Plant Archives Vol. 26, Supplement 1, 2026 pp. 1413-1421 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2026.v26.supplement-1.187

INNOVATIVE STRATEGIES FOR MANAGING PHYTOPLASMA-ASSOCIATED PLANT DISEASES USING ANTIBIOTICS AND ALTERNATIVE ANTIMICROBIAL AGENTS

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(Date of Receiving : 21-09-2025; Date of Acceptance : 26-11-2025)

ABSTRACT

Phytoplasmas are pathogenic bacteria that affect numerous significant crops and ecologically important plant species, resulting in substantial economic and environmental detriment globally. These bacteria, devoid of a cell wall, are susceptible to medications like tetracyclines that interfere with protein synthesis processes. Cultivation of phytoplasma in axenic media has not been accomplished for numerous strains; hence, antimicrobial screening must primarily utilize in vivo materials. Certain research have examined the utilization of in vitro phytoplasma-infected shoots, as well as several antimicrobials, such as tetracyclines, have been evaluated. The evaluation of phytoplasma antimicrobials is crucial for the sustainable management of phytoplasma-related illnesses. It is recommended to utilize compounds with diverse mechanisms of action, including ribosome-inactivating proteins, plant hormones, along with resistance inducers like plasma-activated water, to circumvent the application of antibiotics in agriculture and potential development of resistant microbial strains.
***Keywords*:** phytoplasmas, Plant disease, Chemical control, Induced resistance; Molecular detection

Introduction

Phytoplasmas are mycoplasmas associated with several hundred plant diseases worldwide, including many diseases with important economic or environmental impacts. They are *Mollicutes, i.e*., prokaryotes lacking cell walls, found in plant phloem and insect hemolymph and are transmitted by insects, propagation materials, and seeds. The primary symptoms linked to their presence include stunted plant growth, chlorosis and deterioration, floral virescence, and deformities (Bertaccini *et al.,* 2014; Namba *et al.,* 2019) (Figure 1). Historically, the use of antibiotics allowed the indirect confirmation of the phytoplasma's role in several plant diseases (Ishiie *et al.,* 1967) since they caused the disappearance, often temporary, of microbes and symptoms from the infected plants. The field control of phytoplasma diseases is mainly dependent on the use of insecticides against their insect vectors. However, this strategy is often ineffective because it cannot eliminate the source plants of these diseases (Bertaccini *et al.,* 2010). The culture of phytoplasma in axenic media has not been accomplished for numerous strains; hence, the screening of antimicrobials must primarily utilize in vivo materials. Numerous research have examined the utilization of in vitro phytoplasma-infected shoots, with various antimicrobials, such as tetracyclines, undergoing evaluation (Bertaccini Assunta, 2021). Several research have documented techniques for eradicating phytoplasmas from diseased plants, including shoot-tip culture, heat therapy, callus culture, and hot water treatment; however, these approaches are not applicable in field conditions. Antibiotics like streptomycin and oxytetracycline remain widely used against bacterial plant diseases, raising concerns over antibiotic resistance genes (ARGs), their spread via mobile genetic elements, and cross-transfer to human pathogens (Tn5393 example)(Marie Verhaegen *et al.,* 2023). Since the mid-20th century, streptomycin and oxytetracycline

have been key in controlling plant diseases, but rising resistance and climate-driven epidemics now threaten their efficacy, necessitating careful monitoring and targeted antibiotic use (Ozgur Batuman *et al.,* 2024). Only tetracycline medicines have shown efficacy in mitigating phytoplasma symptoms and proliferation; nonetheless, total eradication of phytoplasma from plants remains challenging. Furthermore, the utilization of antibiotics for agricultural purposes is banned in some nations, and their prolonged exposure may pose risks to human health. For these reasons, non-antibiotic molecules were tested to assess whether they directly or indirectly reduce the phytoplasma presence and symptoms in contaminated plants. The deployment of antibiotics substitutes, like resistant inducers in open fields poses issues. In vitro systems can effectively produce phytoplasma-free germplasm, which can then be propagated in insect-proof environments before being introduced into open fields. This strategy is reducing the environmental impact of insecticides/pesticide to manage the phytoplasma-associated diseases. In this review, approaches used for phytoplasma elimination in plants and *in vitro* shoots are summarized. The reduction of the presence of insect vectors in the field is almost ineffective as it is not feasible to eliminate all of them from the environment. Utilization of healthy plants is essential for eradication or mitigation of phytoplasma-associated illnesses.

