BMC Plant Biology

RESEARCH Open Access

Plasma-activated water promotes and finely tunes arbuscular mycorrhizal symbiosis in *Lotus japonicus*

Filippo Binci[1†], Enrico Cortese[1†], Erfan Nouri[1,2,7], Arianna Capparotto[1], Giacomo Guarneri[1], Alessio G. Settimi[3], Manuele Dabalà[3], Vanni Antoni[4,5], Andrea Squartini[2], Marco Giovannetti[6] and Lorella Navazio[1*]

Abstract

Background Plasma-activated water (PAW) is a recently developed cutting-edge technology that is increasingly gaining interest for its applications in medicine, food industry and agriculture. In plant biology, PAW has been shown to enhance seed germination, plant growth, and plant resilience against biotic and abiotic stresses. Despite increasing knowledge of the beneficial effects exerted by PAW on plants, little information is currently available about how this emerging technology may affect mutualistic plant-microbe interactions in the rhizosphere.

Results In this work we investigated the impact of irrigation with PAW, generated by a plasma torch, on arbuscular mycorrhizal (AM) symbiosis. Roots of the model legume *Lotus japonicus* expressing the bioluminescent Ca^{2+} reporter aequorin responded to treatment with PAW 5′ (obtained by 5 min water exposure to plasma) with the immediate induction of cytosolic and nuclear Ca^{2+} signals, indicating that Ca^{2+}-mediated signalling is one of the earliest cellular responses to PAW. The long-lasting elevations in intracellular Ca^{2+} levels were not found to alter cell viability. Quantitative analyses of AM fungal accommodation in the host plant roots along with phosphate accumulation in leaves, as well as chemical analysis of N, C, S in shoots, showed that treatments with PAW play a modulatory role on plant AM symbiotic performance, in a manner dependent on the time interval of water exposure to the plasma and on the duration of plant treatment with PAW. In particular, irrigation with PAW 5′ increased fungal colonization after 4 weeks, leading to a significant increase in leaf phosphate content after 7 weeks.

Conclusions Our findings reveal that PAW enhances AM symbiosis by facilitating early fungal accommodation in roots and subsequently increasing phosphate content in leaves at later stages. A better understanding of the mechanisms underlying the effects of PAW on the plant microbiome may drive research towards a fine-tuning of this novel green technology to maximize its beneficial effects in the context of a more sustainable agriculture.

Keywords Arbuscular mycorrhizal symbiosis, Plant-microbe interactions, Plasma-activated water, Calcium signalling, Sustainable agriculture, *Lotus japonicus*

[†]Filippo Binci and Enrico Cortese contributed equally to this work.

*Correspondence:
Lorella Navazio
lorella.navazio@unipd.it

Full list of author information is available at the end of the article

Introduction

The world's increasing demand for food, combined with limited natural resources and rising concerns about the environmental impact of food production, is driving the search for innovative technological solutions in agriculture and food systems. In this context, plasma-activated water (PAW) is emerging as a promising technology with the potential to tackle these pressing challenges. PAW is generated by exposing water (or other liquids) to atmospheric non-thermal plasma, i.e. an ionized gas characterized by relatively weak ionization with electrons at higher temperature than heavy particles (e.g. ions, molecules, radicals). The condition of non-equilibrium favours the presence of excited chemicals which, when interacting with water, result in a wide range of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with varying half-lives, ranging from days or longer for long-lived species to less than 1 s for short-lived ones [1, 2]. The rich mixture of chemicals has been shown to stimulate biological responses when applied to living systems [3, 4]. In particular, the resulting PAW is increasingly seen as a safe, rather inexpensive, and eco-friendly alternative that may reduce the use of pesticides and fertilizers in agriculture, thanks to its antimicrobial/disinfection properties as well as to its effects on the improvement of seed germination and plant growth [5–7]. Moreover, the ability of PAW to mildly induce plant defence responses, effectively boosting plant resistance against subsequent pathogen attacks (a pre-alert state termed "priming") is leading the research towards the fine-tuning of this novel "green" technology to maximize its beneficial effects in the context of a more sustainable agriculture, according to the Farm to Fork strategy [5, 8, 9].

A relevant subject of potential great impact, but rather unexplored so far, is how PAW treatment may impact beneficial plant-microbe interactions in the rhizosphere. The arbuscular mycorrhizal (AM) symbiosis, established between most land plants and Glomeromycotina fungi [10], and the symbiotic nitrogen fixation, a mutualistic interaction between legumes and rhizobia [11], positively influence plant growth, by improving plant mineral nutrition and increasing plant tolerance to abiotic and biotic stresses [12]. In addition, they offer ecosystem services in natural and agricultural environments. In the current context of growing environmental concerns, AM and nitrogen-fixing symbioses play a key role in ecosystem functioning and environment restoration [10, 12, 13]. The double symbiotic aptitude in staple leguminous crops raises great interest in sustainable agriculture to feed the growing global population. Moreover, increasing threats to crops by the ongoing climate change and their consequences on soil nutrient and water availability made the modulation of plant-microbe symbioses an urgent strategy to strengthen plant tolerance to abiotic stresses and enhance plant development [14]. As these symbiotic associations are crucial to overall plant health and productivity, understanding how PAW and in general plasma-based treatments may affect them is fundamental for large scale agricultural implementation of this innovative technology. It has recently been demonstrated that pre-sowing treatment of soybean, pea and lentil seeds with non-thermal plasmas resulted in the enhancement of nodulation and biological nitrogen fixation [15, 16]. Moreover, novel approaches for industrial-scale nitrogen fixation based on atmospheric plasma, implying lower energy consumption than the traditional Haber-Bosch process, are currently under investigation [17].

Plasma-activated water (PAW), generated by exposure of water to different plasma sources and conditions to span a wide range of doses and chemical mixtures, has recently been shown to induce rapid and remarkable elevations in the intracellular concentration of Ca^{2+} in the model plant *Arabidopsis thaliana* [18, 19]. Unravelling the mechanisms of perception of PAW by plants may offer a solid scientific background to modulate the large variety of effects that PAW play on plants, ranging from the promotion of plant growth to increased resistance to environmental stresses [20].

In this study we aimed to analyse how PAW treatment may impact plant-microbe symbiotic interactions in the rhizosphere, in particular the AM symbiosis. Therefore, the leguminous plant *Lotus japonicus* was used as plant experimental system, being able, unlike *A. thaliana*, to establish symbioses with both AM fungi and rhizobia. We investigated how *L. japonicus* plants respond to treatments with PAW generated by a plasma torch, in terms of cytosolic and nuclear Ca^{2+} signalling, AM fungal accommodation in roots and phosphate accumulation in leaves. The obtained data provide insights into the potential use of PAW to improve plant mineral nutrition and further develop sustainable agricultural practices.

Results

PAW triggers fast and sustained Ca^{2+} signals in the cytosol and nucleus of *Lotus japonicus* root cells

To check the potential Ca^{2+}-mediated perception of PAW, we transformed *L. japonicus* with constructs encoding the bioluminescent Ca^{2+} reporter aequorin, targeted to either the cytosol or the nucleus [21]. Root segments from *L. japonicus* composite plants were challenged with freshly prepared PAW, tested at different dilutions. PAW was generated by exposing 50 ml deionized H_2O for 5 min to a plasma torch operating at 900 W. The Ca^{2+} response of *L. japonicus* roots to treatment with PAW was monitored, by tracking changes in cytosolic ($[Ca^{2+}]_{cyt}$) and nuclear ($[Ca^{2+}]_{nuc}$) Ca^{2+} concentrations. As shown in Fig. 1A-C, the treatment of *L. japonicus* roots with PAW (final dilution 1:2) triggered

