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Disinfection procedure for stem cuttings and *in vitro* production of axillary buds for the Persian lime sanitation

Humberto Estrella-Maldonado [1,*], Jesús R. Mora Solís [2] and Cynthia G. Rodríguez-Quibrera [1]

[1] *National Institute for Research on Agriculture, Forestry and Livestock (INIFAP). Ixtacuaco Experimental Field, Km 4.5. Martínez de la Torre-Tlapacoyan Street, Cong. Rojo Gómez, Tlapacoyan, Veracruz, Mexico.*
[2] *Technological University of the Huasteca Hidalguense. Huejutla - Chalahuiyapa highway S/N, Tepoxteco, Huejutla de Reyes, Hidalgo, Mexico.*

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Abstract

The research work covered the *in vitro* establishment of Persian lime nodal segments using a twig disinfection protocol, and their culture on Murashige and Skoog (MS) culture media supplemented with antibiotics (Estrepto-Ler® and Rifampicin) and fungicide (Chlorothalonil). The axillary buds production was performed cultured the nodal segments without contamination signs on MS culture media enriched with two cytokinins (6-BAP and Kinetin) at different concentrations. Results showed that 52 % of the nodal segments did not show signs of *in vitro* contamination when the twig submerged in 20% chlorine for 15 min during disinfection process and nodal segments were grown in MS culture medium supplemented with 2 mg L^{-1} of Rifampicin and fungicide Chlorothalonil. Interestingly, MS culture medium enriched with 6-BAP (0.5 mg L^{-1}) + Kinetin (0.5 mg L^{-1}) turned out to be the best combination to achieve that 78 % of nodal segments promoted 1 to 2 axillary buds.

Keywords: *Citrus latifolia; In vitro;* Phytohormones; Axillary buds

1. Introduction

In Mexico, citrus fruits are an agricultural product that are part of the usual diet, likewise, they represent an important source of vitamin C, pectin, terpenes, flavonoids, among other components, that positively influencing consumer health [1]. Regarding to Persian lime (*Citrus x latifolia* Tan.), is exported to various countries around the world due to the high quality of the harvested fruits, it is also the crop with the highest demand in United States of America, being Mexico the main exporter with 98% of imports. Therefore, in recent years, Mexico has positioned itself as the second largest producer of this crop worldwide [2]; being Veracruz the main producer and exporter state [3].

However, the Persian lime is not exempt from diseases caused by bacteria, viruses, fungi, mycoplasmas and other similar organisms which cause significant economic losses in citrus cultivation worldwide [4]. Thereby, citrus fruits are facing new challenges and threats related to phytosanitary problems that incur more risk in the citrus fruit national industry. Consequently, at end 2014, 16 of the 23 citrus producing states in Mexico were affected by presence of Huanglongbing (HLB) and Citrus Tristeza Virus (CTV), devastating vascular diseases that have not cure, as well as of fungal diseases caused by different species of *Lasiodiplodia* [3], diseases that cause a decrease in yield and productivity, which lead to mortality in Persian lime.

* Corresponding author: Humberto Estrella-Maldonado
National Institute for Research on Agriculture, Forestry and Livestock (INIFAP). Ixtacuaco Experimental Field, Km 4.5. Martínez de la Torre-Tlapacoyan Street, Cong. Rojo Gómez, Tlapacoyan, Veracruz, Mexico.

To solve this problem, plant tissue culture represents a very valuable biotechnological tool that allows through plant sanitation, the possibility of massively propagate pathogen-free plants, as well as establish germplasm banks whose purpose is safeguard *in vitro* plant species that due to natural disasters and/or diseases can be lost [5]. In this context, *in vitro* micrografting shoot tips can be an efficient for shoots sanitation, rejuvenation of plant material and has proven to be effective for eliminating systemic pathogens in citrus [6-9]. Therefore, this technique is effective for obtained pathogen-free plants transmissible by grafting and to guarantee plant health [4]. However, to perform the *in vitro* micrografting shoot tips technique, is necessary to aseptically establish the nodal segments obtained from the stem cuttings and adapt the necessary conditions to successfully perform the micrografting technique [6, 8]. Unfortunately, to date, not disinfection protocol has been established to obtain nodal segments under controlled *in vitro* conditions, which crucial step for citrus sanitation. Furthermore, optimal culture media still need to be standardized in order to produce *in vitro* axillary buds using different cytokinins, for example, 6-Benzylaminopurine (6-BAP) and Kinetin (Kin).

