Plasma activated water to enhance plant defenses

Assunta Bertaccini[1], Enrico Biondi[1], Alessandro Canel[1], Vittorio Colombo[2,3], Nicoletta Contaldo[1], Matteo Gherardi[2,3], Romolo Laurita[2], Set Perez[1], Augusto Stancampiano[4], Yuri Zambon[1]

Alma Mater Studiorum-Università di Bologna, Bologna, Italy
[1]Department of Agricultural Sciences, Plant Pathology,
[2]Department of Industrial Engineering,
[3]Industrial Research Centre for Advanced Mechanics and Materials,
[4]Department for Life Quality Studies.

Abstract: Plasma activated water is produced treating sterile distilled water by means of an atmospheric pressure dielectric barrier discharge. Tomato plants, belonging to different cultivars and experimentally inoculated with *Xanthomonas vesicatoria* under greenhouse conditions, and phytoplasma infected grapevine plants in vineyards were treated with plasma activated water in order to evaluate its effectiveness as an alternative means to control plant diseases.

Keywords: plasma activated water; atmospheric pressure dielectric barrier discharge; reactive oxygen and nitrogen species; plant diseases.

1. Introduction

The plasma treatment of sterile distilled water (SDW) induces several changes in water chemical composition: in particular, reactive oxygen and nitrogen species (RONS) are produced [1]. These RONS might have an important role in plant defense responses, involving both hypersensitive reaction and systemic acquired resistance. Nowadays, the control of plant bacterial pathogens is addressed with the use of preventive treatments based on copper compounds, or, in the worst cases, on antibiotics. Thus, plasma activated water (PAW) treatment could represent an innovative and alternative tool to control plant diseases due to bacteria and phytoplasmas (bacteria lacking the cell wall). In this study, the effect of PAW application was tested *in planta* on tomato plants experimentally inoculated with *Xanthomonas vesicatoria* (Xv) under greenhouse conditions. Moreover, the use of PAW as a control strategy against phytoplasmas infecting grapevine plants in vineyards.

2. Materials and Methods

2.1 Plasma source and PAW production

DIW was treated by means of a nanosecond pulsed dielectric barrier discharge (Fig. 1), operating in ambient air. 10 min treatment with a peak voltage of 19 kV and a pulse repetition frequency of 1 kHz induced the formation of nitrates and peroxides (measured by means of analytic strips, Quantofix), and a pH decrease (measured with a pH meter, InLab Micro Pro).

2.2 Antimicrobial activity of PAW against Xv

The in vitro antimicrobial activity of PAW was tested with diffusion and dilution methods recommended by the Clinical and Laboratory Standards Institute [2].

Diffusion method: Luria and Bertani (LB) Agar plates were contaminated with 200 µL of bacterial suspension (approx. 107 CFU/mL). A drop of PAW was poured onto an antibiogram disc; the plates were then incubated for 24 h at 27°C. The antibacterial effect was evaluated measuring the inhibition area. The assay was repeated 3 times.

Dilution method: 15 mL of LB broth were contaminated with 150 µL of bacterial suspension (approx. 108 UFC/mL). PAW was added to the bacteria suspension and incubated at 25°C for 24 h at 80 rpm, in a rotative incubator. The bacterial population of each tube was evaluated after 24 of incubation by collecting 1 mL LB broth. Each sample was tenfold diluted, plated and incubated at 27°C for 48. The assay was repeated 3 times.

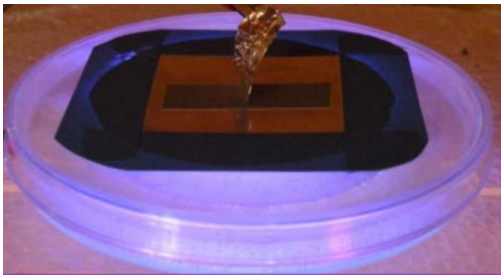

Fig. 1. DBD reactor for the production of PAW [1]

2.3 Greenhouse *in planta* experiment on tomato plants

PAW was assayed to evaluate its ability in inducing resistance in tomato plants against Xv (strain IPV-BO 2684, Xv). Tomato plants 'Moneymaker' and 'VF 10' were individually grown in pots. The plants at 3°-4° leaf stage were uprooted, and the root apparatus was soaked for 10 min into PAW, DIW (control) and acibenzolar-S-methyl (ASM, resistance inducer). The inoculation was carried out by spraying the pathogen in a water suspension (*ca.* 10^7 CFU/mL) on the leaf surfaces; tomato plants were then

sealed in polyethylene (PE) bags overnight. The disease assessments were carried out on 4 to 6 leaves/plant, 21 days after the experimental inoculation. Data were elaborated with ANOVA test using SPSS for Windows; moreover, relative protection (related to the negative control, SDW) were calculated [2].