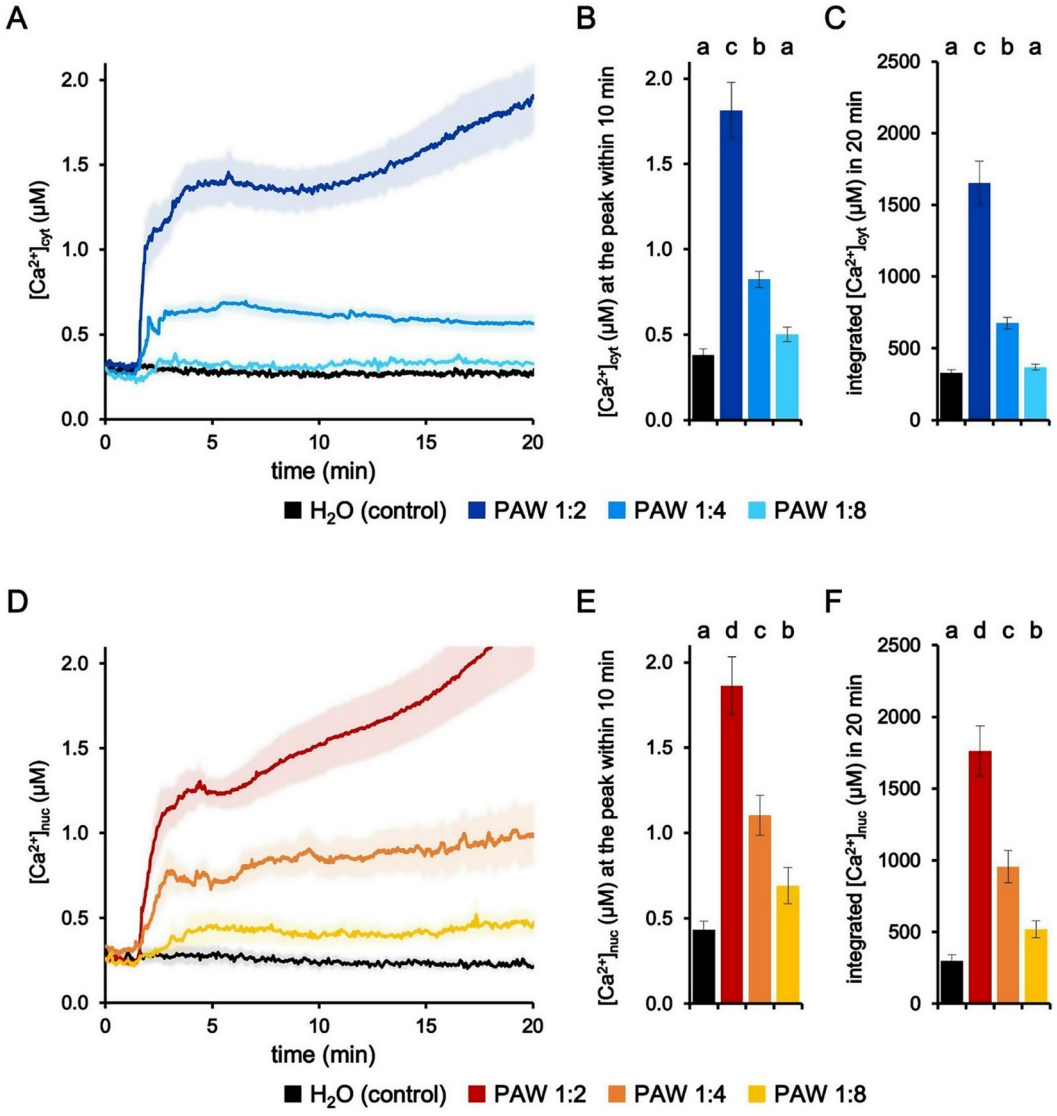

Fig. 1 Monitoring dynamic changes in cytosolic and nuclear Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$) induced by plasma-activated water (PAW) in *Lotus japonicus* roots. *L. japonicus* roots expressing aequorin-based Ca^{2+} probes targeted to either the cytosol (**A-C**) or nucleus (**D-F**) were challenged with progressive dilutions of PAW (lighter colours indicate more diluted PAWs) generated by exposing deionized H_2O to plasma torch-derived atmospheric plasma for 5 min at 900 W. Control samples were subjected to untreated deionized H_2O (black). Data are the means (solid lines) ± SE (shading) of ≥ 9 biological replicates. The arrowhead indicates the time of stimulus application (at 100 s). Statistical analyses were performed on the $[Ca^{2+}]_{cyt}$ peaks recorded in the first 10 min (**B, E**) and on the integrated $[Ca^{2+}]_{cyt}$ dynamics over 20 min (**C, F**). Bars labelled with different letters differ significantly ($p < 0.05$, Kruskal-Wallis test followed by Dunn post-hoc test)

an immediate and sustained elevation in $[Ca^{2+}]_{cyt}$, similarly to what previously observed in *A. thaliana* [18]. No Ca^{2+} increases were detected in control samples, in which deionized H_2O (without activation by plasma) was administered to *L. japonicus* roots. The magnitude of the induced Ca^{2+} signals, in terms of both $[Ca^{2+}]$ peak value (Fig. 1B) and integrated $[Ca^{2+}]$ dynamics (Fig. 1C), were found to correlate with the degree of PAW dilution, demonstrating a dose-dependent effect in the PAW-induced cytosolic Ca^{2+} changes triggered in *L. japonicus* roots. Taken together, these data confirm and extend our previous findings concerning Ca^{2+}-mediated sensing of PAW by plants [18, 19].

To analyse if the treatment with PAW might induce changes in $[Ca^{2+}]$ not only in the cytosol, but also in the nucleus, PAW was administered to *L. japonicus* roots transformed with the construct encoding nuclear-targeted aequorin. As illustrated in Fig. 1D-F, PAW was found to trigger significant elevations in $[Ca^{2+}]_{nuc}$, characterized by an amplitude correlated with PAW dilution. The Ca^{2+} response triggered by PAW 1:8, measured as integrated Ca^{2+} dynamics over 20 min, was significantly higher than the control (Fig. 1F), marking a difference

from the pattern observed in the cytosol (Fig. 1C). These results highlight for the first time the occurrence of nuclear Ca^{2+} changes triggered by PAW and add a further layer of complexity to our understanding of PAW-induced Ca^{2+} responses in plants.

Plant cell viability is not affected by treatment with PAW 5'

Ca^{2+} measurement assays in *L. japonicus* demonstrated that PAW 5' (obtained by 5 min exposure to plasma) induced, at the highest concentration tested, sustained intracellular Ca^{2+} elevations, characterized by remarkable levels of Ca^{2+} (above 1 µM) in both cytosol and nucleus, at least during the first 20 min after administration of the stimulus (Fig. 1A, D). To check whether the long-lasting elevation in intracellular [Ca^{2+}] induced by PAW 5' may affect cell viability, the Evans blue test was applied on *L. japonicus* cell suspension cultures after treatment with PAW. The viability of *L. japonicus* cells was found to remain unaltered following the application of PAW. Indeed, no significant increase in cell death was found either 1 h (Fig. 2A) or even 48 h (Fig. 2B) after PAW administration, in comparison with control samples. The absence of adverse effects on cell viability in response to PAW 5' treatment validates its potential use as a benign intervention in plant-related applications.

PAW irrigation plays a modulatory effect on the establishment and development of the AM symbiosis in *L. japonicus*

To evaluate the impact of the irrigation with PAW in terms of the establishment and development of the AM symbiosis of *L. japonicus* with the AM fungus *Rhizophagus irregularis*, 14-day-old *L. japonicus* seedlings were co-cultivated with propagules of *R. irregularis* for 4 weeks. Plants were weekly irrigated alternating the supply of low-phosphate (20 µM Pi) Long Ashton liquid medium, to boost symbiosis establishment, and PAW 5' (obtained by exposing deionized H_2O for 5 min to the plasma torch). Different concentrations of PAW 5' were tested (undiluted, 1:2, 1:4), whereas deionized H_2O was used as a control. Quantification of the root colonization, according to the Trouvelot method [22], revealed significant differences between PAW-treated plants and the controls (Fig. 3A and B). Treatment of *L. japonicus* plants with undiluted PAW 5' resulted in a significant increase in most symbiotic parameters, i.e. the intensity of mycorrhizal colonization (M%), abundance of arbuscules (A%) and the presence of vesicles (V%). On the other hand, no significant differences were observed when plants were irrigated with diluted (either 1:2 or 1:4) PAW (Fig. 3A). These data demonstrate that PAW positively modulates the establishment of AM symbiosis at an early phase of fungal colonization.

Given the positive impact of PAW 5' on mycorrhizal colonization, subsequent sets of experiments were carried out with extended time durations (5 and 7 weeks post-inoculum) and including, in addition to PAW 5', also a more intense PAW, i.e. H_2O exposed to the plasma for 10 min (PAW 10'). Figure 4A shows that after 5 weeks there were no significant differences between PAW-treated plants and controls, with the only exception of the statistically significant variation in the frequency of mycorrhiza (F%), where the PAW 10' showed a negative effect on the root colonization level. It has to be noted

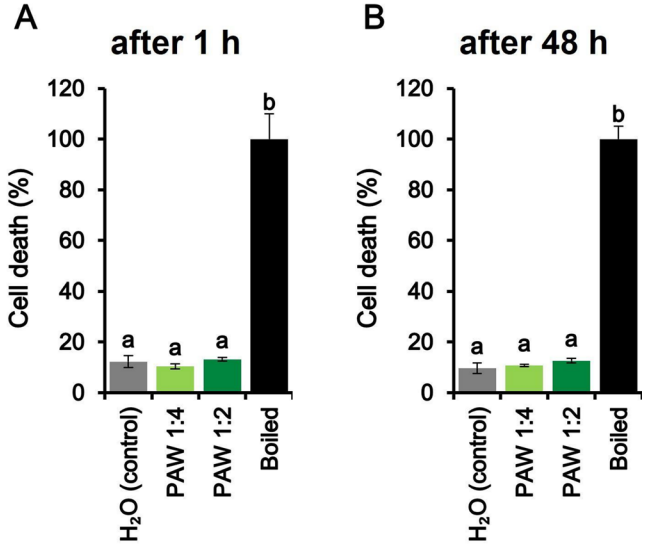

Fig. 2 Viability of *L. japonicus* cell suspension cultures treated for either 1 h (**A**) or 48 h (**B**) with PAW. PAW was generated by exposing deionized H_2O to plasma torch-derived atmospheric plasma for 5 min at 900 W (1:4 diluted, light green; 1:2 diluted, dark green). Control cells were incubated with cell culture medium only (grey). The 100% value corresponds to cells treated for 10 min at 100 °C (black bars). Data are the means ± SE of 3 biological replicates. Bars labelled with different letters differ significantly ($p < 0.05$, ANOVA test followed by Tukey's HSD)