Therefore, the aim of this study was:

- To establish a disinfection protocol from Persian lime stem cuttings and establish nodal segments under *in vitro* conditions.
- To standardize the culture medium enriched with different cytokinins concentrations (Kin and 6-BAP), to induce axillary buds of the nodal segments introduced *in vitro*. This research work is the basis for later performing the micrografting shoot tips, which are necessary for Persian lime sanitation.

2. Material and methods

2.1. Plant material

Persian lime (*Citrus x latifolia* Tan.) trees were grown in Ixtacuaco Experimental Field (CEIXTA) belonging to National Institute for Research on Agriculture, Forestry and Livestock (INIFAP), located at the coordinates N 20° 2´ 35.48" and W 97° 5´ 52.60". A total of 25 five-year-old Persian lime trees grafted on Citrumelo Swingle rootstock were selected. Likewise, with the help of pruning shears (disinfected with 20% commercial chlorine), a minimum of 25 stem cuttings with a length of 10 to 15 cm were cut for each Persian lime tree. To avoid dehydration of the stem cuttings, they were stored in moistened paper inside sterile polyethylene bags and kept in cooler to maintain the temperature close to 4 °C until transfer to laboratory.

2.2. Stem cuttings disinfection

Stage 1. Under laboratory conditions, the stem cuttings were defoliated leaving only leaf petiole in order to protect the axillary buds. Disinfection process consist of washing the stem cuttings with water and mild soap in three repetitions, then, are rinsed in sterile distilled water and submerged in Tween® 20 solution (5 %) for 10 min, later are again rinsed with sterile distilled water in three repetitions. Subsequently, the stem cuttings were submerged in Terra-Vet® 200 (Oxytetracycline) bactericide solution (0.06 g L^{-1}) for 1 h and then in Amistar (Azoxystrobin) systemic fungicide solution (2 ml L^{-1}) for 1 h. Finally, the stem cuttings were rinsed in sterile distilled water and placed on sterile paper for 5 min to dry.

Stage 2. Completed the previous stage, in laminar flow hood, the stem cuttings were disinfected with different concentrations of chlorine (10, 15 and 20 %) for 15 min (**Table 1**). Then, were rinsed in sterile distilled water for 5 min and submerged in 75% alcohol solution for 10 min. Subsequently, were rinsed again in sterile distilled water for 5 min to remove alcohol traces. To finish, stem cuttings were sectioned into segments of 5 to 8 cm (nodal segments) and left to dry on sterile absorbent paper for 5 min until cultured under *in vitro* conditions.

2.3. Nodal segments disinfection under *in vitro* conditions

Nodal segments were cultured in the following medium: 4.43 g L^{-1} MS salts [10] and 20 g L^{-1} sucrose. Culture medium pH was adjusted to 5.8 with NaOH or HCl (1N) and 8 g L^{-1} of agar was added for the correct solidification. The culture medium were sterilized in autoclave for 20 min at 121 °C. Before of the medium culture solidification, two antibiotics [Estrepto-Ler (0.2 MU mL^{-1}): 2 ml L^{-1} and Rifampicin: 2 g L^{-1}] were added to reduce bacterial contamination. Thus, different *in vitro* nodal segments disinfection treatments were performed as following: Control Treatment 1 (C1: 10% chlorine for 15 min), Control Treatment 2 (C2: 15% chlorine for 15 min), Control Treatment 3 (C3: 20% chlorine for 15 min), Treatment 1 (T1: 15% chlorine at 10% for 15 min + Estrepto-Ler), Treatment 2 (T2: chlorine at 15% for 15 min + Estrepto-Ler), Treatment 3 (T3: chlorine at 20% for 15 min + Estrepto-Ler), Treatment 4 (T4: chlorine at 10 % for 15 min + Rifampicin), Treatment 5 (T5: 15% chlorine for 15 min + Rifampicin), Treatment 6 (T6: 20% chlorine for 15 min + Rifampicin) (**Table 1**). In order to reduce fungi contamination, an additional treatment named Treatment 7 (T7) was

