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FULL PAPER

PLASMA PROCESSES AND POLYMERS

The use of plasma-activated water in viticulture: Induction of resistance and agronomic performance in greenhouse and open field

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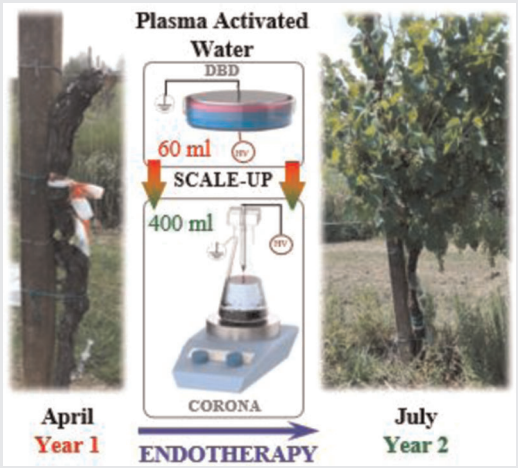
Abstract

In this study, two different cold atmospheric-pressure plasmas are used for the production of plasma-activated water (PAW). To evaluate the effectiveness of PAW as a possible means to control plant diseases, grapevines in an open field and a greenhouse are treated, evaluating qualitative and quantitative yield parameters, phytoplasma presence, and gene expression. The results show the capability of PAW to enhance plant defense mechanisms and, as demonstrated in the field trials, confirm its ability to improve the health status of the treated plants.



KEYWORDS

grapevines, greenhouse, open field, plant pathogens, plasma and agriculture

1 | INTRODUCTION

The exposure of water to cold atmospheric-pressure plasmas (CAPs) induces different reactive oxygen and nitrogen species (RONS) in the liquid phase, leading to the production of plasma-activated water (PAW),[1–3] and it exerts different effects on biological materials, mainly reduction of the bacterial load,[4,5] and in the agricultural field, leading to the increase of seed germination,[6–11] plant growth,[12–14] and induction of disease resistance in plants.[15–17]

Phytoplasmas are wall-less bacteria associated with severe diseases affecting agronomic relevant crops; in grapevines, they are associated with yellows (GY), which occurs in the majority of the grapevine-growing regions.[18] GY diseases are of considerable economic importance, especially in areas where outbreaks occur. In infected plants, flowers and bunches may whiten and desiccate, such that the yield is

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drastically reduced. In addition, the quality of wine is decreased by the high acid and low sugar contents of the infected clusters.[19] Management of these diseases has mainly focused on the use of healthy plant material, removal of infected plants, and control of insect vectors and phytoplasma alternative host plant species. As all these methods present some drawbacks, many efforts are devoted to the development of alternative control strategies. PAW is an innovative and alternative tool to control infectious plant diseases due to bacteria and phytoplasmas' cell wall-lacking bacteria.[15]

In this study, two different methods to produce PAW are compared. In one case, a dielectric barrier discharge (DBD) driven by a nanosecond-pulsed generator was used to treat sterile distilled water, resulting in the production of plasma-activated distilled water (PADW). In the other case, a corona discharge driven by a commercial microsecond-pulsed generator was used to treat tap water (TW), producing plasma-activated tap water (PATW). The latter process was developed to meet the economically sustainable production of large volumes of PAW, overcoming the limitations posed by the use of nanosecond-pulsed generators and sterile distilled water.

To evaluate the effectiveness and applicability in the field of PADW as an alternative means to control GYs, phytoplasma-infected grapevines were treated directly into the vineyard. Moreover, both PADW and PATW were used for the treatment of grapevine plants in a greenhouse, to evaluate the expression of two genes involved in the induction of resistance in plants: phenylalanine ammonia lyase and stilbene synthase.

2 | MATERIALS AND METHODS

2.1 | PADW and PATW production

PADW was produced exposing sterile deionized water (SDW) to a nanosecond-pulsed DBD, as previously described.[15] The treatment induced the production of NO_3^- (5.1 ± 0.2 mM) and H_2O_2 (459 ± 44.2 μM) and the reduction of pH (2.78 ± 0.47), as reported by Perez et al.[15] It is important to note that the concentration of NO_2^- in PADW is below the detection limit due to its reaction with H_2O_2 in an acidic environment, as reported in References [1,7]. The PADW was frozen at −20°C immediately after the treatment.

