ± 0.10	0.34 ± 0.06	0.58 ± 0.10	0.88 ± 0.29
UFGT	1.09 ± 0.26	0.57 ± 0.11	0.57 ± 0.31	1.73 ± 1.49	0.51 ± 0.39	0.44 ± 0.34
CYP90D1	1.09 ± 0.29	2.31 ± 0.68	4.27 ± 1.81	4.89 ± 2.00	4.91 ± 3.27	4.68 ± 1.45
GA3OX2	1.12 ± 0.29	1.24 ± 0.33	1.18 ± 41.36	2.86 ± 0.62	9.30 ± 3.94	6.58 ± 1.08
GA20OX3	1.71 ± 1.12	3.50 ± 1.28	1.55 ± 0.74	1.63 ± 0.79	4.80 ± 4.00	0.30 ± 0.01

Note: The relative expression level (normalized relative quantification) of genes encoding stilbene synthase (*STS*), phenylalanine ammonia-lyase (*PAL*), 1-amino-cyclopropane-1-carboxylic acid oxidase 2 (*ACO2*), UDP-glucose flavonoid 3-O-glucosyltransferase (*UFGT*), pathogenesis-related (PR) protein 1 (*PR-1*), PR protein 2 (*PR-2*), and PR protein 4 (*PR-4*), 3-epi-6-deoxocathasterone 23-monooxygenase (*CYP90D1*), gibberellic acid 3-oxidase 2 (*GA3OX2*), and gibberellic acid 20-oxidase 3 (*GA20OX3*) were assessed for control plants and plants treated with the disturbance vibrational signal (DVS) under greenhouse conditions. Leaf samples were collected at 1, 7, 17, and 27 days of treatment in Experiment 1 (A) and 1, 27, and 50 days of treatment in Experiment 2 (B). Mean and standard error values of three and four replicates (plants) are presented for each treatment and time point of experiment 1 and experiment 2, respectively. No significant differences between DVS-treated and control plants were found at each time point, according to *t*-test, when normal distribution (Shapiro–Wilk normality test, $p > 0.05$) and homoscedasticity (*F*-test, $p > 0.05$) assumptions were respected, Welch's two-sample *t*-test ($p \leq 0.05$) or Wilcoxon rank-sum exact test ($p \leq 0.05$), and when homoscedasticity (*F*-test, $p \leq 0.05$) or normality (Shapiro–Wilk normality test, $p \leq 0.05$) assumptions were not respected (experiment 1: $N = 6$, df = 4, p value > 0.05; experiment 2: $N = 8$, df = 6, p value > 0.05).

plant response to vibrational stimuli is very specific and evolved from a strict correlation between the nature of the stimulus and the biological consequence. Alternatively, the plant response might be subject to habituation, and more complete metabolomic and transcriptomic studies are needed to verify if DVS is responsible for molecular responses in grapevine leaves and berries at different time points, such as a few hours after the application.

The only significant effect found was that DVS treatment increased internodal length under field and greenhouse conditions, with temporary stimulation effects on shoot length during the season. Although no information is available on morphological changes activated by plants in response to DVS, water-borne vibrations can elongate maize roots [15]. Additionally, there is evidence that wind and touch vibrations can lead to stem elongation [13, 17]. However, the expression of genes involved in the biosynthesis of growth-related hormones, such as brassinosteroids (*CYP90D1*) [40] and gibberellic acid (*GA3OX2* and *GA20OX3*) [39], was not affected by DVS, indicating that further metabolic studies are required to analyze hormonal content and to better understand the mechanisms of plant growth stimulation by DVS. Another possible mechanism underlying the stimulation of shoot growth could be the mechanical effect of vibrations on cellulose fibers, which may facilitate their expansion during cell elongation.

5 | Conclusions

The results of this study support that VMD is a sustainable strategy to manage *S. titanus* with no negative effects on grapevine growth, physiological parameters, vine productivity, and grape quality under field conditions. The novel aspect of this work lies in its long-term field and greenhouse assessments, revealing the absence of negative effects of DVS treatment on grapevine physiology and grape quality, while highlighting a stimulation of growth parameters under both field and greenhouse conditions with no modulation of marker genes. Although further studies are required to clarify the possible mechanism of internodal length stimulation, these results demonstrate low risk for the further development of VMD and other pest control strategies that require the application of vibrations in the environment for a long period of time.

Author Contributions

S.G. and R.N. carried out the experiments under greenhouse and field conditions. S.G. and M.F. carried out the assessment of plant growth and photosynthetic parameters. S.G., R.N., and M.V. carried out the assessment of fruit production, grape quality, and berry characteristics. S.G. and M.P. carried out gene expression analyses. S.G., M.P., R.N., V.M., G.A., and M.F. conceived the study and designed the experiments. S.G., M.P., R.N., and V.M. wrote the manuscript.

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Disclosure

All the authors revised and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data are reported in the manuscript as main or supporting information.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. *(Supporting Information)*

Supporting table 1: primer sequences of grapevine genes analyzed by quantitative real-time PCR. Gene name, gene abbreviation, and accession numbers in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov) are reported for the selected grapevine genes. Sequences of forward and reverse primers are indicated for each gene with the respective reference.

Supporting table 2: AIC values of the fitted GLMMs are reported, with the selected model for each variable shown in bold.

Supporting table 3: results of the fitted GLMMs.

Supporting figure S1: overview of experimental field trials. The experimental vineyard (*Vitis vinifera* cultivar Pinot Gris, "pergola doppia" as trellis with a planting distance of 4.5 × 0.5 m, GPS coordinates: 46°12′52″ N and 11°08′15″E) was divided in three zones (A), one zone was treated with the DVS (near the shakers) and two zones were not treated (control; distance of more than 15 m from the shakers). Stand-alone shakers (B) were attached to the pole at 25 m from the end of the row, and the vibration was provided to the plants through the metal wires of the trellis. Twenty replicates (plants) were selected for each treatment, according to the signal amplitude as velocity of substrate (mean ± SE; vibrated plants signal amplitude > 15 μm/s and control plant's signal is not distinguishable from the background noise) and the vigor of the plant

(comparable stem diameter and number of buds after pruning). For each plant, vibrational amplitude and frequency were weekly assessed with an accelerometer on the second wire of the trellis next to the shoot and the shoot in contact with that wire.

Supporting Figure S2: overview of greenhouse trials. Potted grapevine plants (*Vitis vinifera* variety Pinot Noir) were grown under greenhouse conditions (25°C, 60% RH, and 18/6 light–dark photoperiod) on a simplified trellis. A mini shaker was attached to the upper part of one pole and the vibration was provided to the plants through each metal wire of the simplified trellis. Vibrational amplitude and frequency were weekly assessed on apical and basal leaves in proximity of the wires using a laser vibrometer (red dots). Five replicates (plants) were analyzed for each treatment.