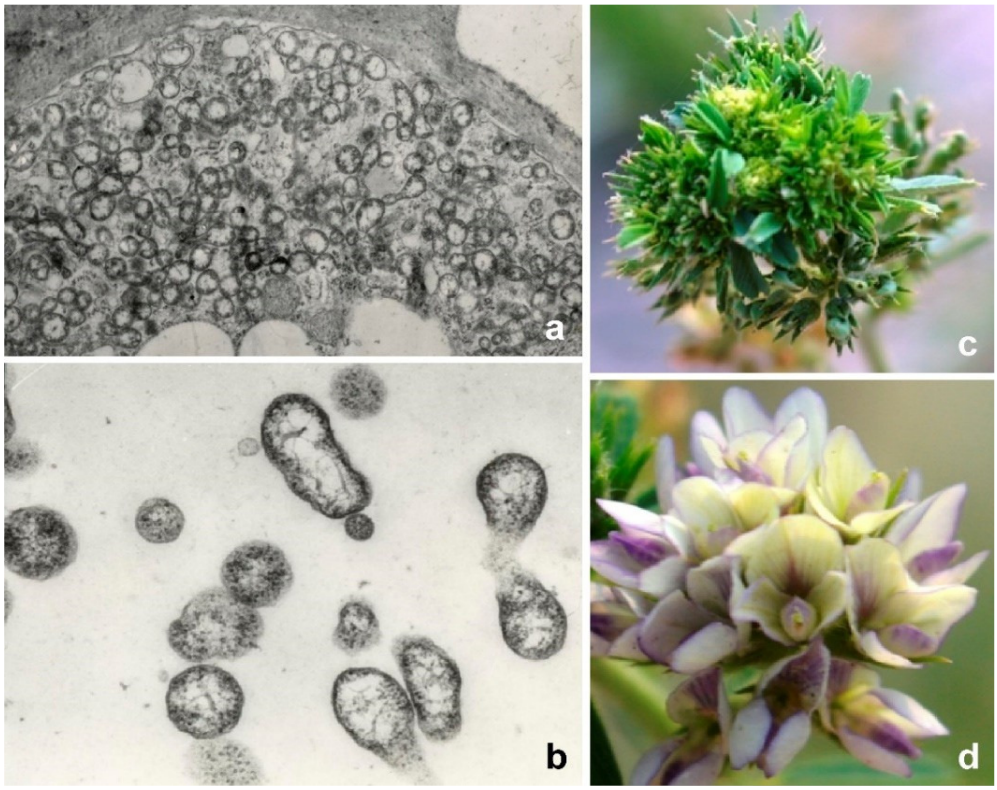

Fig. 1 (a) Transmission electron microscopy of phytoplasmas in sieve tissues (6000× magnification); **(b)** close-up (10,000× magnification); **(c)** fenugreek (Trigonella foenum-graecum) inflorescence exhibiting virescence and phyllody; **(d)** healthy inflorescence (courtesy of J.N. Ahmad).

Antibiotics

Field Application

Common methodologies for antibiotic application in agriculture include root immersion, soil soaking' foliar sprays and trunk injection. However, independently from the method used it was reported symptoms reoccurrence once the antibiotic treatments are suspended. In the urban areas of South Florida, antibiotics have been used in palms of high landscape value (McCoy, 1982). Nyland and Moller (Nyland *et al.,* 1973) first reported that tree decline and leaf curl as typical symptoms of pear decline could be prevented by injecting a solution of oxytetracycline hydrochloride into affected trees. Tetracycline was subsequently employed in the USA to manage pear decline (Nyland *et al.,* 1973). This arduous, costly, and environmentally detrimental treatment has been terminated due to the utilization of rootstocks with reduced disease