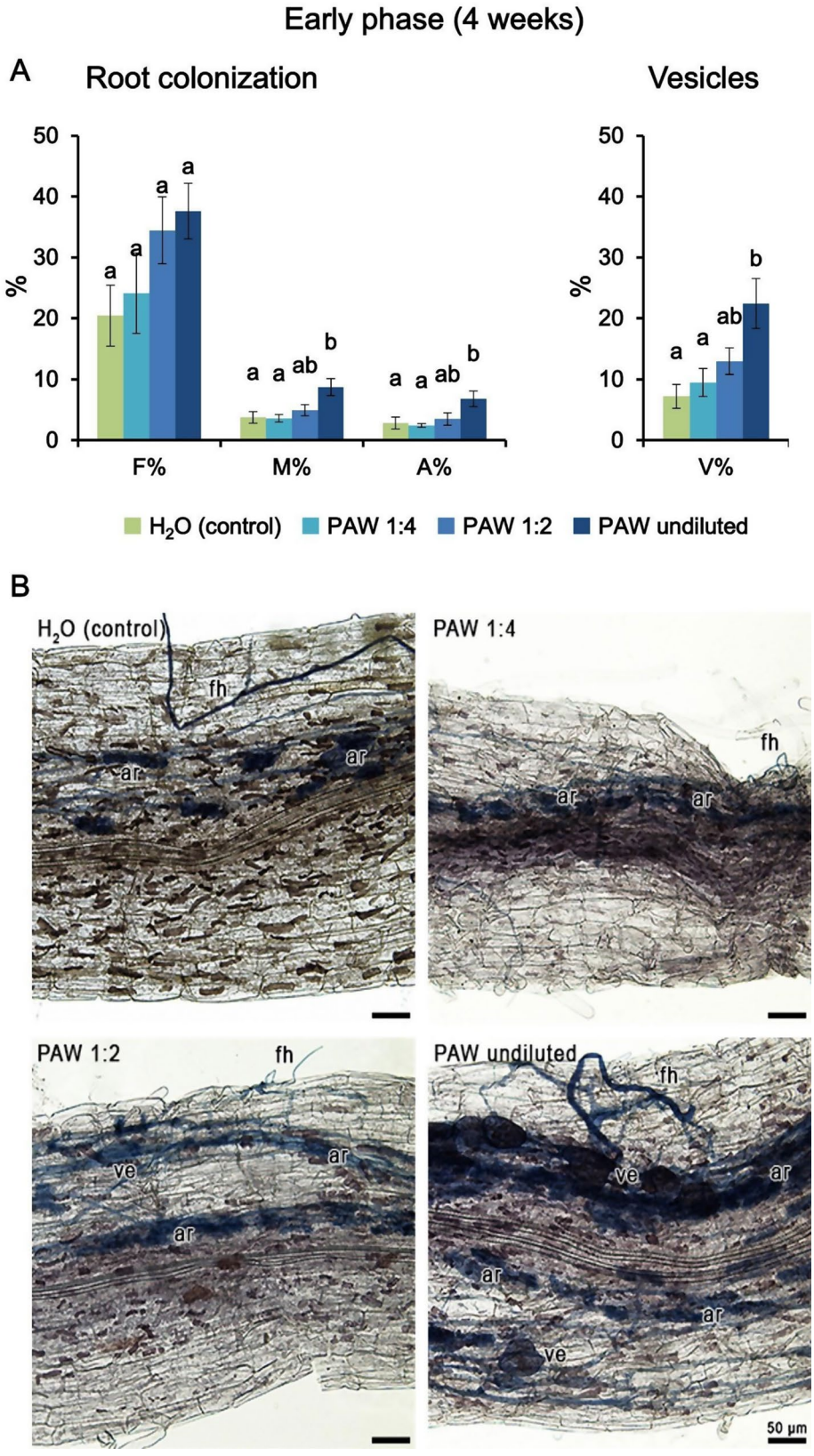

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Phenotypic evaluation of mycorrhizal colonization after 4 weeks (early phase) in *L. japonicus* plants inoculated with the AM fungus *R. irregularis* and irrigated twice per week, alternating low phosphate modified Long-Ashton with PAW (1:4 diluted, light blue; 1:2 diluted, blue; undiluted, dark blue) or H_2O (control, green). PAW was obtained by exposing deionized H_2O for 5 min to atmospheric plasma (PAW 5') generated by a plasma torch operating at 900 W. (**A**) The quantification of the degree of mycorrhization was conducted via the Trouvelot method. F%, frequency of mycorrhiza; M%, intensity of the mycorrhizal colonization; A%, arbuscule abundance. Vesicle abundance (V%) was also determined. Data are the means ± SE of 5 biological replicates. Bars labelled with different letters differ significantly ($p < 0.05$, ANOVA test followed by Tukey's HSD). (**B**) Representative images of the colonized root apparatus of *L. japonicus* plants from the different condition groups. Fungal structures are highlighted in blue by ink-lactic acid staining. ar, arbuscule; fh, fungal hypha; ve, vesicle

that also the other symbiotic parameters in the PAW 10' treatment showed a slight reduction trend, although not statistically significant, after 5 weeks. These effects were not apparent at the earlier time-point (4 weeks) (Fig. S1). The analysis conducted at the late-phase time point (7 weeks) did not reveal significant differences among the various treatment groups, suggesting a recovery phase after the potentially detrimental effect exerted by PAW 10' at the mid-time point (5 weeks). Indeed, all the plant root samples displayed an extremely high level of AM fungal colonization (close to 100% of the root segments), suggesting that saturation was reached (Fig. 4B). Therefore, the beneficial effects played by PAW 5' seem to characterize the early phase of AM interaction, by promoting a faster fungal colonization of the plant root apparatus (Fig. 3), whereas when the symbiotic interaction between the partners is fully established there are no more visible differences in the symbiotic phenotype (Fig. 4).

The effects of PAW on AM colonization levels are reflected in shoot and root biomass

For each above-mentioned experimental conditions, measurements of fresh weight were carried out for both shoot and root at the different timepoints. At the early time point (4 weeks) a positive effect of PAW 5' treatment was observed concerning both shoot and root biomasses in comparison with control (Fig. 5A). On the other hand, no statistically significant differences were observed in samples undergoing PAW 10' treatment. At the mid time point (5 weeks), PAW 10' exhibited a slight inhibitory impact on the fresh weight of the samples, indicative of a negative influence of PAW 10' on the overall growth of the plants at this specific time interval. This is consistent with viability assays on suspension-cultured cells, highlighting an increase in cell death that becomes statistically significant after 48 h of PAW 10' treatment (Fig. S2). Samples treated with PAW 5' did not show significant differences in either shoot or root fresh weight at mid phase (Fig. 5B). When the investigations were extended to the late time point of 7 weeks no significant differences in fresh weight were observed among the three treatments - control, PAW 5' and PAW 10'- suggesting a certain degree of uniformity in growth responses at this later stage (Fig. 5C). These data confirm and extend the findings obtained by Trouvelot analyses (Figs. 3 and 4).

To sum up, the obtained biomass data underscore the nuanced temporal dynamics of the effects of different PAWs on plant growth. Whereas a significant promoting effect is observed in PAW 5'-treated plants at the early phase (4 weeks), a mild depressive effect is detected with PAW 10' treatment at the mid-time point (5 weeks). Both these divergent effects on plant biomasses appear to wane as the plants progress to the late time point of 7 weeks.

PAW increases leaf phosphate content in a manner dependent on AM symbiosis

The analysis of inorganic phosphate levels at different time points provides valuable insights into the effects of PAW treatment on the mineral nutrition of *L. japonicus* mediated by the AM fungus. Soluble inorganic phosphate was extracted from the leaf samples and quantified with a colorimetric assay based on the Malachite green dye. At the early phase and mid-phase time points (4 and 5 weeks), no statistically significant differences were observed among the treatments (Fig. 6). Even if small variations in the root colonization could be observed in plants treated with PAW 5' (Fig. 3) and PAW 10' (Fig. 4), they did not reflect on the accumulation of phosphate in the leaves of the host plant, possibly because of the limited time interval analysed (4–5 weeks). However, at the late phase time point (7 weeks), noteworthy variations emerged; specifically, samples treated with PAW 5' exhibited a significantly higher concentration of leaf inorganic phosphate (Fig. 6). Notably, no differences between PAW-treated and controls were found in non-mycorrhizal plants, i.e. cultivated in the absence of the AM fungus (Fig. S3). This observation points towards an improvement in mineral nutrition facilitated by PAW 5', which might have stimulated phosphate uptake via the AM fungus or plants' assimilation processes in the presence of the fungus.

