In vivo investigation on the effects of plasma activated water against plant pathogenic bacteria

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Abstract: Antibacterial properties of plasma activated water (PAW) were investigated in two plant pathosystems: *Xanthomonas vesicatoria* (Xv) infected tomato plants and '*Candidatus* Phytoplasma asteris' infected periwinkle micropropagated shoots. Bacterial leaf spot severity caused by Xv was reduced and a reduced symptomatology and lack of bacterial colony formation in PAW treated periwinkle micropropagated shoots was observed, indicating the presence of a possible interaction between PAW and these pathogens.

Keywords: dielectric barrier discharge, plasma activated water, phytoplasmas, micropropagation, PCR/RFLP analyses, tomato plants

1. Introduction

The use of plasma activated water (PAW) on plants has been reported as a promising tool for the improvement of their growth [1]; moreover, plasma treatment of water is known to induce the formation of nitrates, nitrites and peroxides and a change in pH, producing PAW with antimicrobial properties [2].

Treatment of infected plants with PAW represents an innovative alternative in the control of plant diseases due to these pathogens; conventional management of plant diseases caused by bacteria has been mainly focused on the use of copper compounds and of a few biofungicides or, in the case of phytoplasmas, on insect vector chemical control and on infected plant rouging.

In this work, the antibacterial properties of PAW were investigated with *in vivo* tests in two model pathosystems: *Xanthomonas vesicatoria* infected tomato plants and '*Candidatus* Phytoplasma asteris' infected periwinkles micropropagated shoots.

In the case of tomato plants, the roots were soaked for 10 min into PAW, SDW (positive control) and a plant resistance inducer (negative control) and then inoculated by spraying a water suspension of *X. vesicatoria* on the leaves. In the second model, periwinkle micropropagated shoots [3] infected with '*Ca*. P. asteris', strains HYD8 (16SrI-B) and KVE (16SrI-C), were treated adding 1 ml of PAW to the surface of the agar contained in the micropropagation tubes.

2. Plasma source

In this work, the plasma source used for the production of the PAW is a dielectric barrier discharge (DBD) type, schematically represented in Fig. 1: it consists of a polystyrene case acting both as liquid container and as dielectric barrier (thickness 1 mm). The volume of the source enables the treatment of up to 80 ml of static liquid in a closed environment; the amount of liquid contained in the reactor determines the thickness of the gas gap. Two aluminum foils are used as electrodes; the liquid-side electrode is connected to a nanosecond pulsed high voltage generator, while the gas-side electrode is grounded. The plasma source is driven by a pulse generator producing high voltage pulses with a slew rate of a few kV/ns and 50 mJ of energy per pulse. All the experiments were carried out treating sterile distilled water (SDW) for 10 minutes in order to produce PAW.

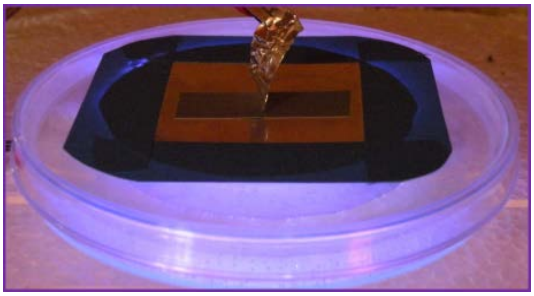

Fig. 1. Photo of the plasma source during operation.

3. Tomato plants

PAW was assayed to evaluate its ability in inducing disease resistance in tomato plants against *X. vesicatoria* (strain IPV-BO 2684, Xv). Tomato plants 'Moneymaker' were individually grown in pots. The procedures implemented for the PAW and the controls are shown in Table 1. The plants to be treated at root apparatus were explanted at third leaf stage and the roots were soaked for 10 min into PAW, SDW (positive control), Bion (negative control); Bion and SDW were also applied at

the leaf surface using a sprayer as well as streptomycin sulfate (negative control).

The inoculation was carried out spraying the pathogen water suspension (about 10^7 CFU/mL) on the surface of the leaves; tomato plants were then sealed in PE bags overnight. The disease assessments were carried out (on 4 to 6 leaves/plant) 21 days after the experimental inoculation. Fig. 2 shows that, PAW applied at the roots induces a slight but significant reduction of leaf spots caused by Xv.