susceptibility and a significant decline in pear production in the affected regions. Efforts to treat peach rosette & X-disease afflicted trees in California with chemotherapy, namely tetracycline injection into the trunk, confirmed initial phytotoxic effects alongside a remission of diseases in only a limited number of treated specimens. Antibiotics have been administered to infected mulberry plants through foliar spray, roots immersion of infected seedlings, pre-planting treatment of shoot cuttings, pre-grafting treatment of budwoods, and therapy of stored & unsprouted shoots, yielding limited effects (Asuyama, 1973). Seidl (Seidl, 1980) indicated that in Europe, apple proliferative plasma can be eradicated from budwood throughout summer by immersing foliated apple bud sticks in a solution of oxytetracycline or chlortetracycline (100–200 ppm) for 24–48 hours before to grafting. Tetracycline application in India led to a transient alleviation of symptoms in brinjal afflicted by small leaf disease; yet, this intervention failed to eradicate pathogen from host plant (Bindra *et al.,* 1972; Anjaneyulu *et al.,* 1972). Tetracycline root dip treatment of affected onion seeds for 15 weeks at 7-day periods resulted in phytoplasmas being present solely in untreated infected plants (Tanaka *et al.,* 1984). Physostegia virginiana (false dragon head) was administered a solution of oxytetracycline hydrochloride at a concentration of 100 ppm. Roots were immersed for 25 hours under darkness, subsequently washed and potted under greenhouse conditions. The plants demonstrated phytotoxicity solely during the initial three months. For two years, the treated plants exhibited no phytoplasma symptoms, and tissue analyses conducted by electron microscopy confirmed the absence of the bacteria (Giunchedi *et al.,* 1986). Plants infected with Ranunculus phytoplasma, exhibiting pronounced stunting and rosette morphology, were administered 300 mL of oxytetracycline per plant thrice weekly for a duration of two months. It exhibited no phytotoxicity at concentrations of 1 mg/L or 100 mg/L. A decrease in symptoms was noted with the emergence of yellow flowers, characteristic of the healthy variety. Two weeks post-treatment, the symptoms recurred, corroborating bacteriostatic effect of antibiotic (Bertaccini *et al.,* 1988). Various compounds, including biophenicol, enteromycelin, chloramphenicol, lycercelin, roscillin, paraxin, camphicillin, chlorotetracycline, oxytetracycline, clove oil, rose oil, and eucalyptus oil, were examined on brinjal cultivars infected with phytoplasmas. The administration of the specified antibiotics shown no notable efficacy in managing brinjal little leaf disease. Furthermore, no blooms or fruits were detected in any of brinjal cultivars subjected to antibiotic treatment (Upadhyay, 2016).

In vitro Utilizations While oxytetracycline antibiotics inhibit phytoplasma proliferation in infected plants cultivated in vitro, elevated dosages of these antibiotics are detrimental to the tissues. This therapy enables phytoplasma eradication regardless of shoot tip size and addresses the challenges associated with excising and regenerating very small meristems. Tetracyclines exhibited a bacteriostatic impact on phytoplasmas in treated plants; however, symptoms predominantly reemerged following transfer of plants to an antibiotic-free medium (Wongkaew *et al.,* 2004; Davies *et al.,* 1994). Pear phytoplasma-infected explants were sustained for over three years using micropropagation on Murashige and Skoog media supplemented with indole butyric acid, gibberellic acid, and benzyl amino purine. Phytoplasmas consistently exhibited larger quantities in micropropagated explants compared to samples from field-grown plants, despite the absence of symptoms in the explants. Phytoplasmas were eradicated by adding 100 µg/mL oxytetracycline to the growing medium for four weeks. The results have ramifications for plant propagation strategies including symptomless phytoplasma-infected explants acquired through micropropagation (Laimer *et al.,* 2019). Significant phytotoxic effects on the growth and multiplication rate of potato explants in vitro were seen with increasing doses (up to 1024 mg/L) of streptomycin, chloramphenicol, & tetracycline in the culture medium (Pereira *et al.,* 2003). No plant regrowth was observed from 1 cm microcuttings of various almond cultivars treated with 50, 100, & 150 µg/mL oxytetracycline in an effort to eradicate almond witches' broom (Chalak *et al.,* 2005). Oxytetracycline exhibited phytotoxic effects on grapevine, significantly impacting axillary buds when cultivated in a medium containing 100 mg/L of the antibiotic (Gribaudo *et al.,* 2007). Carvalho *et al.* (Carvalho *et al.,* 2017) devised a way to eradicate the phytoplasma linked to frog skin disease in cassava using an in vitro shoot culture, integrating thermotherapy along with tetracycline treatments. Cuttings were exposed to different concentrations of tetracycline for several minutes and then subjected to thermotherapy at temperatures ranging from 35 °C to 55 °C. Shoot tips of different lengths (0.2, 0.4, 0.5, and 1.0 mm) were excised and cultured in media supplemented with 0, 5, 10, and 15 mg/L tetracycline for a period of 60 days. PCR