performed, which consisted of using nodal segments previously disinfected with 20% chlorine for 15 min, but Rifampicin (2 mg L^{-1}) and fungicide Chlorothalonil (1 ml L^{-1}) were added to culture medium before solidification (**Table 1**). Subsequently, nodal segments were cultured *in vitro* inside in test tubes (1 nodal segment per tube). All *in vitro* nodal segments were cultured inside growth rooms under the following conditions: T = 25 ºC ± 1, PPFD = 32 µmol photon m^{-2} s^{-1} and 12 h photoperiod supplied by white fluorescent lamps.

2.4. Axillary buds production

After 30 days, the contamination percentage of *in vitro* nodal segments was analyzed. Likewise, nodal segments that did not show signs of contamination were sub-cultured in new culture media (as described above), however, these were supplemented with 6-BAP and Kinetin in order to improve the axillary buds production. The different treatments to produce axillary buds from nodal segments were performed as following: Control Treatment 1 (C1: MS culture medium without phytohormones), Treatment 1 (T1: MS culture medium + 0.5 mg L^{-1} of 6-BAP), Treatment 2 (T2: MS culture medium + 0.5 mg L^{-1} of Kinetin), Treatment 3 (T3: MS culture medium + 0.25 mg L^{-1} of 6-BAP + 0.25 mg L^{-1} of Kinetin) and Treatment 4 (T4: MS culture medium + 0.5 mg L^{-1} of 6-BAP + 0.5 mg L^{-1} of Kinetin), as indicated in **Table 2**.

2.5. Statistical analysis

The nodal segments introduced *in vitro* were analyzed as completely randomized designs. One-way analysis of variance (ANOVA) was used to determine a significant difference among treatments. Tukey multiple comparison test was used to determine significant differences between the means ($p<0.05$) using Statgraphics Plus Ver. 5.1 Software (Statistical graphics Corp., USA). Graphics were performed using the Sigma Plot ver. 11.0 program.

3. Results

3.1. Disinfection protocol

Our results showed that nodal segments from stem cuttings disinfected with 10, 15 and 20 % of chlorine, when were culture in MS medium without antibiotic presence, showed 100 % contamination after 60 days. Similarly, nodal segments disinfected with 10, 15 and 20 % of chlorine, but with the addition of Estrepto-Ler antibiotic (T1 - T3 treatments), showed high contamination percentage (between 74 and 79 %). Interestingly, when to MS medium Rifampicin antibiotic was added, the T4 –T7 treatments showed a lower contamination percentage (between 48 until 69 %). Likewise, the results showed that stem cuttings disinfected with 20% chlorine for 15 min during disinfection process and the subsequent *in vitro* culture of the nodal segments in MS medium supplemented with 2 mg L^{-1} of Rifampicin (T6) turned out to be the best treatment reaching a 35 % disinfection (**Table 1**).

Table 1 Disinfection protocol in nodal segments of Persian lime.