Nitrogen, carbon and sulphur analyses in *L. japonicus* shoot samples

Total nitrogen, carbon and sulphur concentrations within the aerial parts of the mycorrhizal *L. japonicus* plants collected at different time points after the onset of irrigations with either PAW 5', PAW 10', or H_2O only were determined (Fig. 7). At the early stage (4 weeks) neither PAW 5' nor PAW 10' treatment resulted in a nitrogen

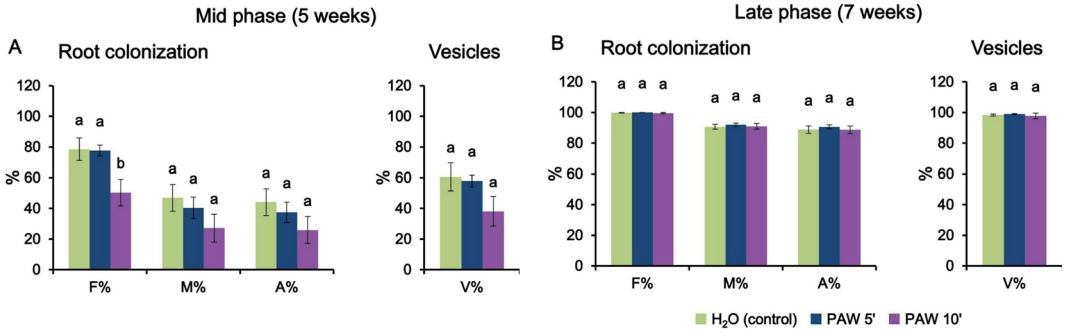

Fig. 4 Phenotypic evaluation of mycorrhizal colonization after 5 weeks (**A**, mid phase) or 7 weeks (**B**, late phase) in *L. japonicus* plants irrigated twice per week, alternating low phosphate modified Long-Ashton with PAW 5′ (blue), PAW 10′ (violet) or H_2O (control, green). PAW was obtained by exposing deionized H_2O for either 5 min (PAW 5′) or 10 min (PAW 10′) to atmospheric plasma generated by a plasma torch operating at 900 W. The quantification of the degree of mycorrhization was conducted via the Trouvelot method. F%, frequency of mycorrhiza; M%, intensity of the mycorrhizal colonization; A%, arbuscule abundance. Vesicle abundance (V%) was also determined. Data are the means ± SE of 5 biological replicates. Bars labelled with different letters differ significantly ($p < 0.05$, ANOVA test followed by Tukey's HSD)

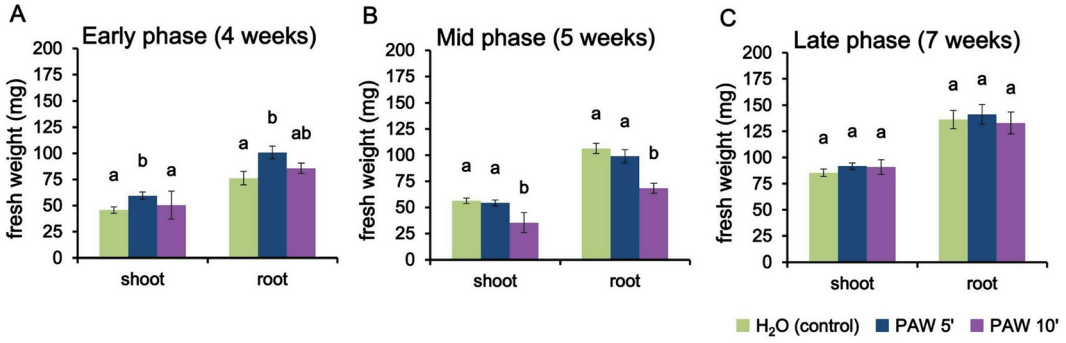

Fig. 5 Shoot and root biomass of *L. japonicus* inoculated with *R. irregularis* and repeatedly irrigated with PAW 5′, PAW 10′ or H_2O. Pots containing *L. japonicus* seedlings were treated twice per week alternating low phosphate modified Long-Ashton with either PAW 5′ (blue), PAW 10′ (violet) or H_2O (control, green) and harvested after 4 weeks (**A**, early phase), 5 weeks (**B**, mid phase) or 7 weeks (**C**, late phase) after the first treatment, respectively. PAW was obtained by exposing deionized H_2O for either 5–10 min to atmospheric plasma generated by a plasma torch operating at 900 W. Data are the means ± SE of 5 biological replicates. Bars labelled with different letters differ significantly ($p < 0.05$, Kruskal-Wallis test followed by Dunn's post-hoc test)

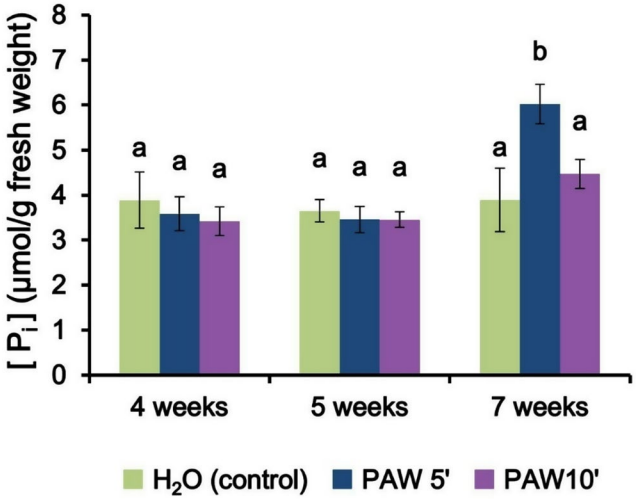

Fig. 6 Inorganic phosphate (Pi) content in leaves of *L. japonicus* seedlings inoculated with *R. irregularis* and repeatedly irrigated with PAW 5′, PAW 10′ or H_2O. Pots containing *L. japonicus* seedlings were irrigated twice per week alternating low phosphate modified Long-Ashton with either PAW 5′ (blue), PAW 10′ (violet) or H_2O (control, green) and harvested after 4 (**A**, early phase), 5 (**B**, mid phase) or 7 weeks (**C**, late phase) after the first treatment, respectively. PAW was obtained by exposing deionized H_2O for either 5–10 min to atmospheric plasma generated by a plasma torch operating at 900 W. Data are the means ± SE of 5 biological replicates. Bars labelled with different letters differ significantly ($p < 0.05$, ANOVA test followed by Tukey's HSD)

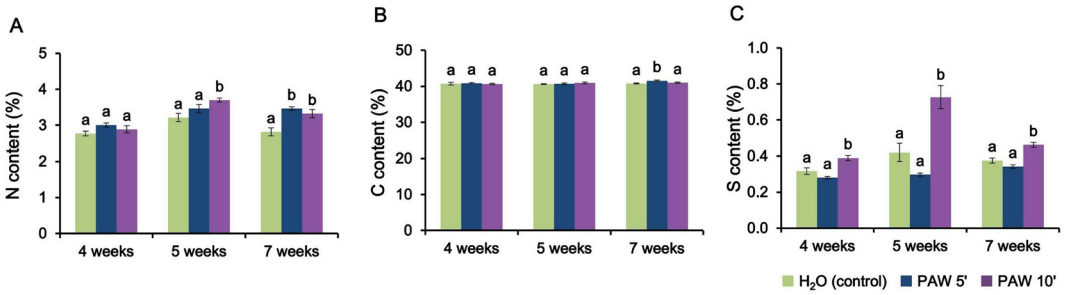

Fig. 7 Quantification of nitrogen, carbon and sulphur content in dry shoots of *L. japonicus* seedlings inoculated with *R. irregularis* and repeatedly irrigated with PAW 5', PAW 10' or H_2O. Pots containing *L. japonicus* seedlings were treated twice per week alternating low phosphate modified Long-Ashton with either PAW 5' (blue), PAW 10' (violet) or H_2O (control, green) and harvested after 4 (early phase), 5 (mid phase) or 7 weeks (late phase) after the first treatment, respectively. PAW was obtained by exposing deionized H_2O for either 5 min (PAW 5') or 10 min (PAW 10') to atmospheric plasma generated by a plasma torch operating at 900 W. The content of nitrogen (panel **A**), carbon (panel **B**), sulphur (panel **C**) is expressed as percentage relative to the dry shoot biomass (% w/w). Data are the means ± SE of 5 biological replicates. Bars labelled with different letters differ significantly ($p < 0.05$, ANOVA followed by Tukey's post-hoc)

content significantly higher than the control. As the experiment progressed into the mid phase, a shift in the relationship between PAW treatments and N content became apparent. Indeed, at 5 weeks the PAW 10' treatment, which is associated with lower fresh weight (Fig. 6), shows the highest N content. This fact could be interpreted as smaller plants having more concentrated N content. As the experiment reached its late phase (7 weeks), both PAW treatments exhibited a statistically significant increase in respect with the control. As a general trend, PAW treatments resulted in increased N accumulation in the shoots (Fig. 7A). Notably, a similar effect of PAW 5' and PAW 10' was obtained in non-mycorrhizal plants at 7 weeks, suggesting a beneficial effect of PAW treatment on the plant content of these elements, independently of fungal colonization (Fig. S4). In terms of carbon content (Fig. 7B), the data exhibit an almost uniform behaviour across treatments and time intervals analysed, in line with the constant role of this element in herbaceous plants of this type and age. The data about sulphur content (Fig. 7C) indicate that mycorrhizal plants undergoing PAW 10' treatments are associated with a statistically significant S accumulation at all time points, whereas in the case of non-mycorrhizal plants this effect is only observable with PAW 5' after 7 weeks.This suggests a potential complex dual role for PAW treatment in enhancing plant sulphur content, both through the direct S uptake pathway and by stimulating mycorrhizal-dependent pathways, depending on the length of plasma exposure time.