PATW was produced exposing TW (directly from water delivery networks of Bologna, Italy; https://www.gruppohera.it/gruppo/attivita_servizi/business_acqua/qualita/qualita_acqua_hera/qualita_media_comuni/-bologna/pagina258.html) to a novel plasma source (Figure 1) composed of a stainless-steel electrode operating in environmental air (relative humidity and temperature in the range of 20%–40% and 20°C–25°C, respectively) and connected to a microsecond-pulsed generator (AlmaPulse; AlmaPlasma s.r.l.). The generator was operated at a peak voltage of 2 kV, frequency of 5 kHz, and duty cycle of 60%. A volume of 500 ml of TW, contained in a borosilicate Erlenmeyer flask on a stirrer (IKA Magnetic Stirrers RCT basic), was directly connected to the ground and exposed to the CAP. A stirrer speed of 200 rpm was set. The voltage (V) and the current (i) were measured using a high-voltage probe (Tektronix

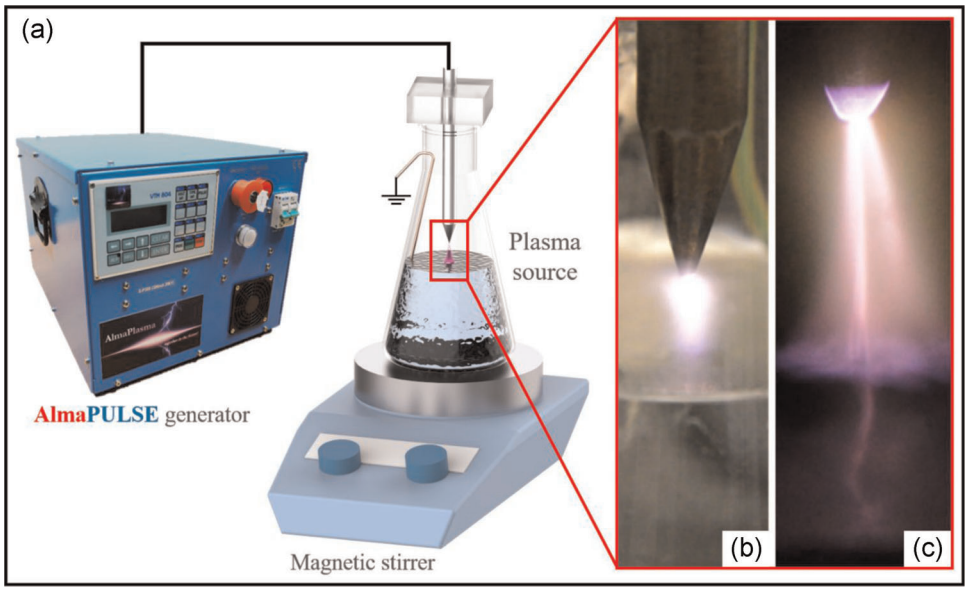

FIGURE 1 (a) Schematic of the experimental setup and pictures of the corona source during the production of plasma-activated tap water with (b) light on and (c) light off

P6015A) and a current probe (Pearson 6585) connected to a digital oscilloscope (Tektronix DPO4034, 350 MHz, 2.5 GSa/s). The average power (P) dissipated in the discharge was determined by applying the following formula:

$$P = \frac{1}{T} DC \int_0^T i(t)V(t)dt, \quad (1)$$

where T is the applied voltage period and DC is the duty cycle.

The concentrations of H_2O_2, NO_2^-, and NO_3^- induced by plasma in the liquid substrate were measured immediately after treatment (about 30 s) by means of Amplex® Red Hydrogen Peroxide Assay Kit (Thermo Fisher Scientific) and the Nitrate/Nitrite Colorimetric Assay (Roche) for three different plasma exposure times: 2.5, 5, and 10 min. Moreover, H_2O_2, NO_2^-, and NO_3^- were also evaluated for 1, 3, 5, and 24 h after the plasma treatment. The measurement of their concentrations was performed according to the manufacturer's protocols and the absorbances were measured photometrically with a microplate reader (Rayto).

2.2 | PADW's effect on phytoplasma-infected and healthy grapevines

2.2.1 | PADW treatments in vineyards

About 100 phytoplasma-infected grapevine plants, 15–20 years old, belonging to cultivars Chardonnay and Glera, located in 17 vineyards in the Treviso province (North-Eastern Italy) at levels ranging from 253 to 13 m above sea level, were selected for field trials. All the selected plants were molecularly tested for phytoplasma presence[20] and then subjected to PADW/SDW treatments for two subsequent years. In each vineyard, an average of six plants was randomly selected and two of them were used as control (SDW), for a total of 70 PADW- and 30 SDW-treated plants. To maintain PADW's physical and chemical properties for a long term, the solution was frozen immediately after its production and gradually defrost (for about 30 min at ambient air temperature) directly in the field, before the treatments. Moreover, to inject 15–20 ml of PADW/SDW directly into the plant vascular tissues, avoiding environmental interference, the endotherapy technique was employed. Three treatments per year (April–June–July) were carried out and samples were collected at the end of each summer season for phytoplasma presence detection.