Treatment	Code	Chlorine (15 min)	Estrepto-Ler	Rifampicin	Chlorothalonil	Contamination
Control 1	C1	10 %	-	-	-	100 %
Control 2	C2	15 %	-	-	-	100 %
Control 3	C3	20 %	-	-	-	100 %
Treatment 1	T1	10 %	+	-	-	79.31 %
Treatment 2	T2	15 %	+	-	-	75.64 %
Treatment 3	T3	20 %	+	-	-	74.09 %
Treatment 4	T4	10 %	-	+	-	69.84 %
Treatment 5	T5	15 %	-	+	-	67.56 %
Treatment 6	T6	20 %	-	+	-	65.09 %
Treatment 7	T7	20 %	-	+	+	48.16 %

(-) without application; (+) with application

In general, although bacterial contamination decreased (T1-T6 treatments), the fungal contamination remained persistent (**Fig. 1a**). Therefore, it was decided to performed a final treatment, which consisted of carrying out the same steps of Treatment 6 but adding Chlorothalonil fungicide (1 ml L^{-1}) to MS medium (T7), the above, to reduce fungal

contamination. Thus, the nodal segments showed 52 % disinfection after 60 days of culture in MS medium under *in vitro* conditions (**Table 1**). As shown in **Fig. 1a**, when not bactericide solution was added to the MS medium, the main contamination of nodal segments (Treatments Control) was by bacteria. Likewise, although both bactericides reduced bacterial contamination, the fungal contamination was persistent even when Chlorothalonil fungicide was added (T7).

3.2. Axillary buds production

Nodal segments cultured after 60 days in MS medium without cytokinins (Treatment Control) showed a low axillary buds number and only the 16 % of these segments produced buds. When the MS medium was enriched with 0.5 mg L^{-1} of 6-BAP, 52 % of the segments produced one axillary buds (T1). Similarly, the results showed that the 56 % of nodal segments produced one axillary buds when were culture in MS medium enriched with 0.5 mg L^{-1} of Kinetin (T2). However, when the MS medium was enriched with 0.25 mg L^{-1} of 6-BAP and with 0.25 mg L^{-1} of Kinetin (T3), only the 32 % of nodal segments produced axillary buds, but a increases of axillary buds was observed (1.67 buds per nodal segments). Also, when the MS culture medium was enriched with high concentrations of 6-BAP (0.50 mg L^{-1}) + Kinetin (0.50 mg L^{-1}) (T3), a best axillary buds production was observed (78 %) (**Table 2**). These nodal segments showed 2.43 buds per nodal segments at 60 days under *in vitro* conditions (**Fig. 1b**).

Table 2 MS culture medium enriched with different concentrations of cytokinins to induce axillary bud production.

Treatment	Code	6-BAP (mg L^{-1})	Kinetin (mg L^{-1})	Axillary bud number	Axillary bud production
Control	C1	-	-	0.34 ± 0.04 a	16 %
Treatment 1	T1	0.50	-	1.15 ± 0.13 b	52 %
Treatment 2	T2	-	0.50	1.06 ± 0.24 b	56 %
Treatment 3	T3	0.25	0.25	1.67 ± 0.11 c	32 %
Treatment 4	T4	0.50	0.50	2.43 ± 0.31 d	78 %