Discussion

Plasma-activated water (PAW) represents a novel and emerging eco-friendly technology with potential broad impact in agriculture, regarding plant fertilization, increased tolerance to abiotic stresses and control of pests [8, 23, 24]. Despite in the last few years there has been a surge of papers in this field, information on how PAW and in general plasma treatments may impact plant-microbe symbiotic interactions is still scarce and only limited to nodulation [15, 16, 25]. In this study, we conducted an experimental investigation into PAW sensing mechanisms and effects on the initiation and progression of the mutualistic symbiosis between the model legume *L. japonicus* and the arbuscular mycorrhizal (AM) fungus *R. irregularis*.

Since PAW perception has recently been demonstrated to occur through Ca^{2+}-mediated signalling in *A. thaliana* [18, 19], in this work we monitored early responses to different dilutions of PAW in *L. japonicus* roots expressing the bioluminescent Ca^{2+} reporter aequorin. Our results demonstrate that PAW triggers fast and sustained Ca^{2+} elevations not only in the cytosol, but also in the nucleus of *L. japonicus* root cells, adding a further layer of complexity in PAW-activated signal transduction pathways. Very recently, non-thermal plasma irradiation has been shown to induce a rapid and transient increase in cytosolic Ca^{2+} in the liverwort *Marchantia polymorpha* expressing the GFP-based Ca^{2+} indicator GCaMP6f [26]. Moreover, exposure of *A. thaliana* plants to the plasma-generated reactive gas N_2O_5 was found to trigger long distance Ca^{2+} signalling within tens of seconds [27]. These findings indicate that fast intracellular Ca^{2+} elevations can be considered as hallmarks of initial cellular responses to both PAW and direct plasma treatments in plants [20].

The experimentalset-up for mycorrhizal experiments involved the inoculation of *L. japonicus* seedlings with the AM fungus *R. irregularis*, followed by weekly irrigation with PAWs, generated by exposing H_2O to the plasma for either 5 min (PAW 5') or 10 min (PAW 10') for different time intervals, and the following analyses of mycorrhizal fungal colonization after 4, 5 and 7 weeks. The obtained results indicate that the treatments with

PAW shape AM development and functioning, in a manner dependent on the time interval of water exposure to the plasma and on the duration of plant irrigation with PAW. Our investigation reveals that the administration of PAW 5' displays a significantly promoting effect on AM symbiosis that leads to an fungal increased colonization after 4 weeks. On the other hand, PAW 10' seems to exert a mild depressive effect in the exponential phase of the colonization process (5 weeks). An intriguing possibility is that a longer exposure of water to the plasma, and the consequent higher content of ROS and RNS in the PAW, may activate a pre-alert state in the plant, mounting a response more directed to the activation of defence mechanisms against environmental stresses, such as potential pathogens [5], rather than the accommodation of fungal symbionts.

The determination of inorganic phosphate levels in the leaves and the chemical analysis of N, C, S content in the entire shoots of PAW-treated plants *versus* control plants allowed to link PAW composition and AM-mediated plant nutrient uptake. PAWs generated by either plasma torches or dielectric barrier discharge sources are enriched in H_2O_2, nitrate (NO_3^-) and nitrite (NO_2^-) [18, 19]. H_2O_2 is a well-known ROS that plays a dual role in plant biology, acting as both a stress inducer and a signalling molecule [28]. Nitric oxide (NO), a signalling molecule derived from $(NO_3)^-$ and $(NO_2)^-$ has multifaceted roles in plants, including mediation of root growth and modulation of stress responses [29]. Moreover, NO has been demonstrated to play a regulatory role during plant interactions with both pathogenic and mutualistic fungi [30, 31]. In both NO- and ROS-based signal transduction pathways, Ca^{2+} has been proposed to act as intracellular mediator [32–34], with mutual interplay resulting in the amplification of the respective signalling mechanisms [35]. Considering the relatively high concentration of $(NO_3^-)^-$ and $(NO_2)^-$ in plasma torch-generated PAW [18], it is likely that treatment with PAW provides the plant with a nutritional advantage as suggested by the N content data, along with the putative activation of H_2O_2- and NO-triggered signalling pathways, leading to either a priming status or facilitating the accommodation of symbiotic fungi. Further investigations are needed to address the importance of these two effects of treatment with PAW on plant physiology and interactions with the plant microbiome. For phosphate, the beneficial effect of PAW was dependent on fungal presence, while PAW treatment inherently enhanced plant nitrogen content, even in the absence of the AM fungus.

The data obtained in this work concerning the effects induced by the application of PAW on AM symbiosis in *L. japonicus* align well with the model proposed by Song and colleagues [36] about the effects of plasma treatments on plant responses, that critically depend on the operational parameters of the plasma source. Indeed, the impact of PAW on AM symbiosis in *L. japonicus* can be explained with the intriguing concept of plant hormesis, which is a distinctive dose-response phenomenon characterized by stimulatory responses at low doses and inhibitory responses at higher doses [37–40]. Based on the obtained results, we propose a model about the hormetic effect of PAW treatment on AM symbiosis, which encompasses the following: i) PAW 5': promotion of AM fungal colonization and plant growth in the early phase, along with an increase of phosphate uptake in the late phase; ii) PAW 10': delay in root colonization and plant growth during the mid-phase, but no long-lasting side effects in the late phase (Fig. 8).

Future investigations should be carried out by using different plasma sources, such as the dielectric barrier

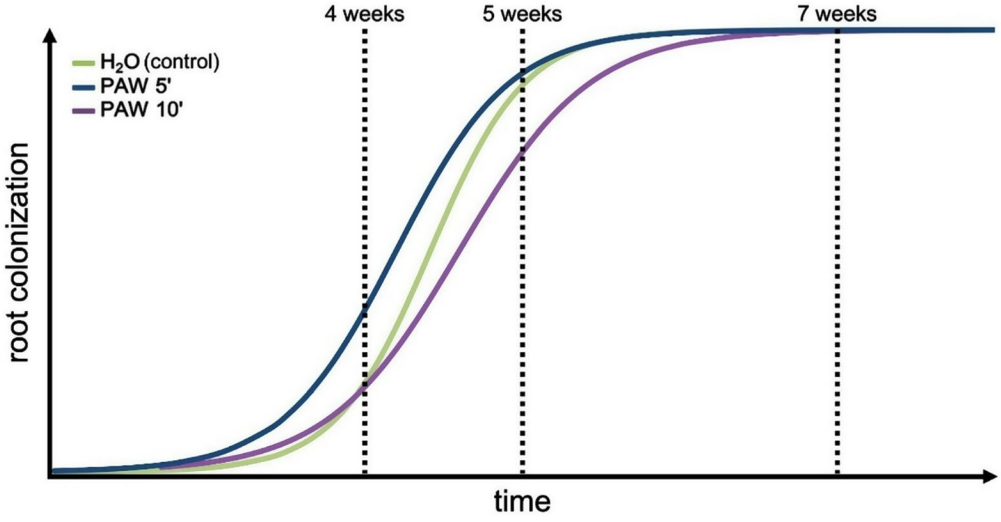

Fig. 8 Proposed model of hormetic effect played by PAW on the establishment and development of the AM symbiosis

discharge, which allows different mix of chemical species to be generated and finely tuned acting on parameters of the plasma generator [26]. Further insights into potential applications of PAW will be obtained by extending these studies to plant species of agronomic interests and to additional beneficial plant-microbeinteractions.