Table 1.Procedures implemented for PAW treatment and controls.

Procedure name	Application mode	Application time
SDW-R	application at roots	6 days BPI
SDW-L	leaf application with sprayer	24 hours BPI
PAW-R	application at roots	6 days BPI
PAW-RW	first at roots and second watering with 50 mL	6 days BPI; 2 days API
Bion-R 75 ppm	application at roots	7 days BPI
Bion-L 75 ppm	leaf application with sprayer	7 days BPI
Streptomycin-L 100 ppm	leaf application with sprayer	24 hours BPI

BPI: before pathogen inoculation; API: after pathogen inoculation.

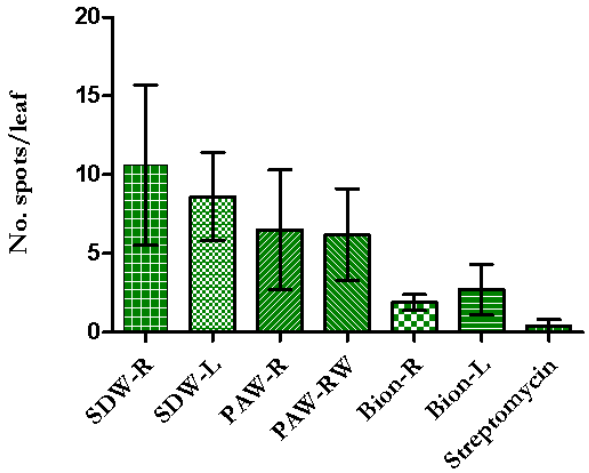

Fig. 2. Results of *in vivo* experiments on tomato plants against bacterial leaf spot caused by Xv.

4.Periwinkles micropropagated shoots

Periwinkles micropropagated shoots infected with phytoplasmas belonging to '*Candidatus* phytoplasma asteris' group, particularly HYD8 (16SrI-B) and KVE (16SrI-C), and maintained in micropropagation were treated with PAW.

Three trials with two thesis each were performed: three infected shoots were treated with the addition of 1 ml of PAW on the surface of the 10 ml agar-solidified culture medium [3] contained inside the micropropagation tubes and three shoots were treated with 1 ml of sterile deionized distilled water (SDDW) as control. After 1 month all shoots were transferred to tubes with fresh medium without PAW.

Three and six months after the treatments, total DNA was extracted from leaves of treated and untreated shoots HYD8- and KVE- and from healthy micropropagated periwinkle shoots using a CTAB method [4]. Meanwhile, phytoplasmas isolation was performed from all the treated and from the KVE untreated shoots (control), according to Contaldo *et al*. [5]. DNA extraction from inoculated tubes was carried out 10 days after isolation using a phenol/chloroform based method [6]. When tubes showed signs of a colour change from orange-red to yellow 100 µl of broth were inoculated onto plates containing 8 ml of solid medium and incubated. The agar surface was observed every 24 hours for up to seven days with optical bifocal microscope in order to verify the colony presence.

PCR assays were carried out tubes to amplify phytoplasma 16S rRNA gene using primers R16F2n/R2 followed by nested PCR with 16Sr group specific primers R16(I)F1/R1 and/or 16Sr general primers 16R758f/16R1232r (=M1/M2) [7]. The phytoplasma identification was then obtained by RFLP analyses using *Tru1*I on obtained amplicons as previously described [5].

Periwinkle shoots treated with PAW did not show toxicity symptoms, indicating that PAW treatment did not negatively affect the physiology of the shoots. On the other hand these shoots showed increased leaf size and bushy appearance compared with the healthy and the untreated ones (Fig. 3).

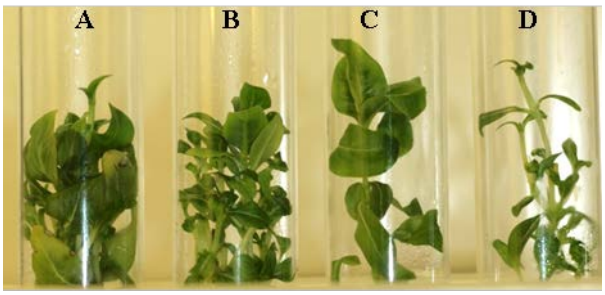

Fig. 3. Periwinkle micropropagated shoot: A and B after PAW treatement on shoots HYD8; C, healthy periwinkle shoot and D untreated periwinkle shoot HYD8.