(-) without application

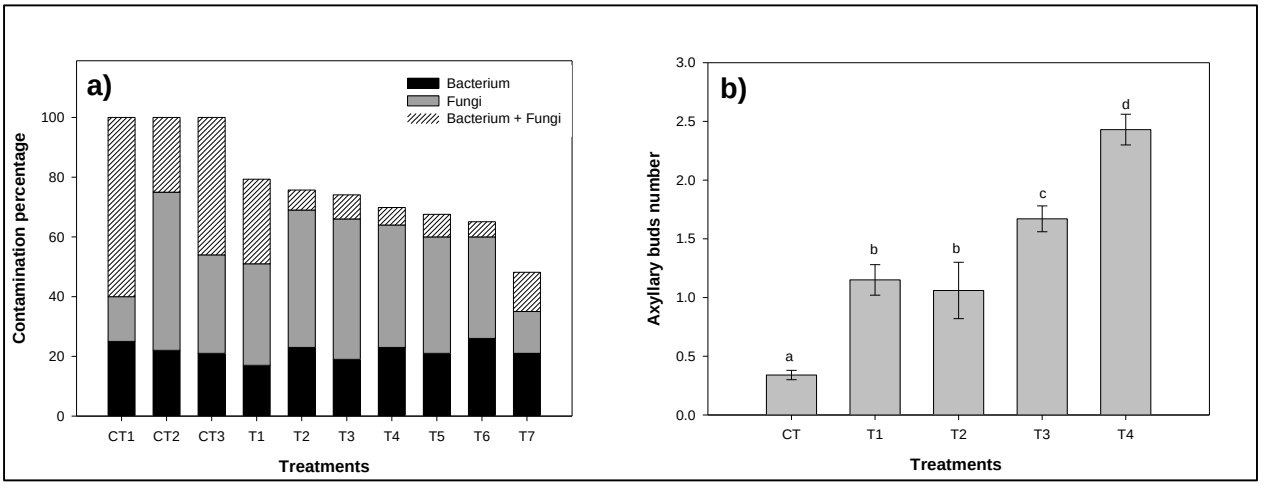

Figure 1 Effect of disinfection treatments on the establishment of nodal segments from Persian lime, after 60 days of culture on MS medium (a). Effect of treatments with 6-BAP and Kinetin, alone or combined, on organogenic response of *in vitro* nodal segments for axillary bud production, after 60 days of culture in MS medium (b). Letters indicate significant differences at p>0.05, bars indicate mean ± SE.

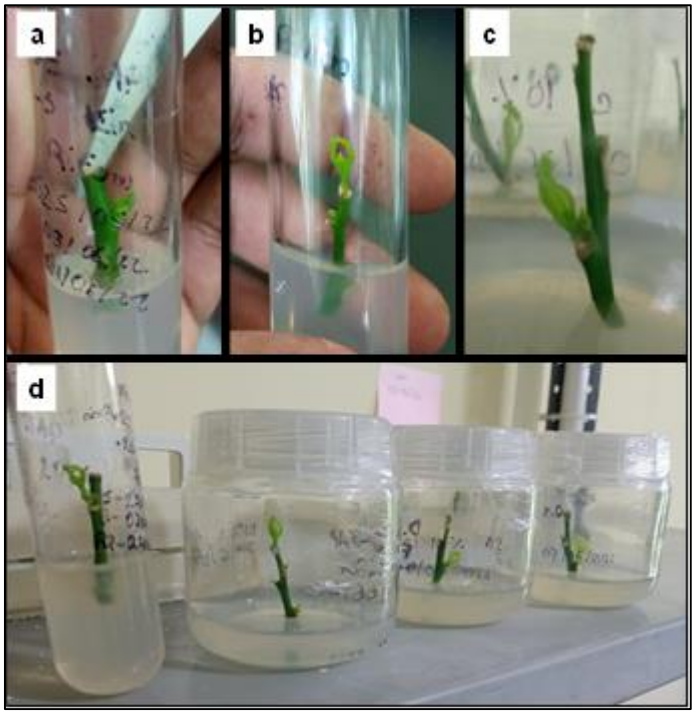

Figure 2 *In vitro* production of axillary buds obtained of Persian lime nodal segments. a-c) buds produced from T1-T3 treatments. d) Buds produced with T4 treatment (0.5 mg L^{-1} of 6-BAP + 0.5 mg L^{-1} of Kinetin).

4. Discussion

Over the years, several sanitation methods has been described to establish *in vitro* plants, all with the same goal, optimize the process and propagate pathogen-free plants. CEIXTA currently has a germplasm bank that holds a wide variety of Persian lime trees rescued in the region, as well as introduced from United States of America. Due to their agronomic importance, the producers selected all these Persian lime varieties introduced in the CEIXTA germplasm bank, however, in the last decade, others varieties have been of interest to the producers, therefore, these varieties should be included in the CEIXTA germplasm bank collection and thus have greater varietal diversity. For this reason, the aim of this study was establish *in vitro* nodal segments for achieve an adequate number of axillary buds per nodal segment. In this way, we introduced new varieties of Persian lime to germplasm bank; so that micrografts shoot tips can be carried out in the medium term with the purpose of massively propagate pathogen-free Persian lime plants.