Conclusions

This study underscores the potential of PAW technology to finely tune the ability of plants to engage in symbiotic relationships with beneficial microorganisms of the rhizosphere, in particular AM fungi. As AM symbiosis is crucial to overall plant health and productivity, understanding how PAW may affect them is fundamental for large scale agricultural implementation of PAW. Our results suggest that plasma treatments may be used to improve plant mineral nutrition and/or induce a prealert state (priming) through a controlled generation and administration of PAW to plants. Further investigations are needed to analyse the fine balance, under different PAW treatment regimes, between accommodation and defence responses during the dynamic relationships of the plant with its associated microbiome.

Materials and methods

Sterilization and germination of *Lotus japonicus* seeds

Lotus japonicus Gifu ecotype seeds were scarified in a mortar with sandpaper. The sterilization was performed in a 2-mL tube with 2 mL of 0.5% NaClO for 11 min. Seeds were washed five times with sterile H_2O and left swollen in water for about 30 min. Seeds were plated in half-strength B5 (Duchefa Biochemie, Haarlem, The Netherlands) medium (pH 5.5, adjusted with KOH), supplemented with 1% (w/v) Plant Agar (Duchefa) in 12 × 12 cm squared Petri dishes and were vertically incubated covered with an aluminum foil. After 3 days, the aluminum foil was removed and seedlings were left growing in the same conditions.

L. japonicus hairy root transformation by *Agrobacterium rhizogenes*

Hairy roots transformation was performed according to Boisson-Dernier et al. [41]. -80 °C stocked *Agrobacterium rhizogenes* AR1193 strains carrying the plasmid encoding either cytosolic or nuclear-targeted aequorin-based Ca^{2+} chimeras [21] were plated in LB medium containing 1% Bacto-Agar and supplemented with 50 µg/ml rifampicin, 100 µg/ml ampicillin and 50 µg/ml kanamycin. Bacteria were grown for 2 days at 28 °C, and then plated again to have a full Petri dish of actively growing cells. 7-day-old *L. japonicus* seedlings were cut with a scalpel in the hypocotyl region to remove the root. The scar at the base of the remaining shoot was then dipped into the bacterial film on the plate and placed in a new squared Petri plate provided with half-strength B5, 1% Plant Agar. The shoots were then co-cultivated with bacteria at 22 °C for 3 days in the dark and for additional 3 days at 16 h/8 h light/dark cycle. The shoots were then transferred to new squared 12 × 12 cm Petri dishes containing half-strength B5, 1% Plant Agar and 300 µg/ml cefotaxime (Duchefa) and let grow for 4–5 weeks (22 °C, 50% humidity, 16 h/8 h light/dark). Transformed roots were then identified at the fluorescence stereomicroscope MZ16f (Leica, Wetzlar, Germany) via the fluorescence of the YFP-aequorin chimeras [21].

Generation of plasma-activated water (PAW)

Plasma-activated water (PAW) was produced by exposing deionized H_2O to the atmospheric plasma generated by a plasma torch operating at 900 W power and 3 bar pressure. The torch used for this study was a single rotating FLUME Jet RD1004 with an FG 1001 plasma generator (Plasmatreat, Elgin, IL, USA) with a maximum power of 2.7 kW (230 V, 12 A). Throughout the study, the reported results employed 50 mL of deionized H_2O with its surface exposed for 5 to 10 min at 1.5 cm from the torch nozzle. H_2O was kept in beakers immersed in an ice and salt cooling bath (Fig. S5) to keep the water temperature constant during the exposure. Once generated, PAW was divided into single-use aliquots, rapidly cryogenically frozen through immersion in liquid nitrogen, and stored at -80 °C.

Evaluation of cell viability

Cell viability was assessed using the Evans blue method [42] using *L. japonicus* suspension cell cultures, established and subcultured as previously described [43]. Cultured-cells were either maintained in control conditions or treated with PAW 5' and PAW 10' for 1–48 h during their mid-exponential growth phase (4 days). Following a 15 min incubation with 0.05% (w/v) Evans blue dye (Merck, Darmstadt, Germany), unbound dye was removed by thorough washing with H_2O. The dye bound to dead cells was then solubilized in a solution containing 1% (w/v) SDS and 50% (v/v) methanol, maintained at 55 °C for 30 min and then collected. The extent of cell death was determined by measuring the absorbance at 600 nm. As positive control representing 100% cell death, a separate cell aliquot was incubated at 100 °C for 10 min.

Aequorin-based Ca^{2+} measurement assays

Ca^{2+} measurement assays were conducted in *L. japonicus* roots using the recombinant expression of aequorin chimeras targeted to either the cytosol or nucleus [21]. 5-mm-long transformed root segments from composite plants were reconstituted overnight with 5 µM coelenterazine (Prolume, Pinetop, AZ, USA). On the following day, the root segments were extensively washed to remove

excess coelenterazine and transferred in a custom-built luminometer (ET Enterprises Ltd, Uxbridge, UK) containing a 9893/350A photomultiplier (Thorn EMI). Each root segment was placed in 50 µl H_2O and challenged by injection of an equal volume of PAW produced through a 5 min long exposure to plasma torch at different dilutions (1:2, 1:4, 1:8). Ca^{2+} dynamics were recorded for 20 min and each experiment was terminated with the injection of 100 µL of the discharge solution (30% v/v ethanol, 1 M $CaCl_2$). The light signal was recorded and later converted into Ca^{2+} concentration values using an algorithm based on the Ca^{2+} response curve of aequorin [44]. The Ca^{2+} peak (i.e. the highest $[Ca^{2+}]$ value obtained over time as means of the Ca^{2+} response intensity) and the Ca^{2+} integral (i.e. sum of all $[Ca^{2+}]$ values over time, as means of the total mobilized Ca^{2+} in the cell) were used as parameters to compare the Ca^{2+} signals activated by different concentrations of PAW.

PAW treatment of *L. japonicus* seedlings co-cultivated with the AM fungus *Rhizophagus irregularis*

14 days after sterilization, *L. japonicus* seedlings were transplanted into plastic pots (220 ml volume) with 4 seedlings per pot. The pots were filled with washed and autoclaved river sand characterized by a granulometry of 1–5 mm, which was subsequently inoculated with 3000 propagules of the AM fungus *Rhizophagus irregularis* (Agronutrition, Carbonee, France). A set of plants was cultivated in the absence of the fungus (non-mycorrhizal control). Afterwards, each pot was irrigated using 20 ml of a modified Long Ashton liquid medium (1.5 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.75 mM $MgSO_4$, 0.1 mM Fe-EDTA, 10 µM $MnCl_2$, 50 µM H_3BO_3, 1.75 µM $ZnCl_2$, 0.5 µM $CuCl_2$, 0.8 µM Na_2MoO_4, 1 µM KI, 0.1 µM $CoCl_2$) with low phosphate content (20 µM KH_2PO_4). Starting from the 10^{th} day of pot cultivation, a bi-weekly irrigation regimen was initiated. The first irrigation involved applying 20 ml of the appropriate treatment, either H_2O (control samples) or freshly prepared PAW activated by plasma torch for 5 min (PAW 5') or 10 min (PAW 10'). The second irrigation, conducted 3 days later in the same week, employed 20 ml of modified liquid Long Ashton, containing 20 µM Pi. Plants were cultivated in pots for 4, 5 and 7 weeks in order to assess the impact of PAW treatment at distinct stages of the AM symbiosis establishment and functioning. The full experimental setup is depicted in Fig. 9. All irrigations were performed from the bottom of the pot. At the end of each irrigation period *L. japonicus* plants were harvested by pooling together the 4 seedlings growing in each pot, representing a single biological replicate. The roots were separated from the shoots of each plant and rinsed gently with H_2O to remove excess sand debris. For each plant, the fresh biomasses of the root apparatus and of the entire shoot were evaluated with a precision digital balance. For each plant, roots and aerial parts (either entire shoots or only leaves) were processed as described in the next sections.

Evaluation of AM fungal colonization

Roots of inoculated plants were ink-stained to highlight intraradical fungal structures and quantify them according to the Trouvelot method [22]. Before staining, the roots were clarified with 10% KOH at 96 °C on a heating block for 6 min. After incubation, KOH was discarded and 2 washing steps were performed with 5% lactic acid. Roots were then incubated in the staining solution (5% black ink, 5% lactic acid) at 96 °C for 11 min. The staining solution was then discarded and stained roots were washed in 5% lactic acid, replacing the washing solution until the liquid was clear. From each biological replicate (1 pot, 4 plants) five microscope slides were prepared, each containing 20 root fragments of 1 cm in length. Consequently, for each pot approximately 100 cm of root apparatus were analysed.