2.2.2 | Phytoplasma detection

Total nucleic acids were extracted from 1 g of the fresh plant tissue (leaf midribs) and ground in liquid nitrogen using a phenol/chloroform protocol.[21] The plant nucleic acid was diluted in SDW to a final concentration of 20 ng/μl, and nested polymerase chain reaction/restriction fragment length polymorphism analyses on the 16Sr gene were carried out following the protocols described in the study of Zambon et al.[20]

2.2.3 | Field trials: PADW effect on grapevine production

The experiment was carried out in five vineyards, with grapevine plants belonging to cultivar Glera, located in Treviso province. At the beginning of the year, 20 healthy grapevines per vineyard, with the same age (15 years old), pruning system, pedological, and light exposure conditions, were selected and pruned with the same number of buds (30). None of these vineyards were irrigated, and they had fertilizers distributed in winter and additional green pruning applied in spring and summer, as are the normal practices for these areas. An integrated pest management program was applied to control the main fungal diseases (downy mildew, powdery mildew, and gray mold) and insects (moths). In every vineyard, the selected plants were divided into two experimental plots (PADW/SDW) and treated three times (April–June–July) by endotherapic techniques, as described above.

Grapevine berries from each plant/treatment were separately harvested to evaluate qualitative and quantitative yield parameters. The number of berries clusters/plant and the weight of 100 berries per grapevine cluster per thesis were measured using a digital balance (model Kern DS; Kern & Sohn GmbH). The analysis of qualitative parameters was carried out on juice samples from the grapevine berries collected from the five vineyards. Each juice sample was obtained by blending 200 g of berries. In all, about 25 ml of filtered juice was collected and centrifuged at 4200g for 5 min in 50-ml Corex tubes. The soluble solid content was determined using a Brix refractometer (Atago), and the titratable acidity was measured by titration with NaOH to an endpoint pH of 8.2, with the pH determined using a pH meter (model HI222; Hanna Instruments). The data for the quantitative parameters were compared by Student's test[22] at $p \leq .05$ and analyzed by box and whiskers plot.[23]

2.3 | PAWs treatments in a greenhouse

Under greenhouse conditions, 2-year-old grapevine plants (three plants/treatment) were partially uprooted, the roots were washed, and special irrigation devices were applied directly to them to acclimatize the plants before treatments. After 3 weeks, the root apparatus was drenched into 450 ml of PADW and PATW, and the fourth, fifth, and sixth leaf of each plant/treatment were collected at three time points: 16, 26, and 36 h. Three untreated plants at each time point were then used as a negative control and calibrator for further data normalization. The tissues were flash-frozen in liquid nitrogen and stored at −80°C until RNA extraction.

2.3.1 | Gene expression

Total RNA from grapevine samples was extracted from 200 mg of the frozen plant material, as described in Gambino et al.[24] All RNA samples were diluted at 250 ng/μl for quantitative reverse-transcription polymerase chain reaction assays performed using the Real-Time PCR ABI PRISM StepOne sequence detection system (Applied Biosystem). M-MLV reverse transcriptase (RT; Promega) was used to synthesize complementary DNAs (cDNAs) with a random hexamer primer (Fermentas), following the manufacturer's instructions. Genes coding phenylalanine ammonium lyase (*VvPAL1*) and stilbene synthase (*VvSTS*) were studied using ~1.5 ng of cDNA template for quantitative PCR (qPCR), with expression normalized to the actin, ubiquitin, and glyceraldehydes 3-phosphate dehydrogenase as internal control genes. All qPCR reactions were performed using the SYBR® Green Master Mix (Applied Biosystems), with three technical replicates and a minimum of three biological replicates per experiment (treatment). The specificity of primers (listed in Table 1) was evaluated on RNA extracts by the dissociation curves analysis, to exclude nonspecific amplifications or primer dimers presence. The thermal profile was set up as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Dissociation curves were observed at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The efficiency of each primer pair was determined using LinRegPCR software.[25] All gene transcription levels were reported as mean normalized transcription, relative to the genes used as the reference gene, with the equation $2^{-\Delta CT}$, where ΔCT is CT target gene − CT geometric mean of the three reference genes. The statistical significance was determined using a Student's *t* test (Excel) for pairwise comparisons and one-way analysis of variance.