In addition, from tomato plants (cv. Moneymaker) pretreated with PAW, SDW (negative control), ASM (resistance inducer, positive control) and jasmonic acid (JA, resistance inducer, positive control), and from plants inoculated with the pathogen, the total RNA was extracted and the increase of phenylalanine ammonia-lyase (*pal*) gene (defense-related gene) transcripts was also evaluated up to 48 hours from root treatment by using RT-qPCR. ß-actin gene was used as reference gene. All gene transcription levels were reported as Mean Normalized Transcription relative to *ß-actin*, used as the reference gene. Gene transcription was determined by the $2^{-\Delta\Delta CT}$ method [4]. Standard errors were calculated to evaluate statistically significant differences among samples.

2.4 Phytoplasma infected grapevine plants in vineyards

During 2015 and 2016, preliminary trials using PAW were carried out on grapevine plants in five 'Glera' vineyards; the PAW treatment was performed on selected symptomatic and asymptomatic plants previously analyzed for phytoplasma presence by nested PCR/RFLP assays, Experiments were carried out injecting at three different times (April, June and July) 10-20 ml of PAW or SDW in each selected phytoplasma-infected and phytoplasma-free grapevine plant; the remaining plants of the vineyards were used as untreated control to verify the natural behaviour of the disease. Specially adapted syringes were used to inject the liquids into the plant vascular tissue.

3. Results and Discussion

3.1Chemical analysis of PAW

The plasma treatment of DIW induced the production of nitrites, nitrates and hydrogen peroxide. As reported by the Authors [1], nitrites completely disappear few minutes after plasma treatment, due to their reaction with hydrogen peroxide.

Hydrogen peroxide and nitrate concentrations in PAW (reported in Table 1) are stable for 2 days when stored at room temperature; so the delay time between PAW production and PAW treatment of plants was mantained below 2 d in order to have stable concentrations of hydrogen peroxide and nitrate.

H_2O_2 [mM]	NO_3^- [mM]	pH
1,5-3	8-4	2,5

Table. 1 RONS concentration in PAW and pH after 10 min of plasma treatment.

3.2 Antimicrobial activity of PAW against Xv

Plasma activated water did not show antimicrobial activity against Xv either in diffusion and dilution *in vitro* experiments. This can be appointed to the delay time (30 min) adopted in the experiments, which caused the water to contain no more nitrites at the moment of application.

Indeed, this correlation between the loss of antimicrobial efficacy and the post discharge is well known; as an example, it was previously reported in [1], where PAW was tested against *Candida albicans* and *Staphylococcus aureus* using the dilution method.

3.2 Greenhouse *in planta* experiment on tomato plants

In Fig. 2, results on the disease assessment are reported. PAW treatment significantly reduced the number of leaf spots caused by Xv in tomato plants 'Moneymaker' and 'VF 10'. The relative protection provided by PAW treatment was approx. 38% in both tomato cultivars, independently from the disease pressure, depending in this case only by the susceptibility of the host to the pathogen. ASM, as positive control, confirmed its ability in reducing disease severity giving a relative protection of approx. 83%.

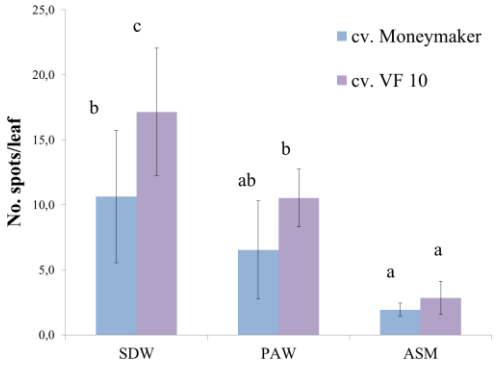

Fig.2 Results of the *in planta* efficacy of PAW applied 6 days before pathogen inoculation at root apparatus (PAW-R), compared to the negative (SDW) and positive controls (ASM), against Xv, strain IPV-BO 2684, on tomato plants cv. Moneymaker and cv. VF 10 grown under greenhouse conditions. Different letters indicate significant differences according to the Duncan's test (p 0.05).

