International Journal of Science and Research Archive
eISSN: 2582-8185
Cross Ref DOI: 10.30574/ijsra
Journal homepage: https://ijsra.net/

(RESEARCH ARTICLE)

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In vitro establishment from mature nodal segments of Persian Lime (*Citrus latifolia* Tan.)

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International Journal of Science and Research Archive, 2024, 12(01), 854–861

Publication history: Received on 02 April 2024; revised on 15 May 2024; accepted on 18 May 2024

Article DOI: https://doi.org/10.30574/ijsra.2024.12.1.0868

Abstract

In vitro propagation is a technique that has been used to produce large-scale, healthy, pathogen-free plants. However, the presence of endophytic microorganisms in explant tissues is one of the limiting factors for the *in vitro* establishment of any species. In this sense, we studied the effectiveness of PPM® biocide (Plant Preservative Mixture) and Chlorothalonil fungicide to control and/or reduce microbial and fungal contamination in mature nodal segments of different Persian lime clones under *in vitro* conditions. The results showed that explants cultured in MS medium (Murashige and Skoog 1962) supplemented with 0.2 mL L^{-1} of PPM biocide + 1.0 mL L^{-1} of Chlorothalonil were adequate to achieve a higher percentage of disinfection (44%) in mature nodal segments in the Persian lime clones. Furthermore, it was observed that PPM concentrations above 0.3 mL L^{-1} resulted in greater phytotoxicity in mature nodal segments at 60 days.

Keywords: Biocide; *Citrus latifolia*; Contamination; Disinfection; Endophytic microorganisms

1. Introduction

In vitro propagation is a reliable technique to produce great number of pathogen-free plants with high uniformity in a short period of time [1]. However, a limiting factor for *in vitro* propagation is the severe contamination of explants during *in vitro* establishment phase by endophytic microorganisms [2-4]. Microbial and fungal microorganisms are the most common contaminants during the *in vitro* establishment phase because, being systemic, they are difficult to detect and eliminate in disinfection protocols [5-6].

Therefore, a protocol must be in place that ensures the disinfection of microbial and fungal microorganisms in the first phase of the *in vitro* establishment, this because the culture medium provides a favorable environment for these microorganisms, causing the mortality of the introduced plant material [7-8]. Consequently, the use of antibiotics, fungicides, and biocides is necessary during the *in vitro* introduction phase in order to control the development of these microorganisms. In this sense, PPM is a broad-spectrum biocide for *in vitro* tissue culture that reduces fungal and bacterial contamination of explants and enables aseptic establishment of mature nodal segments [9-10]. PPM has been used to disinfect *in vitro* explants of species such as bamboo, strawberry, red ginger, etc. [11-14]. Likewise, Chlorothalonil is a broad-spectrum fungicide with a polychlorinated aromatic component that delays mycelial growth

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and inhibits spore germination. Chlorothalonil affects the respiration of fungal cells, affecting the Krebs cycle by reducing ATP synthesis, leading to cell death [15]. In *Eucalyptus grandis,* chlorothalonil fungicide has a high inhibitory effect on the growth of endophytic microorganisms on *in vitro* culture medium [16].

For Persian lime, to date, explants introduced *in vitro* have been particularly affected by microorganisms that limit their development, and therefore, no disinfection protocols has been established for obtaining aseptic Persian lime explants [17]. Furthermore, in Persian lime, there are few reports on the use of this biocide for the *in vitro* establishment of mature nodal segments.

Therefore, the aim of this study was:

- Develop an effective disinfection protocol for *in vitro* establishment of mature nodal segments using different concentrations of PPM biocide and Chlorothalonil fungicide to evaluate the efficiency in the control of endophytic microorganisms in different Persian lime clones.

2. Materials and Methods

2.1. Plant material

The work was developed in Ixtacuaco Experimental Field belonging to National Institute for Research on Agriculture, Forestry and Livestock (INIFAP), located in the northern region of Veracruz, Mexico (N 20° 2´ 35.48" and W 97° 5´ 52.60"). The plant material used in this study was obtained from 7-year-old trees of the Persian lime clones: "Arbolito", "Doble Persa", Persa común", "Peruano" and, "Chino". Using pruning shears that have been pre-sterilized with 20% commercial chlorine, were cut stem cuttings per each Persian lime clone. The stem cuttings were stored in moist paper inside sterile polyethylene bags and placed in a cooler to maintain a temperature close to 4 °C until transferred to the laboratory.