3 | RESULTS AND DISCUSSION

3.1 | Electrical characterization and chemical analysis of PATW

Figure 2 shows voltage and current waveforms related to five subsequent periods representative of the electrical steady-state behavior of the plasma source during the treatment of the TW. For each period (0.2 ms), four voltage peaks can be distinguished; the first and the third are more intense than the second and the fourth. The average discharge power calculated from the measured voltage and current is 79.47 ± 3.62 W.

The RONS concentrations contained in PATW after 2.5, 5, and 10 min of CAP treatment are reported in Figure 3a. More specifically, during the plasma treatment time, H_2O_2, NO_2^-, and NO_3^- concentrations increase linearly, reaching maximum values of 406 ± 67.74, 768 ± 117.33, and 4007

TABLE 1 Primers used with relative reference genes, GenBank accession number, and references

Gene	Primer	Sequence 5′–3′	GenBank accession number	Literature
VvPAL1	Forward	TGCTGACTGGTGAAAAGGTG	X75967	[26]
	Reverse	CGTTCCAAGCACTGAGACAA		
VvSTS	Forward	GTGGGGCTCACCTTTCATT	AF274281	[26]
	Reverse	CTGGGTGAGCAATCCAAAAT		
VvACT	Forward	TCAGCACTTTCCAGCAGATG	TC30205	[26]
	Reverse	TAGGGCAGGGCTTTCTTTCT		
VvUBQ	Forward	GTGGTATTATTGAGCCATCCTT	TC32075	[27]
	Reverse	AACCTCCAATCCAGTCATCTAC		
VvGAPDH	Forward	TTCTCGTTGAGGGCTATTCCA	CB973647	[22]
	Reverse	CCACAGACTTCATCGGTGACA		

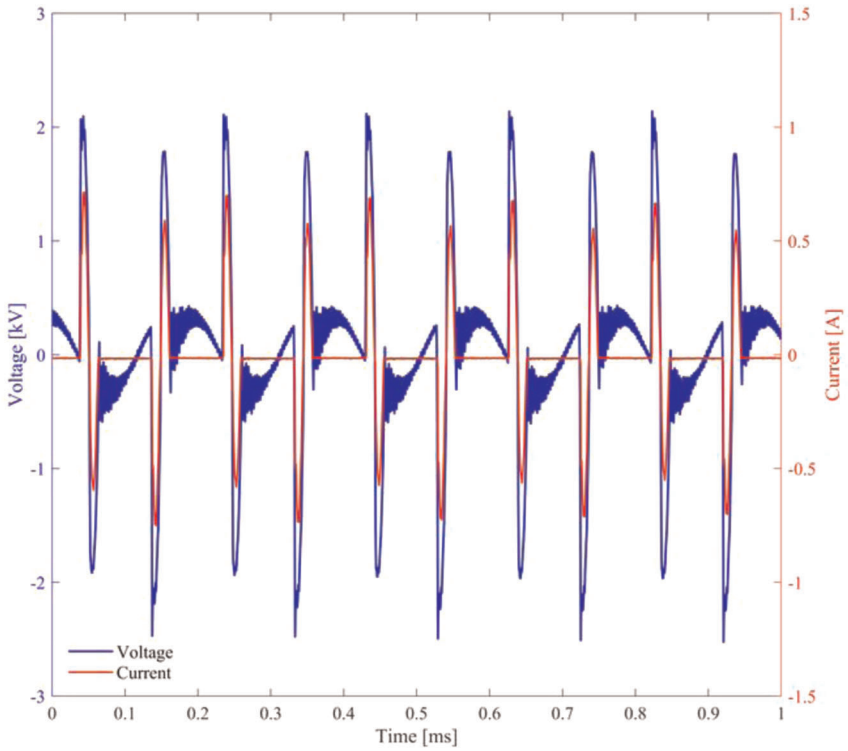

FIGURE 2 Voltage and current waveforms during plasma-activated tap water production

$\pm$ 158.33 μM, respectively. In addition, H_2O_2, NO_2^-, and NO_3^- concentrations were also evaluated 1, 3, 5, and 24 h after the CAP treatment (Figure 3b), and a low decrease of their concentrations is observed, reaching the minimum values of 372.04 $\pm$ 42.96 μM for H_2O_2, 677.33 $\pm$ 96.77 μM for NO_2^-, and 3738.67 $\pm$ 233.94 μM for NO_3^-. Moreover, a low increase of the pH value after CAP treatment with respect to the untreated solution was observed, as shown in Figure 4.