In Fig. 3, the abundance of *pal* gene transcripts are reported. Transcriptomics results highlighted a significant increase of *pal* gene transcripts starting from 1 h and reaching its highest transcription level after 48 h. In every assessment, the *pal* transcript abundance resulted significantly higher than the one detected in negative control plants: untreated (NT) or treated with SDW (SDW-R). On the other hand, tomato plants treated with JA started *pal* transcription 7 h after the treatment and after 48 h, the transcript abundance resulted significantly higher than that of negative control plants, thus confirming the JA role in the defense response. Moreover, tomato plants inoculated with Xv began the *pal* transcription after 48 h from the inoculation, demonstrating the involvement of *pal* gene during the pathogenetic process. 48h after the treatment, the *pal* gene transcription abundance for tomato plants treated with PAW and then inoculated with Xv was significantly higher than that for plants only inoculated with the pathogen, but similar to those only treated with PAW.

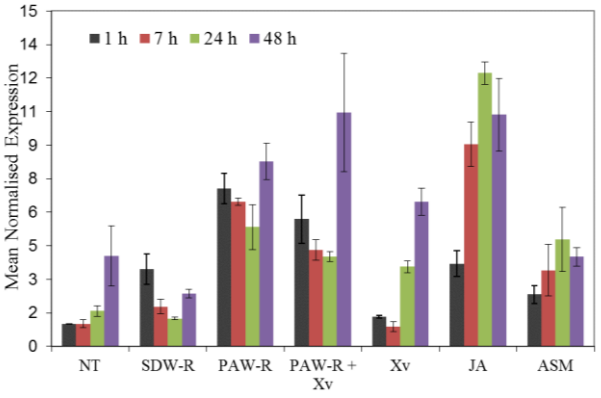

Fig.3 Transcription abundance of *pal* gene present 3 young leaves of plants treated by root drenching in PAW, SDW, ASM and JA for 10 min. The histograms show the genes transcription at 1h, 7h, 24h and 48h after the treatment.

3.3 Phytoplasma infected grapevine plants in vineyards

In a relevant number of cases, the infected plants treated with PAW showed reduction of symptoms and a production increase (Fig. 4), while the SDW and untreated plants did not show any symptom reduction.

Fig. 4. Phytoplasma infected grapevine treated with PAW showing an increasing performance during the two years of treatment. From the left: April 2015; April 2016; June 2016 and July 2016. The same plant in the previous year was not able to unfold leaves and set fruits.

PCR/RFLP analyses carried out on leaves of PAW treated plants showed a reduction of phytoplasma positive plants (62%); it is worth noting that a reduction (33%) of phytoplasma positive plants was observed also for plants treated with SDW. No phytotoxicity was observed in the phytoplasma-free plants treated with PAW. At this stage of the research, results are very promising and have prompted to increase the number of tested grapevine plants, in order to achieve statistically valuable data.

4. Conclusions

The presented results demonstrated a reduction of the disease severity both in tomato and grapevines plants, but no direct antimicrobial activity of PAW against Xv was observed; taken together, these results demonstrate that PAW works as resistance inducer for plants, triggering plant defenses against both bacteria and phytoplasmas. Furthermore, PAW did not induce any phytotoxic effect in tomato and grapevine plants.

5. References

[1] R. Laurita, D. Barbieri, M. Gherardi, V. Colombo, and P. Lukes, Clin. Plasma Med. **3**, 61 (2015).
[2] A. Bertaccini, E. Biondi, V. Colombo, N. Contaldo, M. Gherardi, R. Laurita, *et. alii*, 22nd International Symposium on Plasma Chemistry, Antwerp, Belgium, (2015).
[3] Clinical and Laboratory Standards Institute. Antimicrobial Susceptibility Testing Standards (2012).
[4] J. Livak and D. Schmittgen, Methods, **25**, 402 (2001).