2.2. Explant preparation

Upon arrival at the laboratory, the stem cuttings were sterilized following the pre-sterilization protocol established by Estrella-Maldonado et al. [18], in order to minimize the number of contaminants (mainly bacteria and fungi) before establishment under *in vitro* conditions. After pre-disinfection of the stem cuttings, these explants were cut into 5–7 cm pieces and named mature nodal segments. Finally, the mature nodal segments were exposed to 20% chlorine for 20 minutes, then were submerged in bactericide solution (Terra-Vet® 200, 1 g L^{-1}) for 1 hour and immediately submerged in systemic fungicide solution (Amistar, 2 mL L^{-1}) for 1 hour.

2.3. *In vitro* culture of mature nodal segments

Once the pre-disinfection was completed, we proceeded to work under aseptic conditions in laminar flow hood. Under these conditions, the nodal segments were submerged in 85% alcohol solution for 10 min and finally, they were submerged for 5 min in sterile distilled water. The basal medium used for the mature nodal segments establishment was 4.43 g L^{-1} MS salts [19], supplemented with 20 g L^{-1} sucrose and 6-BAP at 0.5 mg L^{-1}. The culture medium pH was adjusted to 5.8, and 8 g L^{-1} of agar was added for the medium solidification. The medium was dispensed in 150 mm x 25 mm culture tubes and sterilized in an autoclave at 121 °C for 20 min. After, sterile culture medium was allowed to cool and two experiments were designed. Experiment 1 included treatment control without PPM biocide (treatment control) and four treatments with different PPM biocide concentrations (0.1, 0.2, 0.3 and 0.4 mL L^{-1} PPM). Experiment 2 consisted of 4 treatments: MS culture medium supplemented with 0 mL L^{-1} PPM + 0.5 mL L^{-1} Chlorothalonil (treatment 1), 0 mL L^{-1} PPM + 1 mL L^{-1} Chlorothalonil (treatment 2), 0.2 mL L^{-1} PPM + 0.5 mL L^{-1} Chlorothalonil (treatment 3), and 0.2 mL L^{-1} PPM + 1 mL L^{-1} Chlorothalonil (treatment 4). Furthermore, 1 mature nodal segment (3-4 cm long) was culture for each culture flask. These *in vitro* introduced mature nodal segments were stored for 30 days in a growth chamber under the following conditions: T = 25 °C, PPFD = 32 µmol m^{-2} s^{-1}, and a 16-h photoperiod provided by white fluorescent light. After 60 days, contamination percentage was evaluated.

2.4. Statistical analysis

All mature nodal segments from different Persian lime clones under *in vitro* conditions were analyzed as completely randomized designs. Each experiment was repeated three times using twelve mature nodal segments per each condition. One-way analysis of variance (ANOVA) with Tukey multiple range test at $p < 0.05$ was performed using Statgraphics Plus Ver. 5.1 Software (Statistical graphics Corp., USA) (http://www.statgraphics.com) to determine a significant difference among treatments. The graphics were performed using the Sigma Plot ver. 11.0 program.

3. Results

3.1. Disinfection of mature nodal segments using PPM biocide

The results showed that when PPM biocide was not added to MS medium, mature nodal segments of all Persian lime clones were contaminated at day 60, with fungi and fungi + bacterium contamination accounting for 100% (Fig. 1a). However, when 0.1 mL L^{-1} and 0.2 mL L^{-1} of PPM were added to MS medium, the results showed that the contamination rate of these mature nodal segments was of 70-80%, mainly caused by fungi (Fig. 1b-c). After 60 days, culture of mature nodal segments of Persian lime in MS medium supplemented with 0.3 and 0.4 mL L^{-1} of PPM caused phytotoxic effects on all explants, which high oxidation rates (over 95%) (Fig. 1 d-e). In this context, at 0.3 mL L^{-1} of PPM, Doble Persa clone had a lower contamination percentage (60%), but 35% of this contamination was caused by fungi and 25% by PPM phytotoxicity (Fig. 1 d). When 0.4 mL L^{-1} of PPM was added to MS medium, the mature nodal segments of all Persian lime clones showed high PPM phytotoxicity (70-83%) (Fig. 1e).