3.2 | Effects of PADW and PATW on plants

3.2.1 | PADW application in vineyards

The endotherapic treatment applied to grapevines in vineyards showed a greater performance in terms of liquid absorption/time (20 ml/15 min) in the early morning and late evening, when the plant transpiration rate is higher. In the PADW-treated plants only it has been observed a slight reduction in symptoms associated to phytoplasma presence and a delay in their appearance that in some cases allowed the plants produce. A similar phenomenon has been observed in grapevines, *Arabidopsis thaliana*, periwinkle, and *Chrysantemum* treated with benzothiadiazole (BTH) or auxin.[28–30] Moreover, nested PCR analyses showed a reduction in the number of phytoplasma-positive PADW-treated plants. In particular, after 2 years of treatment, 27 out of 76 phytoplasma-positive plants resulted negative, whereas only two SDW-treated plants showed the same behavior (Figure 5). The improvement of plant fitness, however, was registered mainly in plants with mild symptoms, whereas the plants severely symptomatic at the beginning of the trial did not show improvements. Furthermore, the statistical analysis highlighted an odds ratio index of 3.93, indicating that PADW phytoplasma-positive treated plants have a 3.93 higher probability to show symptom reduction than the untreated controls with a statistical significance of $p < .005$.

These results are in agreement with previous studies conducted with plant resistance inducers on phytoplasma-infected grapevines,[28] where researchers reported an average of 35%–57% of recovered plants after 13 treatments/year, depending on the elicitors used (chitosan and BTH, respectively). However, the negative controls have shown a recovery rate 50% lower than the BTH-treated plants. The comparison of these data with the results presented here, where a higher reduction of positive plants after PADW treatment (34.3 $\pm$ 1.25% and 15.5 $\pm$ 3%, respectively) was registered, supports the hypothesis that PADW acts as a plant resistance inducer. The quantitative yield parameters measured after 1-year treatment on 50 asymptomatic plants showed, in all the five selected vineyards, a plant fitness improvement in terms of the number of grapevine clusters per plant and 100 berry weights (Figure 6a,b). In particular, PADW-treated plants