Measurements of phosphate concentration

Each leaf sample (second leaf triplet from the shoot apex) was weighted in an analytical balance and stored at -80 °C until use. Frozen samples were homogenized with the help of a metal bead in a TissueLyser II (Qiagen, Hilden, Germany) via two rounds of shaking at 30 Hz for 20 s. The tissue powder was resuspended in 1 ml ultrapure H_2O, vortexed for 10 s and incubated at 100 °C for 1 h. After 10 min incubation on ice, samples were centrifuged at 4 °C for 15 min at 13,200 rpm. The supernatant was used for measurement of the concentration of soluble phosphate using the Phosphate Assay Kit (Merck, Darmstadt, Germany), following the manufacturer's instructions, as previously described [45]. Standards were prepared to make a calibration curve ranging from 0 to 40 µM phosphate. Both standards and samples were mixed with an equal volume of the dye Malachite green in a transparent 96-well plate and incubated for 30 min at room temperature in the dark. Absorbance at 620 nm was measured using a Tecan multiwell plate reader (Männedorf, Switzerland). Each sample was analysed in two technical replicates. Absorbance data were converted into concentrations of phosphate using the calibration curve and normalized to the fresh weight of the leaf sample.

Chemical analyses of *L. japonicus* shoot samples

Shoots from harvested seedlings were used to quantify N, C and S concentrations. To this aim, the samples were dried in an oven set at 60 °C until a constant weight was achieved (2–16 h). The dried plant samples were ground into a fine powder to ensure homogeneity. Chemical

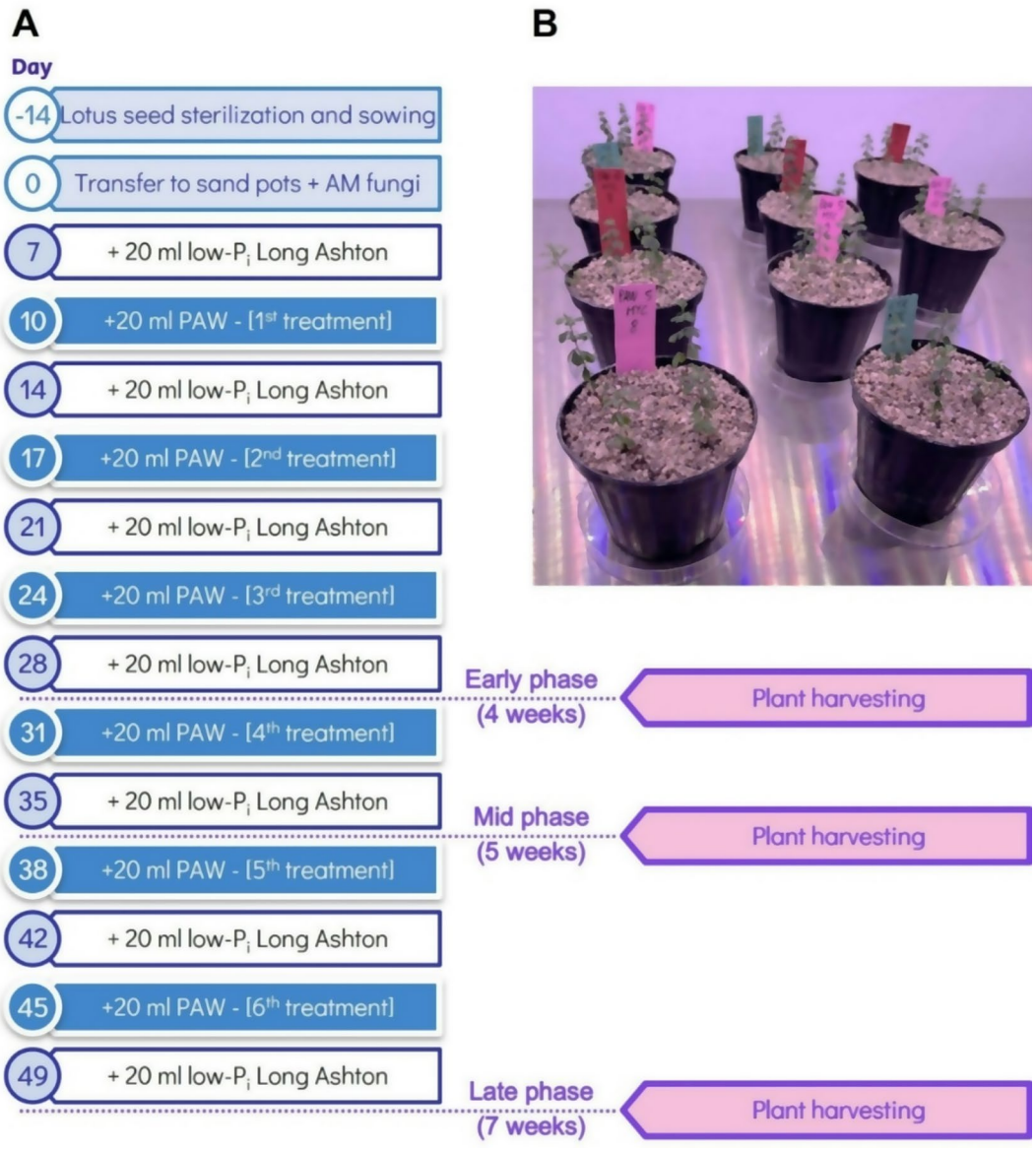

Fig. 9 Experimental set-up for the evaluation of the effects of PAW treatment on the co-cultivation of *L. japonicus* seedlings with the AM fungus *R. irregularis*. (**A**) Schematic representation of the irrigation regime alternating low Pi (20 µM KH_2PO_4) Long-Ashton with PAW. (**B**) Representative picture of *L. japonicus* seedlings in pots

analyses of N, C and S were conducted using a CNS Macrovario combustion analyzer (Macrovario, GmbH).

Statistical analyses

Statistical analyses were performed via R-Studio software, using the packages: tidyverse, multcomp, ggpubr, rstatix, patchwork and ggplot2. After verifying that the data presented normal distribution and homogeneity of variances, an ANOVA was performed, followed by Tukey's HSD (Honestly Significant Difference) post-hoc test. In case data did not exhibit normal distribution or homoscedasticity, the Kruskal-Wallis non-parametric test was performed, followed by Dunn's post-hoc test. In each figure letters are used to indicate statistically different groups (p-value < 0.05).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06563-1.

Supplementary Material 1: Fig. S1. Phenotypic evaluation of mycorrhizal colonization after 4 weeks (early phase) in L. japonicus plants inoculated with the AM fungus ***R. irregularis*** after repeated irrigations with PAW 10′ or H2O only. Fig. S2. Viability of ***L. japonicus*** cell suspension cultures treated for either 1 h (A) or 48 h (B) with PAW 10′. Fig. S3. Inorganic phosphate (Pi) content in leaves of non-mycorrhizal ***L. japonicus*** seedlings after repeated irrigations with PAW 5′, PAW 10′ or H2O only. Fig. S4. Quantification of nitrogen, carbon and sulphur content in dry shoots of non-mycorrhizal ***L.***

japonicus seedlings after repeated irrigations with PAW 5', PAW 10' or H2O only. Fig. S5. Representative image of the generation of plasma-activated water (PAW) by exposing water to a plasma torch.

Acknowledgements
We thank the Plant Genome Editing facility of the Department of Biology, University of Padova (Italy) and the central Chemical Laboratory (La-Chi) of the Department of Agronomy, Food, Natural resources, Animals and Environment, University of Padova (Italy) for skillful technical assistance.

Author contributions
F.B., E.C., E.N., A.C., G.G. and A.G.S. conducted the experiments. F.B. and E.C. analysed the data and prepared the figures. L.N. and M.G. designed the study, supervised the experiments and secured the funding for this project. F.B. and L.N. wrote the manuscript and M.G., A.S., V.A. and M.D. revised and edited it. All authors read and approved the manuscript.

Funding
Open access funding provided by Università degli Studi di Padova.
This work was supported by grants from the European Union - Next Generation EU (PRIN prot. 2022NW97JX to L.N.; PRIN PNRR prot. P2022WL8TS and 2021 STARS Grants@Unipd programme P-NICHE to M.G.) and from the University of Padova (PRID prot. BIRD214519 to M.G.).