ANOVA showed that the disinfection percentage of mature nodal segments of Persian lime cultured was low (less than 5%) when cultured in MS without PPM. When mature nodal segments were exposed at 0.2 mL L^{-1} of PPM, contamination levels reached 68 to 82%, particularly by fungi contamination. Furthermore, when mature nodal segments were cultured in MS medium supplemented with 0.3 mL L^{-1} of PPM, the disinfection rate of Doble Persa clone reached 40%, followed by Peruano and Arbolito clones (35 and 37% respectively), the Persa Común clone with 30%, and Chino clone with 22% disinfection (Fig. 2).

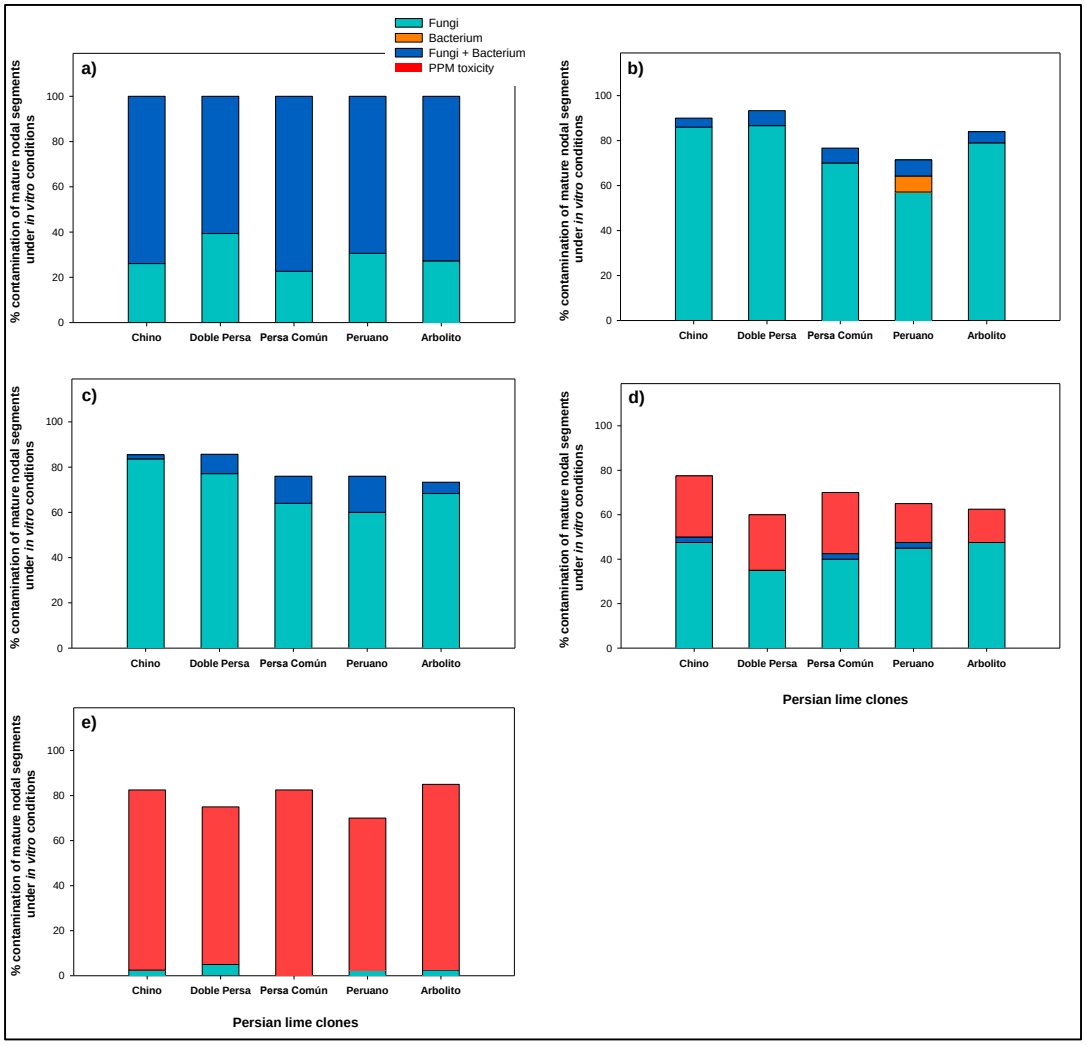

Figure 1 Contamination percentage of mature nodal segments from different Persian lime clones under *in vitro* conditions, after 60 days of culture on MS medium supplemented with a) 0 mL L^{-1} PPM, b) 0.1 mL L^{-1} PPM, c) 0.2 mL L^{-1} PPM, d) 0.3 mL L^{-1} PPM and e) 0.4 mL L^{-1} PPM

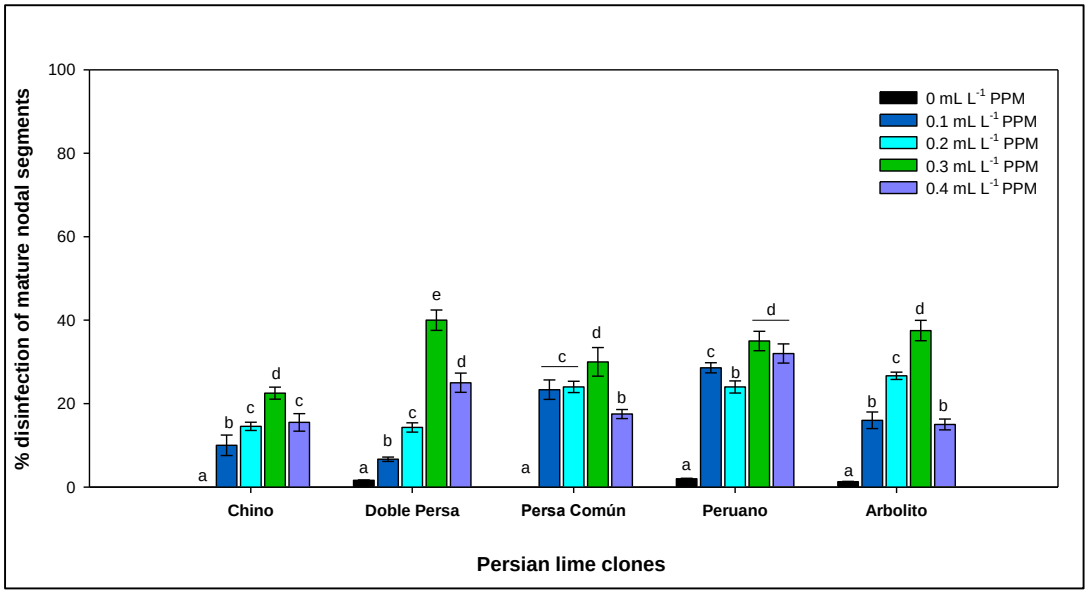

Figure 2 Disinfection percentage of mature nodal segments of different Persian lime clones at 60 days under *in vitro* conditions. MS medium was supplemented without PPM or with different concentrations (0.1, 0.2, 0.3 and 0.4 mL L^{-1}). Letters indicate significant differences at *p>0.05* and bars represent mean ± SE