analysis confirmed that the phytoplasma infection was completely eliminated in all previously infected plants seven months after being transferred to field conditions. In another study, micropropagated periwinkle shoots infected with *'Candidatus Phytoplasma rubi*' (strain RuS) were maintained for 90 days on a solid culture medium. A volume of 0.5 mL sterile distilled water containing tetracycline hydrochloride (molecular weight 480.9) diluted at a 1:100 ratio, or 9 CH homeopathic dilutions of tetracycline, was applied weekly. All treatment groups experienced high mortality rates; however, the surviving shoots remained phytoplasma-positive when analyzed using nested PCR with primers specific to the phytoplasma 16SrV group. Interestingly, shoots treated with the 9 CH dynamized tetracycline did not express visible symptoms, whereas those grown in media with diluted tetracycline or plain distilled water showed clear signs of infection. This suggests that the dynamized tetracycline may have enhanced the plants' intrinsic defense responses (Cantagallo *et al.,* 2002).

Additionally, a comprehensive screening protocol was developed using a co-culture system of plant tissues and phytoplasmas to evaluate antibiotics at reduced concentrations (100–120 ppm). This approach was designed to protect plant tissues from damage while maintaining their defensive response against phytoplasma infection (Tanno *et al.,* 2018). The system was used to test more than 40 antibiotics belonging to different classes such as peptide, quinolone, sulfonamide, rifamycin, tetracycline, phenicol, and macrolide. Several of these antibiotics were found to significantly reduce the phytoplasma load in micropropagated shoots infected with the onion yellows strain. Furthermore, complete phytoplasma elimination was achieved after a continuous four-month treatment with a combination of tetracycline and rifampicin, which disrupt phytoplasma proteins and RNA respectively.

An initial assessment of the in vitro antibacterial efficacy in phytoplasma colonies

Figure 2 was performed using seven antibiotics against two phytoplasma isolates from coconut plants infected by lethal yellowing disease (Contaldo *et al.,* 2016, Contaldo *et al.,* 2019). The standard disc diffusion method was employed using a selection of the antibiotics previously demonstrated able to reduce the phytoplasma presence in micropropatated shoots (Tanno *et al.,* 2018). Rifampicin, 5-fluorouracil, tetracycline, tobramycin, polymyxin B, and cephalexin hydrate inoculated with 10^8 CFU/mL of phytoplasma isolates had been used. Results of antibiotic susceptibility tests revealed that tobramycin exhibited the maximum of activity against the tested phytoplasma isolates, followed by polymyxin B and tetracycline. The isolates displayed intermediate susceptibility to 5-fluorouracil but were completely resistant to cephalexin hydrate and rifampicin.

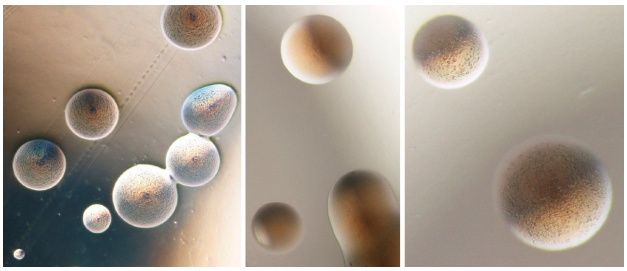

Fig. 2 : Phytoplasma colonies in solid medium CB (31) seen with an optical microscope at 40× magnification (courtesy of N. Contaldo).