Different reports have described that the important aspects for achieve the citrus production under controlled *in vitro* conditions is based on the adequate rootstocks selection [11], as well as an efficient protocol for explants disinfection from field, nursery and greenhouse [12]. However, to the day, disinfection protocol for obtaining aseptic explants in citrus fruits is not established. In addition, under natural field conditions, citrus fruits have a high group of parasitic microorganisms that lead to the development of pests and diseases, mainly caused by bacteria, fungi and virus. Therefore, the few *in vitro* buds that it can be obtained present defoliation problems and although it can be controlled with $AgNO_3$, the buds length not develop adequately to be used as explants source [12]. In this context, Varela [13] described the sanitation of 35 different citrus varieties, including Persian lime, their experimental design consisted in stem cuttings cut into nodal segments of approximately 3 cm and then were submerged by immersion in 70% chlorine for 2 min. After, these nodal segments were submerged in different $NaClO_2$ solutions at different exposure times and treated with bactericide (Oxytetracycline; 2.0 g/L) and fungicide (Amistar; 2.0 g/L) solution for 1 h. However, Varela [13] reported nodal segments death under *in vitro* conditions due to the presence of a large contaminants number and the short time of immersion of the nodal segments in $NaClO_2$ solution. Similarly, García [14] reported high contamination rate of nodal segments of Persian lime under *in vitro* conditions, of which 20 % corresponded to fungi and 10 % to bacteria. Furthermore, Herrera-Flores et al. [15] reported a disinfection protocol for the *in vitro* establishment of Persian lime nodal segments; however, they showed a percentage asepsis close to 34 %. Due to the above, we confirm that the plant material disinfection is a complex process because the stem cuttings brought directly from field have high fungi and bacteria rates, which highlights the importance to establish a disinfection protocol in suitable explants of Persian lime. Although some works present a disinfection protocol to follow, they are not standardized due to the difficulty of *in vitro* establishment. The results obtained in our research work coincide with

those reported because also finding high contamination percentages during the disinfection process in Persian lime nodal segments under *in vitro* conditions. In this way, we believe that although the use tissue culture techniques is a viable alternative for the sanitation and Persian lime micropropagation, the large number of microorganisms present in explants from field, limits it's *in vitro* establishment. Therefore, is necessary to continue conducting research that allows us to reduce bacterial and fungal contamination, however, at 60 days, we obtained 52% of nodal segments without contamination signs under *in vitro* conditions.

Respect to axillary buds production, most reports indicate the use of cytokinins such as 6-BAP and Kinetin, as well as, auxins (AIA and ANA) in order to produce a greater number of axillary buds under *in vitro* conditions using nodal segments as explants in species different. Thus, Singh et al. [16] reported cultured stem cuttings in MS medium enriched with 1 mg L^{-1} of 6-BAP and 0.5 mg L^{-1} of Kinetin to induce buds in *Citrus reticulata* Blanco and *Citrus limon* Burm.f. Likewise, García [14] reported that cultured Persian lime nodal segments in MS medium enriched with 6-BAP (1 mg L^{-1}) and Indole-acetic acid (0.5 mg L^{-1}) produced 1.19 axillary buds, however, this treatment not showed difference significant in relation to other treatments; this variable was related to cell dedifferentiation caused by cytokinins-auxins interaction. In addition, Herrera-Flores et al. [15] reported that achieved 42% of axillary buds 15 days after *in vitro* establishment when used a combination of 1 mg L^{-1} of 6-BAP and 0.5 mg L^{-1} of Naphthalene-acetic acid, obtained 1.2 axillary buds per explant. Our results showed that culture the nodal segments during 60 days (two sub-cultured of 30 days) in MS medium enriched with 6-BAP (0.5 mg L^{-1}) + Kinetin (0.5 mg L^{-1}), was sufficient for that 78 % nodal segments induced at least two axillary buds (**Fig. 2**). Therefore, this MS medium enriched with 6-BAP and Kinetin is promising for use in the *in vitro* propagation of Persian lime. Finally, our showed the disinfection protocol using Persian lime nodal segments from stem cuttings, as well as, disinfection process of these nodal segments under *in vitro* conditions, also, is shown the axillary buds obtained when the MS medium was enriched with 6- BAP and Kinetin (**Fig. 3**).