3.2. Disinfection of mature nodal segments using PPM biocide + Chlorothalonil fungicide

To minimize fungi contamination, we performed a second experiment in which mature nodal segments were treated with PPM biocide + Chlorothalonil fungicide (Fig. 3). The results showed that the treatment where only 0.5 mL L^{-1} of Chlorothalonil fungicide was added to MS culture (0 mL L^{-1} PPM + 0.5 mL L^{-1}), not able to decrease the contamination rate, because the mature nodal segments showed contamination between 67% to 80%, mainly by fungi, bacteria, or fungi + bacteria (Fig. 3a). When the Chlorothalonil fungicide concentration was increased to 1 mL L^{-1} (0 mL L^{-1} PPM + 1 mL L^{-1}), the mature nodal segments still showed a high contamination rate (67% to 73%) (Fig. 3b). Contamination of mature nodal segments of Persian lime clones decreased at 0.2 mL L^{-1} PPM + 0.5 mL L^{-1} Chlorothalonil (57% to 66 %), however, some mature nodal segments still showed symptoms of PPM phytotoxicity (Fig. 3c). Interestingly, treatment with 0.2 mL L^{-1} PPM + 1 mL L^{-1} Chlorothalonil was effective in further reducing both bacteria or fungi contamination (Fig. 3d). Peruano was the only clone that showed no PPM phytotoxicity in the mature nodal segments using 0.2 mL L^{-1} PPM with 0.5 or 1 mL L^{-1} of Chlorothalonil (Fig. 3c-d).