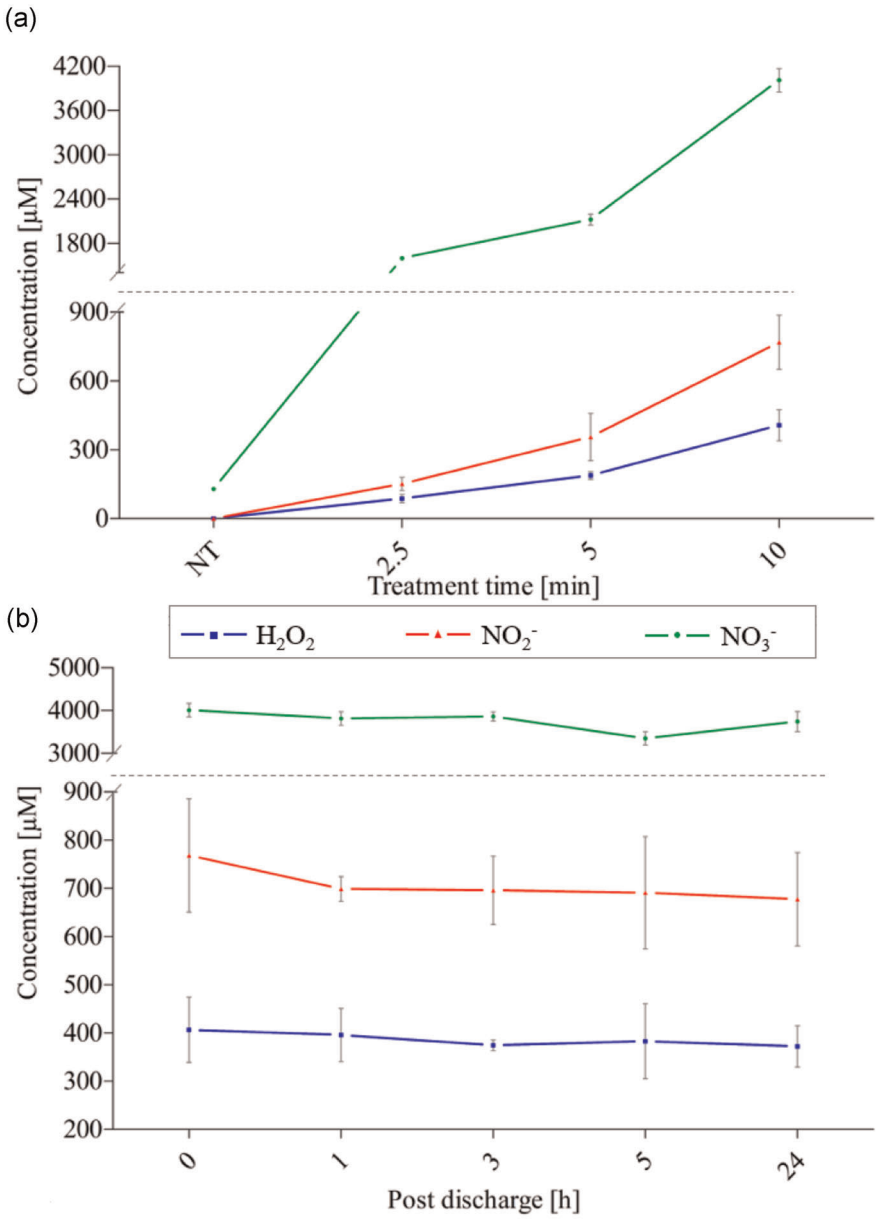

FIGURE 3 (a) The chemical analysis of the reactive oxygen and nitrogen species concentration in untreated tap water (NT) and after 2.5, 5, and 10 min of plasma treatment, and (b) its decay kinetics at room temperature

registered an average number of cluster significantly higher than controls (37.67 and 32.54, respectively), together with an increasing berry weight average of 212.80 g, compared with 194.60 g (controls). Moreover, the analysis of the main qualitative production parameters for the grape juice did not show a significant difference for the real and total acidity and Babo degrees (data not shown).

These findings were unexpected, considering the lack of positive correlation in resistance induction and plant fitness reported for other elicitors; it is known, in fact, that the secondary metabolite activation under stress has an energetic cost for the plant and leads to a reduction in terms of growth and production.[28] The overall field trial analysis highlighted a PADW effect on the enhancement of the plant fitness. This has been preliminary reported for grapevines[20] and for other plant and seed species where a higher growth and germination rate were registered after PADW exposition.[6,31]

3.2.2 | PAW effect on grapevine under controlled conditions

The results obtained by the qRT-PCR analysis showed a different behavior of PADW- and PATW-treated plants. In particular, the effect of PADW on *VvPAL1* and *VvSTS* genes relative expressions was higher at the first time point (16 h pt,T1), dropping drastically soon after, while PATW showed an effect after 26 h, in the case of *VvSTS* gene and 36 h for *VvPAL1* gene (Figure 7a,b). These results indicate that both PAWs have an effect on the expression of the studied grapevine genes. The *VvPAL1* gene encodes the

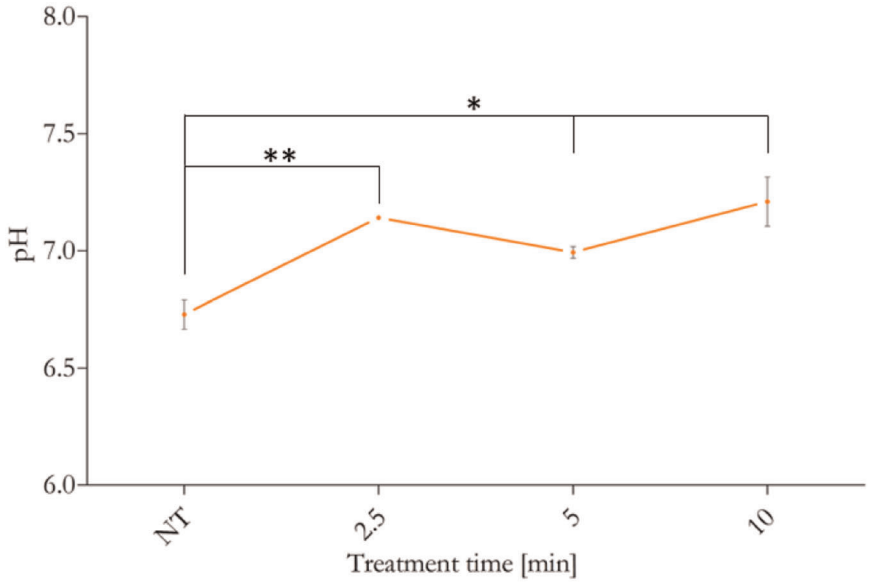

FIGURE 4 The pH values of the untreated solution (NT) and 2.5, 5, and 10 min after plasma treatment. Data are expressed as mean ± *SD*, and statistical significance is specified with asterisks (*$p \leq .05$, **$p \leq .001$, as determined by the paired Student's *t* test)