All the infected tested shoots gave positive results for phytoplasma presence. In particular, after direct PCR with R16F2n/R2 primer pair, amplicons of the expected lengths were obtained from all the treated shoot tested (Table 2), while only after nested-PCR with R16(I)F1/R1 group specific primers amplification was obtained from HYD8 and KVE untreated shoots (Table 1). RFLP identification verify that the HYD8 phytoplasmas belong

to 16SrI-B while phytoplasmas in KVE before PAW treatment, were 16SrI-C and after PAW treatment 16SrI-B (Tables 2 and 3).

Table 2. Phytoplasma detection before PAW treatment.

Phytoplasma strains	PCR /RFLP on shoots	Culture colour change	PCR /RFLP on DNA isolation tubes
KVE + PAW	16SrI-C	Strong yellow, cloudy	Bacteria
KVE + SDDW	16SrI-C	Orange	16SrI 16SrI+16SrXII 16SrI+16SrXII
HDY8 + PAW	16SrI-B	No colour change (red)	Negative
HDY8 + SDDW	16SrI-B	No colour change (red)	Negative

Acid colour change occurred in a number of isolation tubes after up to 15 days, according to previous reports [8]. However, there were differences in the colour observed in the various tubes; in particular, it was possible to detect heavy growth, together with strong medium acidification or no colour change in some KVE tubes (Table 3). After colour change, aliquots of liquid medium were inoculated in Petri dishes, to check for colony formation.

One week after plating, some heavy bacterial growth was observed in dishes inoculated with medium from KVE untreated shoots, while no growth was observed from media containing all the PAW treated periwinkles. DNAs extracted from KVE isolation tubes after 3 months were amplified in nested PCR assays using M1/M2 primers, resulting in one case in bacterial amplification, while in the majority of other tubes the presence of 16SrXII-A or 16SrI was detected (Table 2). From all the HYD8-PAW isolation tubes 16SrI-B phytoplasmas were identified by RFLP on R16(I)F1/R1 amplicons (Table 3).

The results show some interaction between PAW treatment and phytoplasma presence consisting mainly in the improvement of phytoplasma detection in both shoots and isolation medium.

5. Conclusions

PAW treatment did not show phytotoxicity effects on both tomato plants and periwinkles micropropagated shoots.

When applied on tomato root apparatus, PAW showed a reduction of number of the leaf spots caused by Xv [9]. The reduction of the bacterial leaf spot severity after PAW root treatment suggested an indirect action mediated through the host. Further *in vivo* experiments are ongoing on a high number of plants/leaves to confirm these results and to assay different times of application before the experimental inoculation with Xv.

The PAW treatment on periwinkles phytoplasma-infected shoots allowed an improved detection of their presence in shoots and in liquid phytoplasma isolation medium [5, 10].

Table 3. Phytoplasma detection after PAW treatment.

Phyto-plasma strains	PCR/ RFLP on shoots	Culture colour change	PCR/ RFLP/3 months	PCR /RFLP/6 months
KVE-PAW	16SrI-B	Red	Negative	Nt
KVE-PAW	16SrI-B	Yellow	Bacteria	Nt
KVE-PAW	16SrI-B	Orange	16SrXII-A	Nt
KVE-SDDW	16SrI-B	Orange	16SrI	Nt
KVE-SDDW	16SrI-B	Orange	16SrXII-A	Nt
KVE-SDDW	dead	-	-	-
HDY8-PAW*	16SrI-B	Orange	16SrI-B	16SrI-B
HDY8-PAW*	16SrI-B	Orange	16SrI-B	16SrI-B
HDY8-PAW*	16SrI-B	Orange	16SrI-B	16SrI-B

*HDY8-SDDW shoots were contaminated and not tested.

The reduced bacterial leaf spot symptoms by Xv and the lack of colony formation on dishes inoculated with media from all the PAW treated shoots induce to speculate that PAW might enhance plant resistance or could interact with phytoplasmas and/or endophytes viability.

A deeper investigation on the interaction between infected plants and PAW is required to understand the effectiveness of its antimicrobial activity.

6. References

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