ANOVA analysis showed that Chino clone showed better disinfection rate when the mature nodal segments were cultured in MS medium supplemented with 0.2 mL L^{-1} PPM + 1 mL L^{-1} Chlorothalonil (42% disinfection). Likewise, Doble Persa clone showed the best disinfection rate when its explants were cultured in MS medium supplemented with 0.2 mL L^{-1} of PPM + 0.5 mL L^{-1} Chlorothalonil (35%) and 0.2 mL L^{-1} of PPM + 1 mL L^{-1} Chlorothalonil (52%). Persa Común clone reached 54 % disinfection rate when the mature nodal segments were cultured in MS medium supplemented with 0.2 mL L^{-1} of PPM + 1 mL L^{-1} Chlorothalonil. Interestingly, Peruano clone reached 42 % disinfection rate when their explants were cultured in MS medium supplemented also with 0.2 mL L^{-1} of PPM + 0.5 mL L^{-1} Chlorothalonil, but increased their disinfection rate with 0.2 mL L^{-1} of PPM + 1 mL L^{-1} (52%). Likewise, Arbolito clone, reached 48% disinfection rate with 0.2 mL L^{-1} of PPM + 0.5 mL L^{-1} Chlorothalonil (Fig. 4).

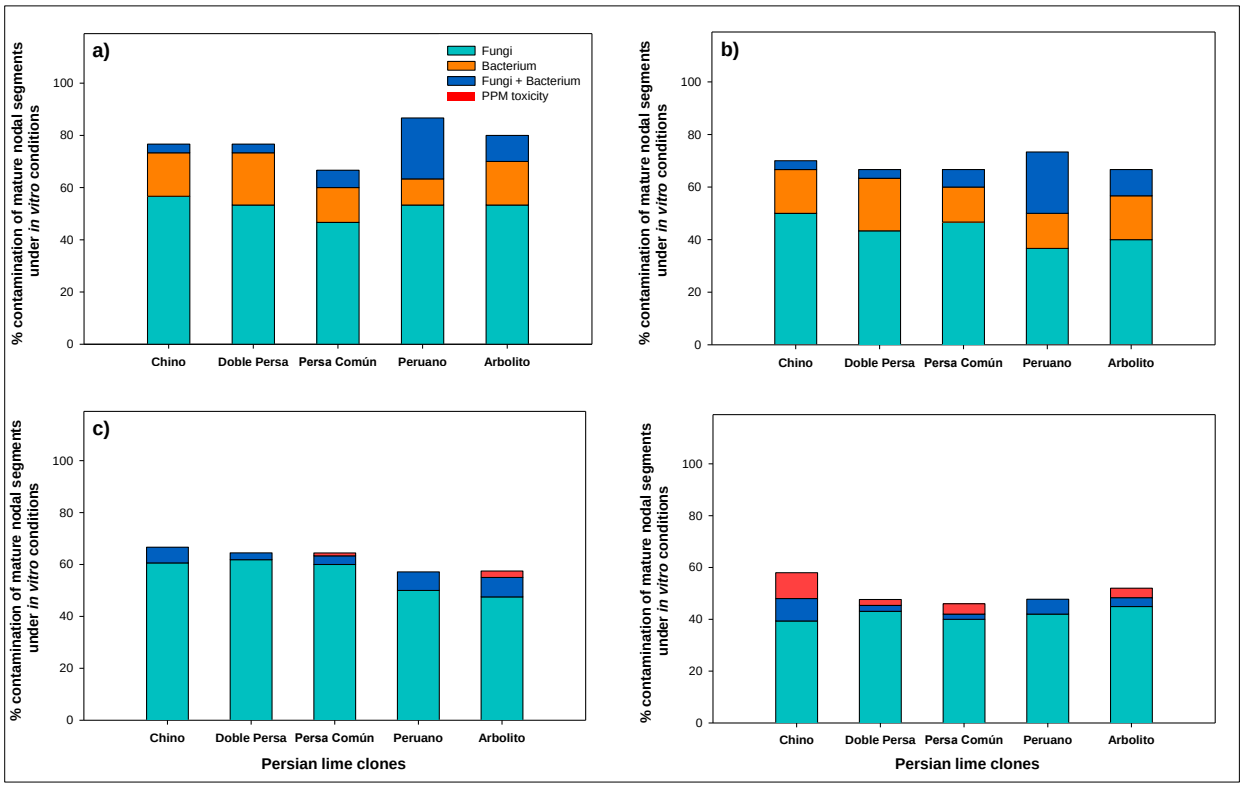

Figure 3 Contamination percentage of mature nodal segments from different Persian lime clones under *in vitro* conditions, after 60 days of culture on MS medium supplemented with a) 0 mL L^{-1} PPM + 0.5 mL L^{-1} Chlorothalonil, b) 0 mL L^{-1} PPM + 1 mL L^{-1} Chlorothalonil, c) 0.2 mL L^{-1} PPM + 0.5 mL L^{-1} Chlorothalonil, and d) 0.2 mL L^{-1} PPM + 1 mL L^{-1} Chlorothalonil