Data availability
All data generated during this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Biology, University of Padova, Via U. Bassi 58/B, 35131 Padova, Italy
[2]Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Viale dell'Università 16, 35020 Legnaro, Italy
[3]Department of Industrial Engineering, University of Padova, Via Marzolo 9, 35131 Padova, Italy
[4]National Research Council, Institute for Plasma Science and Technology (CNR-ISTP), Corso Stati Uniti 4, 35127 Padova, Italy
[5]Consorzio RFX, Corso Stati Uniti 4, 35127 Padova, Italy
[6]Department of Life Sciences and Systems Biology, University of Torino, Viale Mattioli 25, 10125 Torino, Italy
[7]Present Address: Earth and Life Institute, Université catholique de Louvain, Croix du S, 1348 Ottignies-Louvain-la-Neuve, Louvain, Belgium

Received: 4 January 2025 / Accepted: 15 April 2025
Published online: 25 April 2025

References

1. Zhou R, Zhou R, Wang P, Xian Y, Mai-Prochnow A, Lu X, et al. Plasma-activated water: generation, origin of reactive species and biological applications. J Phys D: Appl Phys. 2020;53:303001.
2. Fridman A, Kennedy LA. Plasma physics and engineering. third ed. Boca Raton: CRC; 2021.
3. Thirumdas R, Kothakota A, Annapure U, Siliveru K, Blundell R, Gatt R, et al. Plasma activated water (PAW): chemistry, physico-chemical properties, applications in food and agriculture. Trends Food Sci Technol. 2018;77:21–31.
4. Kaushik NK, Ghimire B, Li Y, Adhikari M, Veerana M, Kaushik N, et al. Biological and medical applications of plasma-activated media, water and solutions. Biol Chem. 2018;400:39–62.
5. Adhikari B, Pangomm K, Veerana M, Mitra S, Park G. Plant disease control by non-thermal atmospheric-pressure plasma. Front Plant Sci. 2020;11.
6. Starič P, Vogel-Mikuš K, Mozetič M, Junkar I. Effects of nonthermal plasma on morphology, genetics and physiology of seeds: a review. Plants. 2020;9:1736.
7. Konchekov EM, Gusein-Zade N, Burmistrov DE, Kolik LV, Dorokhov AS, Izmailov AY, et al. Advancements in plasma agriculture: a review of recent studies. Int J Mol Sci. 2023;24:15093.
8. Holubová Ľ, Kyzek S, Ďurovcová I, Fabová J, Horváthová E, Ševčovičová A, et al. Non-thermal plasma - A new green priming agent for plants? Int J Mol Sci. 2020;21:9466.
9. Pańka D, Jeske M, Łukanowski A, Baturo-Cieśniewska A, Prus P, Maitah M, et al. Can cold plasma be used for boosting plant growth and plant protection in sustainable plant production? Agronomy. 2022;12:841.
10. Genre A, Lanfranco L, Perotto S, Bonfante P. Unique and common traits in mycorrhizal symbioses. Nat Rev Microbiol. 2020;18:649–60.
11. Gage DJ. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol Mol Biol Rev. 2004;68:280–300.
12. Field KJ, Daniell T, Johnson D, Helgason T. Mycorrhizas for a changing world: sustainability, conservation, and society. Plants. People Planet. 2020;2:98–103.
13. Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, et al. Towards an ecological Understanding of biological nitrogen fixation. Biogeochemistry. 2002;57:1–45.
14. Trivedi P, Batista BD, Bazany KE, Singh BK. Plant-microbiome interactions under a changing world: responses, consequences and perspectives. New Phytol. 2022;234:1951–9.
15. Pérez-Pizá MC, Cejas E, Zilli C, Prevosto L, Mancinelli B, Santa-Cruz D, et al. Enhancement of soybean nodulation by seed treatment with non–thermal plasmas. Sci Rep. 2020;10:4917.
16. Abeysingha DN, Dinesh S, Roopesh MS, Warkentin TD, Thilakarathna MS. The effect of cold plasma seed treatments on nodulation and plant growth in pea (*Pisum sativum*) and lentil (*Lens culinaris*). Plasma Process Polym. 2024;21:2400015.
17. Aceto D, Ambrico PF, Esposito F. Air cold plasmas as a new tool for nitrogen fixation in agriculture: underlying mechanisms and current experimental insights. Front Phys. 2024;12.
18. Cortese E, Settimi AG, Pettenuzzo S, Cappellin L, Galenda A, Famengo A, et al. Plasma-activated water triggers rapid and sustained cytosolic Ca^{2+} elevations in *Arabidopsis thaliana*. Plants. 2021;10:2516.
19. Cortese E, Galenda A, Famengo A, Cappellin L, Roverso M, Settimi AG, et al. Quantitative analysis of plant cytosolic calcium signals in response to water activated by low-power non-thermal plasma. Int J Mol Sci. 2022;23:10752.
20. Antoni V, Cortese E, Navazio L. Plasma-activated water to foster sustainable agriculture: evidence and quest for the fundamentals. Plants People Planet. 2025. in press. https://doi.org/10.1002/ppp3.70025
21. Binci F, Offer E, Crosino A, Sciascia I, Kleine-Vehn J, Genre A, et al. Spatially and temporally distinct Ca^{2+} changes in *Lotus japonicus* roots orient fungal-triggered signalling pathways towards symbiosis or immunity. J Exp Bot. 2024;75:605–19.
22. Trouvelot A, Kough J, Gianinazzi-Pearson V. Evaluation of vesicular arbuscular mycorrhizal infection levels in root systems. Research for Estimation methods having a functional significance. In: Gianinazzi-Pearson V, Gianinazzi S, editors. Physiological and genetical aspects of mycorrhizae. France: INRA-; 1986. pp. 217–21.
23. Guo D, Liu H, Zhou L, Xie J, He C. Plasma-activated water production and its application in agriculture. J Sci Food Agric. 2021;101:4891–9.
24. Gao Y, Francis K, Zhang X. Review on formation of cold plasma activated water (PAW) and the applications in food and agriculture. Food Res Int. 2022;157:111246.
25. Abeysingha DN, Dhaliwal HK, Du L, De Silva C, Szczyglowski K, Roopesh MS, et al. The potential of cold plasma-based seed treatments in legume–rhizobia symbiotic nitrogen fixation: a review. Crops. 2024;4:95–114.
26. Tsuboyama S, Okumura T, Watanabe K, Koga K, Shiratani M, Kuchitsu K. Real-time live imaging of cytosolic hydrogen peroxide and Ca^{2+} of *Marchantia polymorpha* Gemmalings reveal immediate initial responses of plant cells triggered by nonthermal plasma irradiation. Plant Physiol Biochem. 2024;216:109172.

27. Sasaki S, Iwamoto H, Takashima K, Toyota M, Higashitani A, Kaneko T. Induction of systemic resistance through calcium signaling in Arabidopsis exposed to air plasma-generated dinitrogen pentoxide. PLoS ONE. 2025;20:e0318757.
28. Smirnoff N, Arnaud D. Hydrogen peroxide metabolism and functions in plants. New Phytol. 2019;221:1197–214.
29. Besson-Bard A, Pugin A, Wendehenne D. New insights into nitric oxide signaling in plants. Annu Rev Plant Biol. 2008;59:21–39.
30. Calcagno C, Novero M, Genre A, Bonfante P, Lanfranco L. The exudate from an arbuscular mycorrhizal fungus induces nitric oxide accumulation in *Medicago truncatula* roots. Mycorrhiza. 2012;22:259–69.
31. Martínez-Medina A, Pescador L, Terrón-Camero LC, Pozo MJ, Romero-Puertas MC. Nitric oxide in plant–fungal interactions. J Exp Bot. 2019;70:4489–503.
32. Besson-Bard A, Courtois C, Gauthier A, Dahan J, Dobrowolska G, Jeandroz S, et al. Nitric oxide in plants: production and cross-talk with Ca^{2+} signaling. Mol Plant. 2008;1:218–28.
33. Gilroy S, Suzuki N, Miller G, Choi W-G, Toyota M, Devireddy AR, et al. A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci. 2014;19:623–30.
34. Gilroy S, Białasek M, Suzuki N, Górecka M, Devireddy AR, Karpiński S, et al. ROS, calcium, and electric signals: key mediators of rapid systemic signaling in plants. Plant Physiol. 2016;171:1606–15.
35. Marcec MJ, Gilroy S, Poovaiah BW, Tanaka K. Mutual interplay of Ca^{2+} and ROS signaling in plant immune response. Plant Sci. 2019;283:343–54.
36. Song J-S, Kim SB, Ryu S, Oh J, Kim D-S. Emerging plasma technology that alleviates crop stress during the early growth stages of plants: a review. Front Plant Sci. 2020;11:988.
37. Agathokleous E, Kitao M, Calabrese EJ. Hormesis: a compelling platform for sophisticated plant science. Trends Plant Sci. 2019;24:318–27.
38. Schirrmacher V. Less can be more: the hormesis theory of stress adaptation in the global biosphere and its implications. Biomedicines. 2021;9:293.
39. Erofeeva EA. Environmental hormesis of non-specific and specific adaptive mechanisms in plants. Sci Total Environ. 2022;804:150059.
40. Erofeeva EA. Plant hormesis: the energy aspect of low and high-dose stresses. Plant Stress. 2024;14:100628.
41. Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant-Microbe Interact. 2001;14:695–700.
42. Baker CJ, Mock NM. An improved method for monitoring cell death in cell suspension and leaf disc assays using Evans blue. Plant Cell Tiss Organ Cult. 1994;39:7–12.
43. Moscatiello R, Sello S, Ruocco M, Barbulova A, Cortese E, Nigris S, et al. The Hydrophobin HYTLO1 secreted by the biocontrol fungus *Trichoderma longibrachiatum* triggers a NAADP-mediated calcium signalling pathway in *Lotus japonicus*. Int J Mol Sci. 2018;19:2596.
44. Ottolini D, Calì T, Brini M. Methods to measure intracellular Ca^{2+} fluxes with organelle-targeted aequorin-based probes. Methods Enzymol. 2014;543:21–45.
45. Ames BN. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. 1966;8:115–8.

Publisher's note