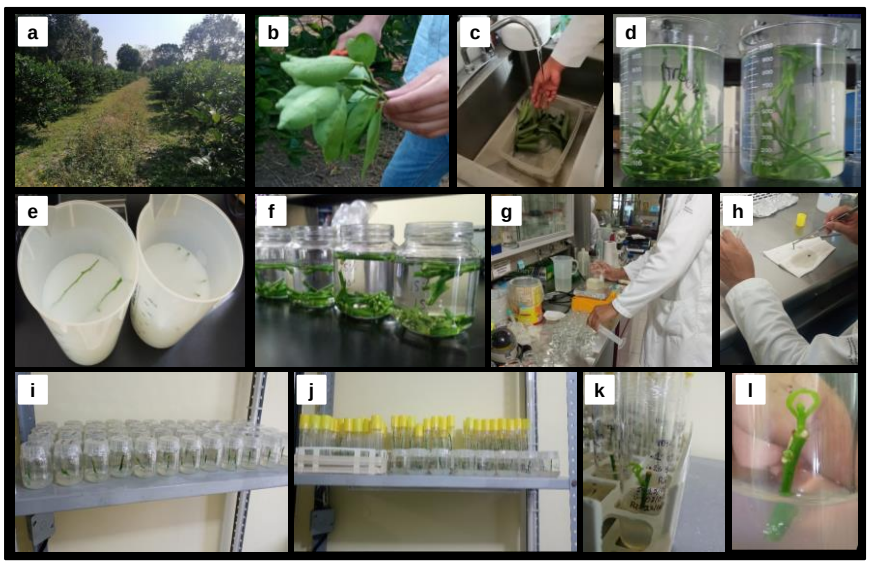

Figure 3 Disinfection of Persian lime nodal segments obtained from stem cuttings and *in vitro* axillary buds production. a) Persian lime tree of CEIXTA. b) Stem cuttings from 10-15 cm. c-d) Disinfection process of stem cuttings. e) Stem cuttings treated with bactericide and fungicide. f) Stem cuttings disinfection with different chlorine concentrations. g) MS culture medium enriched with 6-BAP and Kinetin, alone or combined, for axillary buds production. h) Stem cuttings cut by segments and *in vitro* culture of these nodal segments. i-j) *In vitro* establishment of nodal segments in culture room. k-l) Axillary buds production from *in vitro* nodal segments at 60 days.

5. Conclusion

We must develop new strategies which allow us reduce the contaminants growth in nodal segments of Persian lime during its *in vitro* establishment. The results of our research showed that 52% of the nodal segments did not show signs of *in vitro* contamination when the stem cuttings were disinfected with 20% chlorine for 15 min and when the nodal segments were cultured with Rifampicin bactericide (2 mg L^{-1}) and Chlorothalonil fungicide (1 ml L^{-1}). Furthermore, the use of cytokinins as 6-BAP (0.5 mg L^{-1}) and Kinetin (0.5 mg L^{-1}) in culture medium promoted that 78% of the nodal segments produced of 1 to 2 axillary buds. The disinfection and production of axillary buds protocols will allow us to medium term perform the sanitation of Persian lime varieties using *in vitro* micrografting shoot tips technique.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that no conflict of interest exists.

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