Antimicrobial Molecules

Experiments performed employing diverse kind of molecules as alternative to antibiotics such as kinetins showed that they were ineffective in phytoplasma elimination (Plavsic *et al.,* 1986; Plavsic *et al.,* 1988). Also, a β-aminobutyric acid treatment of phytoplasma-infected periwinkle shoots (Curkovic-Perica *et al.,* 2007) proved to be ineffective, while putrescine, spermidine or spermine caused various alterations in the phytoplasma ultrastructure, which could account for reduced multiplication and movement of the pathogens in the infected plants (Curkovic-Perica *et al.,* 2007). A slower de- velopment of symptoms was also observed in polyamine treated shoots compared to infected controls (Musetti *et al.,* 1999). The effect of exogenously supplemented auxins was investigated on greenhouse-grown and shoot-tip cultures of periwinkle infected with *Spiroplasma citri* and phytoplasmas (Chang, 1998) and in *in vitro*-grown '*Ca.* P. trifolii'-related strain infected plants of the same species (Pertot *et al.,* 1998). An increase in the endogenous concentration of indole-3-acetic acid (IAA) in phytoplasma-infected plants and a reduced number of phytoplasmas in ultrathin sections of infected plant cells was observed after treatment with high hormone concentrations. When *in vitro* shoots of periwinkle infected with different strains of '*Ca.* Phytoplasma' such as '*Ca.* P. pruni' (strain KVI, clover phyllody) and '*Ca.* P. asteris' (strain HYDB, hydrangea phyllody) were exposed to IAA or indole-3-butyric acid, both auxins induced recovery of the symptoms in phytoplasma infected

shoots. Latter was more effective (Plavsic *et al.,* 1988). Recovery was contingent upon the 'Ca. Phytoplasma' strain, treatment time, & concentration along with type of auxin. Conversely, 'Ca. P. ulmi' (strain EY-C) & 'Ca. P. solani' (strain SA-1) remained in host tissues, even if shoots were asymptomatic. (Curkovic-Perica *et al.,* 2008). The susceptibility of '*Ca*. P. mali' to several chemical or synthetic antimicrobial agents as nisin, esculetin, pyrithione and chloramphenicol as molecules having different target activities was also evaluated. The activity of these molecules was compared with the one of two antibiotics (tetracycline and enrofloxacin) in *in vitro* grown infected apple shoots by adding them to the medium at 100, 500, 1000 ppm; nisin and pyrithione which were tested at 10, 100 and 500 ppm. The qPCR results indicated that phytoplasma was undetectable after a period of one and two months solely in the presence of pyrithione at concentrations of 10 and 100 ppm. Additionally, several other products diminished concentration of phytoplasmas after a two-month period. Shoots died or withered on media enriched with essential oils; especially when they were used at concentration of 500 and 1000 ppm (Aldaghi *et al.,* 2008) Experiments carried out with PAPII (Houston *et al.,* 1983), a ribosome-inactivating protein (RIP) extracted from *Phytolacca americana,* showed some efficacy in phytoplasma elimination in micropropagated infected plant shoots. RIPs are specific N-ß-glycosidases isolated from plants and share the ability to hydrolyze the N-glycosidic bond of a single adenosine present in a conserved sequence of the major RNA of ribosomes. Micropropagated periwinkle shoots contaminated with a 'Ca. P. asteris' strain (hydrangea virescence, HyV strain) had been utilized. Initial experiments conducted on shoots submerged in sterile water with diminishing concentrations of PAPII revealed a heightened incidence of necrotic shoots following the initial 48 hours of exposure. Nonetheless, the introduction of PAP-II to the medium did not elicit any measurable phytotoxicity, irrespective of the duration of exposure. Groups of shoots measuring 1–3 cm in length were subjected to serial dilutions of PAPII for a duration of 15 to 150 days. Only 4% of infected shoots cultivated on a medium supplemented with PAPII at dilutions of 1:10 & 1:100 for 15 to 150 days was determined to be devoid of phytoplasmas. The elimination rate seemed to correlate with both PAPII strength and time of exposure. The proportion of phytoplasma-free shoots varied from 40% to 50% throughout dilutions of 1:10 to 1:1000 and durations of 50 to 150 days. The optimal treatment for phytoplasma eradication was 1:1000 dilutions over a duration of approximately three months (Veronesi *et al.,* 2000).