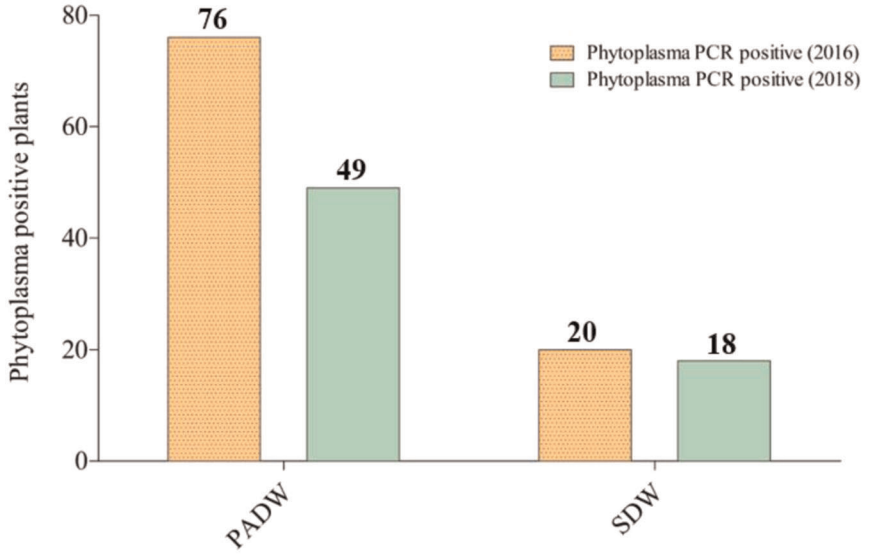

FIGURE 5 PCR phytoplasma-positive plants before (orange) and after (green) 2 years of treatment. PADW, plasma-activated distilled water; PCR, polymerase chain reactive; SDW, sterile deionized water

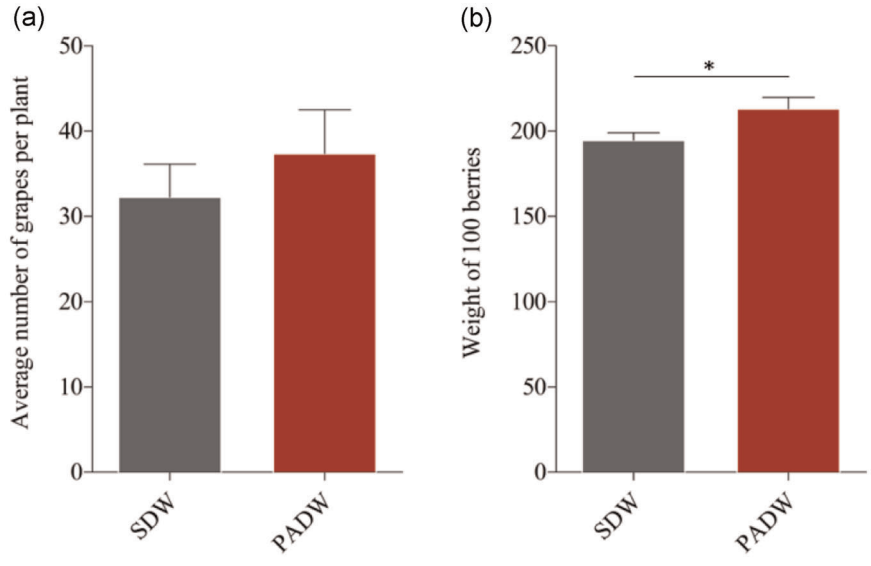

FIGURE 6 (a) The average number of grape clusters, with relative standard error and (b) weight of 100 berries, obtained after three plasma-activated distilled water (PADW) and sterile deionized water (SDW) treatments. *Statistically significant $p < .05$