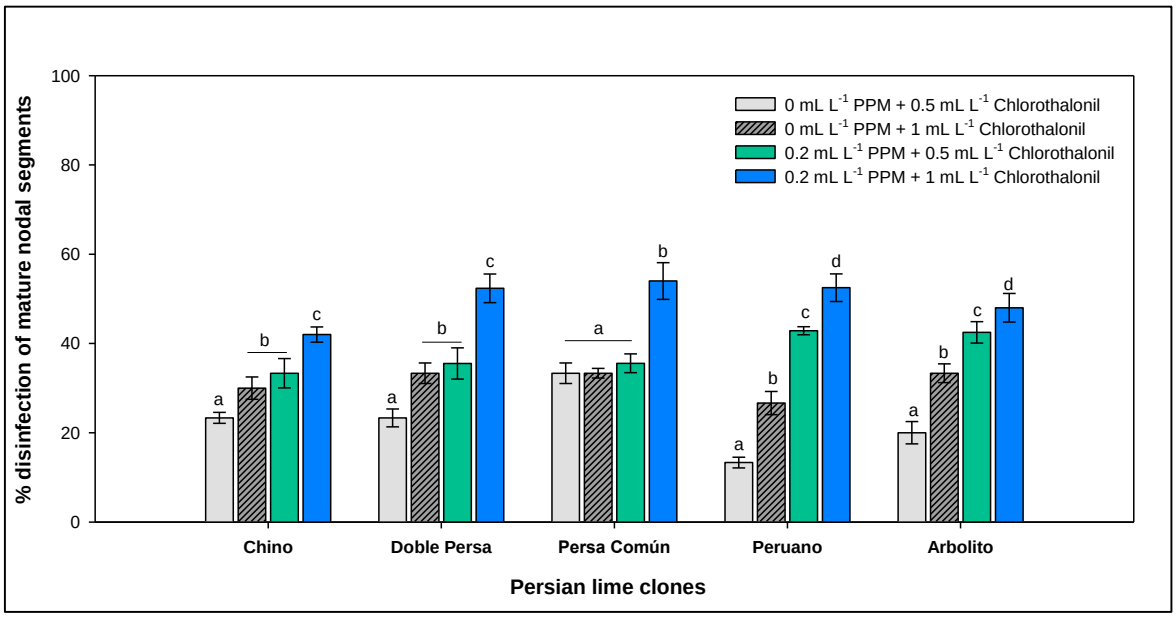

Figure 4 Disinfection percentage of mature nodal segments of different Persian lime clones at 60 days under *in vitro* conditions. MS medium was supplemented with two different concentrations of Chlorothalonil (0.5 and 1 mL L^{-1}) and with addition of 0.2 mL L^{-1} of PPM. Letters indicate significant differences at *p>0.05* and bars represent mean ± SE

4. Discussions

Due to the great economic potential of the Persian lime, research has focused on obtaining plants free of vascular diseases. To achieve this aim, it is necessary to establish disinfected explants *in vitro* for subsequent successful micropropagation of this specie. However, the difficulty of disinfecting Persian lime explants during the *in vitro* establishment phase is limited, due to the high rate of microbial, fungal, and necrotic contamination (phenol exudate products) [20]. Therefore, it is necessary to use biocides, fungicides, antibiotics, whose functions have been described as alternatives to control the growth of fungi and bacteria endophytes during *in vitro* establishment [21].