Additional experiments aimed at eradicating phytoplasmas from infected shoots were conducted utilizing PAPII on micropropagated periwinkle shoots infected with 'Ca. P. asteris' (FE1 and O-1), 'Ca. P. pruni' (RA), along with 'Ca. P. rubi' (Rus) strains preserved in the collection (Bertaccini *et al.,* 2000, Bertaccini *et al.* 2020). PAPII was applied at three dilutions (1:10, 1:100, and 1:1000), when treated shoots had compared to infected shoots of strains cultivated in sterile medium (Plavsic *et al.,* 1986). The shoots were maintained in a growth chamber at 24°C with 16 h light for 90 days and tested by nested PCR to verify phytoplasma presence. After this period the great majority of the survived shoots was still positive for phytoplasma presence and shoots were therefore subject to a second 90-day treatment under the same conditions as before. The highest growing shoot percentage was observed for the '*Ca*. P. rubi' strain grown on a 1:10 dilution of PAPII. The rate of phytoplasma clearance rose with second treatment and at elevated PAPII concentrations. No elimination of phytoplasma was detected in the shoots cultivated in 1:1000 dilution during either the short-term or long-term treatments. An additional experiment was conducted on two more strains of 'Ca. P. asteris' (HyV & O-1) over a duration of 90 days with the same methodology. Additional experiments aimed at eradicating phytoplasmas from infected shoots were conducted utilizing PAPII on micropropagated periwinkle shoots infected with 'Ca. P. asteris' (FE1 & O-1), 'Ca. P. pruni' (RA), and 'Ca. P. rubi' (Rus) strains preserved in the collection (Bertaccini *et al.,* 2000, Bertaccini *et al.,* 2020). PAPII was utilized at 3 dilutions (1:10, 1:100, and 1:1000), while treated shoots were compared with infected shoots of same strains cultivated in sterile medium (Plavsic *et al.,* 1986). The shoots were maintained in a growth chamber at 24°C with 16 h light for 90 days and tested by nested PCR to verify phytoplasma presence. After this period the great majority of the survived shoots was still positive for phytoplasma presence and shoots were therefore subject to a second 90-day treatment under the same conditions as before. The highest growing shoot percentage was observed for the '*Ca*. P. rubi' strain grown on a 1:10 dilution of PAPII. The rate of phytoplasma elimination improved following a second round of treatment and with the use of higher concentrations of PAPII. However, no elimination of phytoplasma

was detected in shoots treated with the 1:1000 dilution, regardless of whether the treatment duration was short or extended. An additional study was conducted over a 90-day period on two others *'Candidatus Phytoplasma asteris'* strains (HyV and O-1) using the same tetracycline dilutions. The results showed that only a limited number of shoots tested negative for phytoplasma infection (Figure 3) (Cantagallo *et al.,* 2002).

Plant Resistance Inducers

A technology based on plasma-activated water (PAW), characterized by the presence of reactive oxygen and nitrogen species (RONS) in liquid, was tested on phytoplasma- infected micropropagated shoots and plants, in orchards and in greenhouse cultivation systems, to evaluate its effectiveness as a resistance inducer (Perez *et al.,* 2019). The exposure of sterile distilled water (SDW) to a cold atmospheric pressure plasma (CAP) caused a reduction in pH and the production of RONS that induced plant defense responses. To evaluate the effectiveness of PAW to control the phytoplasma-associated disease grapevine yellows, infected plants were treated in open-field and greenhouse conditions. The qualitative and quantitative yield parameters, the phytoplasma presence, and the gene expression were evaluated. The results showed that PAW enhanced the plant defense mechanisms and, as demonstrated in the field trials, improved the health status of the treated plants (Laurita *et al.,* 2021). In a preliminary field trial performed on 120 plants from 17 vineyards, with treatments performed in April, June, and July for three years, treated plants showed a slight reduction and a delay in the phytoplasma symptom appearance, which allowed the plants to carry their productive load (Figure 3).