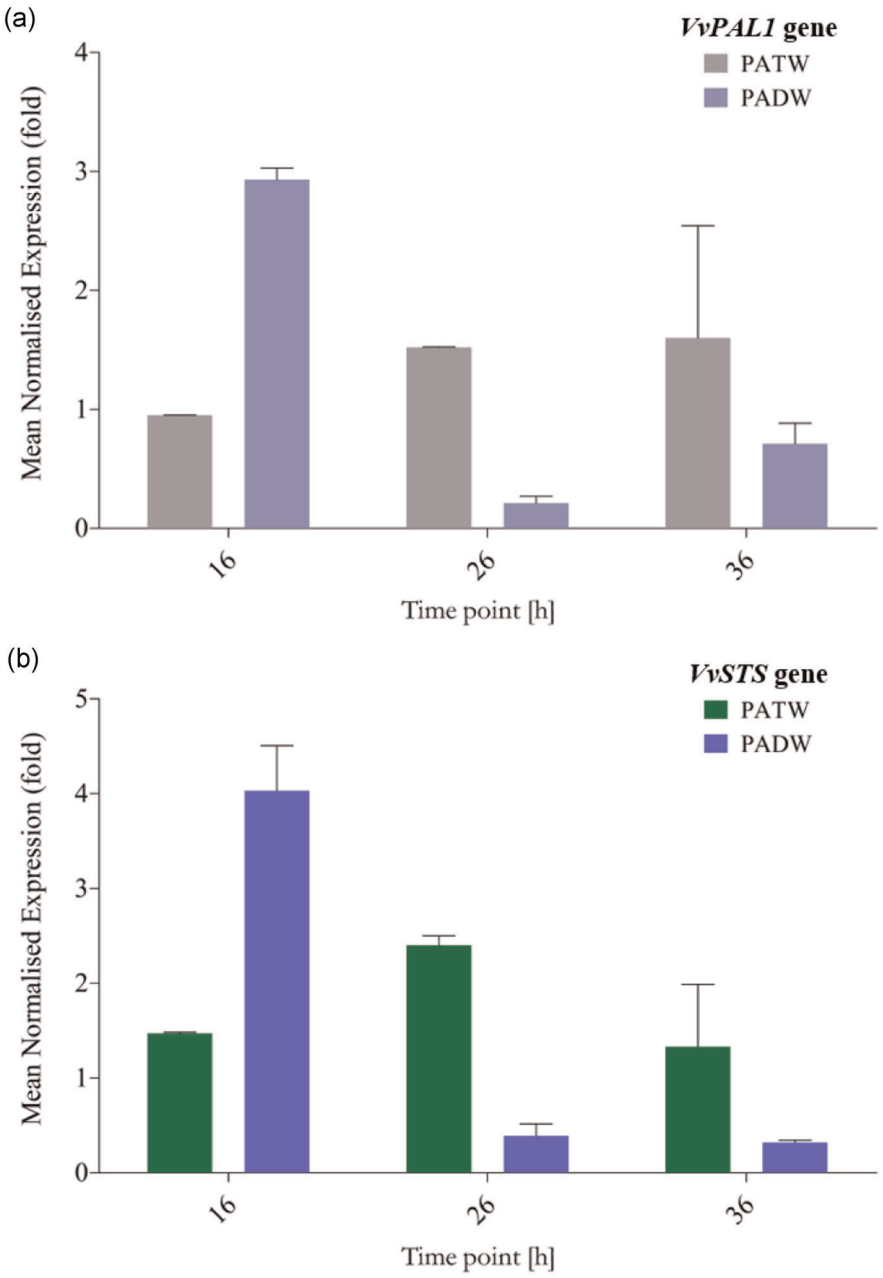

FIGURE 7 (a) Phenylalanine ammonia lyase, (b) stilbene synthase expression kinetics in grapevine plants maintained under a controlled condition 16, 26, and 36 h after plasma-activated distilled water and plasma-activated tap water treatment. The graph shows the gene induction at each time point and the standard error (±*SE*)

phenylalanine ammonium lyase enzyme, precursor of scikimate and phenylpropanoid metabolic pathways[32] through the formation of resveratrol (catalyzed by stilbene synthase [STS]).[32–34] These metabolic pathways have been extensively studied in grapevine as they contribute to the pigmentation of flowers, fruits, seeds, and leaves and are involved in different physiological and biochemicals processes such as UV protection, insect attraction, defense against herbivores and pathogens.[35–37] Resveratrol and its derivatives such as 3- and α-viniferin, pterostilbene, and piceatannol represent the main phytoalexins in grapevine.[38,39] Besides their role as defense inducers, these compounds show a strong antioxidant activity that makes them important molecules in human medicine. Moreover, among them, resveratrol has antioxidant properties used to prevent cardiovascular diseases.[40–42]

The results obtained from grapevines confirm the positive PAW effect on the phytoalexin pathway, confirming the same behavior registered in tomato and periwinkle plants.[15,43] The presence of RONS in the PAW solution generates an oxidative stress that triggers the stilbene metabolic pathway, leading the production of compounds that are able to neutralize the oxygen reactive forms due to their strong antioxidant properties.[44] Similar results were recently obtained from salt-stressed barley seedlings where nitrogen metabolism-related transcripts were positively induced by PAW, confirming its protective effect against biotic and antibiotic stress.[45]

4 | CONCLUSION

The results obtained from the open field and greenhouse showed the capability of PAW to enhance the defense mechanisms in grapevine plants and to increase plant fitness. The treatment of plants using PAW can, therefore, be considered as an innovative approach to trigger plant resistance. Future studies on the validation of the use of PATW in open fields and the analysis of the effect of the main constituent of PATW on the treated plants will open the possibility for the field application of this technology. Moreover, additional efforts will be devoted to the evaluation of the economic impact of PAW-assisted irrigation systems as compared with standard methods, to quantify the possible benefits of using PAW in open fields and greenhouses.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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