Although Vega-Pérez et al. [22] reported that mature nodal segments are ideal explants for *in vitro* introduction; these are particularly susceptible to microbial invasion, which limits their development. In addition, there are currently no established disinfection protocols for these explants. This has led to generate new alternatives to reduce the presence of microorganisms during the first phases of the *in vitro* establishment of mature nodal segments of Persian lime. Chlorothalonil fungicide and PPM biocide are highly recommended reagents in plant tissue culture media to avoid excessive bacterial and fungal loads during *in vitro* culture of different plant species [23]. Chlorothalonil action is rapid, its general effects on fungal cells include the inhibition of mycelial and spore growth, which is due to its broad spectrum of action and the lack of development of genetic resistance [24]. Likewise, PPM (Plant Preservative Mixture) type biocide used to control contamination and its effect on *in vitro* growth in tropical species [25].

In this context, Herrera-Flores et al. [26] used nodal segments of Persian lime with the aim of obtaining a disinfection protocol of this species under *in vitro* culture conditions; however, they obtained a low percentage of asepsis (34%). Similarly, Méndez-Jiménez et al. [27] with the aim of establishing nodal segments of Persian lime *in vitro*, used methanolic extracts of *Hibiscus sabdariffa*, *Cinnamomun zeylanicum*, *Ruta graveolens* and *Thymus vulgaris*, however, only 20% sterile explants were obtained with the *Hibiscus sabdariffa* extract; after 48 hours the explants showed contamination with bacteria and fungi. In a previous study performed by our working group [18], we used different concentrations of bactericide (Terra Oxitetracycline) + fungicide (Chlorothalonil), however, the disinfection rate was 52% during the *in vitro* establishment of mature nodal segments of Persian lime. Therefore, more research is needed to achieve the disinfection percentage using nodal segments mature of Persian lime for the aseptic establishment of these explants under *in vitro* conditions.

In our study, a double disinfection was performed with the purpose of minimizing contamination in the nodal segments coming from the field. For this purpose, during the pre-disinfection phase, as mentioned by Estrella-Maldonado et al. [18], the mature nodal segments of Persian lime were treated with chlorine, fungicides and bactericides for a long time. In the second disinfection phase, the mature nodal segments were cultured in MS medium supplemented with PPM and Chlorothalonil, in order to eliminate even more endophytic microorganisms that can cause the death of the explants. Interestingly, addition of 0.3 and 0.4 mL L^{-1} of PPM reduced contamination, however, mature nodal segments showed oxidative characteristics of PPM phytotoxicity. As mentioned by Carneiro et al. [28], high concentrations of PPM can cause the medium to become more acidic due to the formation of HCO_3. Therefore, we hypothesize that concentration greater than 0.3 mL L^{-1} of PPM caused the culture medium where the nodal segments of Persian lime were cultured to be acidic. Thus, the PPM phytotoxicity is because these explants lost the ability absorb water and nutrients.

5. Conclusions

Although PPM is an advantageous biocide that acts to control both bacteria and fungi on the *in vitro* establishment phase, in our study, it was evident that at concentrations greater than 0.3 mL L^{-1} PPM, the nodal segments were affected by the increase in the rate of oxidation produced by PPM phytotoxicity. The best disinfection procedure was to use 0.2 mL L^{-1} PPM + 1.0 mL L^{-1} Chlorothalonil, with this treatment, the mature nodal segments did not show PPM phytotoxicity and the development of contaminating microorganisms in different Persian lime clones was reduced.

Compliance with ethical standards

Acknowledgments

The authors are grateful at Ixtacuaco Experimental Field, belonging to the National Institute for Research on Agriculture, Forestry and Livestock (INIFAP), for providing facilities to carry out this research.

Disclosure of conflict of interest

The authors declare that no conflict of interest exists.

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