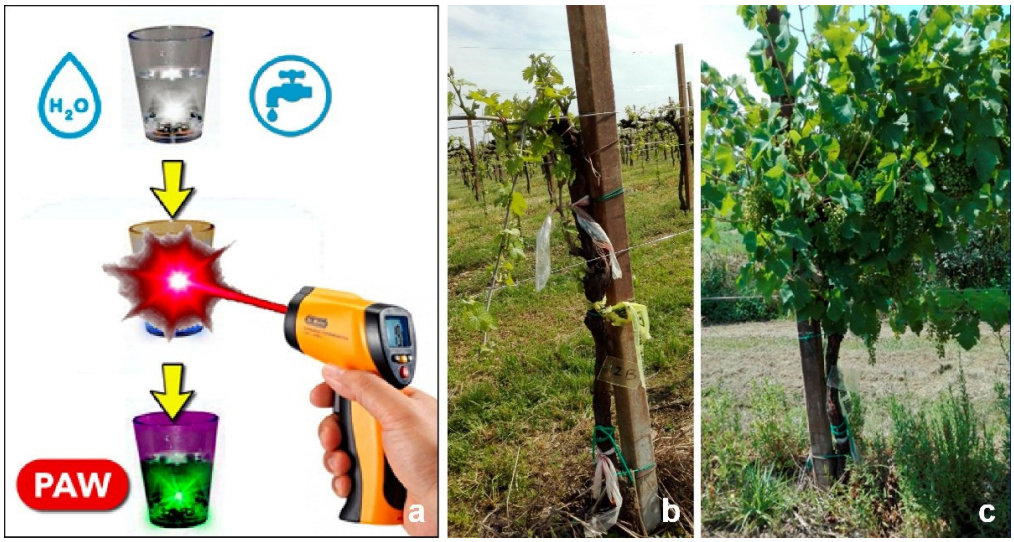

Fig. 3 : (**a**) scheme to produce PAW; (**b**) grapevine plant before and (**c**) after the PAW application for a period of three years.

The analyses confirmed that PAW treatment diminished quantity of plants with infections (Zambon *et al.,* 2017; Zambon *et al.,* 2018). Quantitative yield evaluations performed one-year post-treatment on 50 contaminated grapevine specimens revealed a significant enhancement in plant health, as evidenced by the quantity of grape bunches per plant along with the mass of 100 berries. Plants treated with PAW (Plasma Activated Water) showed a significantly higher average cluster count and increased berry weight compared to control plants (Laurita *et al.,* 2021). These outcomes were unexpected, as previous studies on plant elicitors had not shown a substantial correlation between induced resistance and improved plant fitness. Stimulation of secondary metabolic pathways during stress conditions generally incurs an energetic expense for the plant, frequently leading to diminished growth and production. Due to the complexity of environmental variables in field conditions, the full practical effectiveness of this treatment approach is yet to be determined; however, it holds promise as part of an innovative and environmentally sustainable strategy for managing phytoplasma-related diseases. Molecular analysis at both transcriptional and post-transcriptional levels confirmed that PAW enhances expression of genes involved in biosynthesis of phytoalexins-liks alkaloids & stilbenes-in grapevine as well as periwinkle, while also modulating stress-responsive genes through miRNA regulation (Zambon *et al.,* 2020).

Discussion and Conclusions

Eradication of phytoplasmas via in vitro shoot culture employing antimicrobial drugs like tetracycline and rifampicin is essential for generating disease-free planting material, safeguarding elite germplasm, and promoting global biodiversity conservation. The most efficient method for achieving phytoplasma-free plants involves the combination of in vitro chemotherapy with the micropropagation of sanitized shoots, ensuring effective plant recovery and sustainable disease management. This strategy facilitates the cultivation of plants for maintaining pristine plantations. This strategy necessitates the employment of sensitive and reliable testing procedures (Wang *et al.,* 1996; Heinrich *et al.,* 2001; Bertaccini *et al.,* 2019) to validate the eradication of phytoplasma from plant material post-treatment, which is crucial for the successful production of these plants. The utilization of plant resistance inducers like PAW facilitates the development of asymptomatic shoots and plants, wherein a mutualistic host-pathogen relationship occurs, resulting in the generation of visually healthy plants. This condition may also become permanent, particularly in the absence of additional stress on the plants; however, the existence of insect vectors in the environment and propagation methods like grafting allow for the transfer of phytoplasmas to other host plants or species. In such instances, the presence of germs may result in perilous, undesirable, and unpredictable epidemics. The evaluation of anti-phytoplasma agents or instruments is a crucial step toward sustainable field management of phytoplasma-related disorders. Employing molecules with diverse mechanisms of action, including plant hormones, ribosome-inactivating proteins, and plant resistance inducers (PAW), is recommended to circumvent the application of antibiotics in agriculture and the potential development of resistant microbial strains (Stockwell *et al.,* 2112). The comparison of proteins targeted by antimicrobials and conserved among various phytoplasma strains, may provide an indication of the potential effectiveness of new or already used molecules against a range of different phytoplasmas.

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