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Original article

Contamination Control of *In Vitro* Cultures of *Passiflora* Species for Multiplication Purpose

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Abstract

This study was conducted to obtain biological material regenerated from *Passiflora caerulea* and *Passiflora quadrangularis* by direct and indirect organogenesis, in order to enrich the assortment of flowering plants in Romania. The endogenous latent contamination of the plant material used for in vitro culture initiation is one of the biggest problems, demanding a special approach. The explants disinfection steps was organized as a trifactorial experience which included two variants of NaOCl concentrations (0, 5%, 10%), three immersion times in the sterilizing solution (10, 15 and 20 minutes) and the four types of explants (apical buds, fragments of young and mature leaves, and flower explants represented by pedicel, receptacle and sepals) taken from mature plants, in the stage of active growth. The explants were pretreated with 70% EtOH solution with a few drops of Tween 20, for 1 minute, and rinsed with distilled water, then disinfected according to the experimental variants. The explants were initiated on Murashige and Skoog, (1962) medium in order to stabilize the culture. The leaves explants reacted best to the treatment with 5% hypochlorite for 15 minutes. P. caerulea registered an average contamination rate of 52.78%, lower than P. quadrangularis in which case the explants obtained an average contamination rate of 58.24%.

Keywords: *Passiflora caerulea, Passiflora quadrangularis*, explants, contamination, hypochlorite

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INTRODUCTION

Passiflora genus, native to America, is emblematic of the *Passifloraceae* family, consisting of more than 600 species (Vanderplank J, 2000). *Passiflora* has been used for medicinal purposes since ancient times, being highly valued in traditional medicine due to bioactive compounds (Bandara et al., 2018). Spanish conquistadors learned from the Aztecs that Passionflower was used as a sedative for insomnia, anxiety and tranquilizers. The plant was introduced into European medicine and widely cultivated there (Watson, 2013). The ornamental potential of passion flowers is unexploited in Brazil, but in Europe has been used to decorate European greenhouses and gardens since 1625 (Abreu et al., 2009).

Conventional seed propagation of *Passiflora* often raises problems due to the germination and the long dormancy period of the seeds (Faleiro et al., 2019).

Micropropagation is an extraordinary technological facility for the production of ornamental, medicinal or fruit plants of *Passiflora*. The restrictions imposed on the marketing of ornamental plants on the health of plants meet the need to establish *in vitro* multiplication protocols in *Passiflora* in order to obtain a high quality biological material, free of diseases and pests, produced at the lowest possible cost.

Contamination of biological material for *in vitro* culture initiation is a major challenge given that passionflower secretes a sweet sap that is a nutrient medium for many microorganisms (Ozarowski & Thiem, 2013). *Passiflora* plants are often infected with *Xanthomonas axonopodi*s pv. *passiflorae* (Boro et al., 2011) and PWV (passion fruit woodiness virus) (Arogundade et al., 2018), two of the most important pathogens of the genus, reducing the period of commercial exploitation and implicitly the ornamental value.

In order to obtain aseptic biological material for inoculation, several protocols for surface disinfection of seeds or explants have been developed so far. Otahola & Diaz, 2010 performed asepsis of *P. quadrangularis* and *P. edulis* explants using the following protocol: after washing the explants with water, they were kept in a solution of 70% ethyl alcohol for one minute, then for 20 minutes in a 5% sodium hypochlorite solution. Subsequently, the explants were rinsed 3 times. In the research conducted by Rathod et al. (2014) the explants (young leaves from shoots harvested from *P. caerulea* plants rooted in the field) were disinfected as follows: the explants were washed with 5% Savon (surfactant) for 30 minutes and then rinse with distilled water 3 times. Then, they were subjected to a solution of 5% sodium hypochlorite for 10 minutes and rinsed three times with distilled water. The last step was disinfection with 0.1% mercuric chloride for 10 minutes then rinsed three times with distilled water. In the experience of Prithviraj et al. (2015), the explants were washed under running water and then sterilized with 0.1% mercury chloride for 3-5 minutes, then rinsed three times with sterile distilled

water. To evaluate the disinfection potential of sodium hypochlorite on the seeds of *P. caerulea*, Jafari et al. (2016) tested six different concentrations of sodium hypochlorite (0, 5, 10, 15, 20 and 25%) and three times intervals for immersion of explants in the solution to be sterilized (5, 10 and 15 minutes). The seeds were inoculated on MS medium. The lowest contamination rate (0%) was obtained in treatments containing 15% sodium hypochlorite for the immersion time of 10 and 15 minutes and for 20% and 25% sodium hypochlorite at the immersion times of 5, 10 and 15 minutes. The highest seed germination rate (86.66%) was obtained in the case of treatments with 10% sodium hypochlorite at a 10-minute immersion.

The present study represents a preliminary stage of research aimed at micropropagation in *P. caerulea* and *P. quadrangularis* by direct and indirect organogenesis. To minimize the depreciation of the explants, it is necessary to choose carefully the concentration of the sterilizing agent and the exposure time.

MATERIALS and METHODS

The research was carried out in the Micropropagation laboratory within the Institute of Advanced Horticultural Research of Transylvania (ICHAT), USAVM Cluj-Napoca, Romania.

The biological material comes from mature plants one year aged, *P. caerulea* and *P. quadrangularis* on vegetative growth stage in the didactic and experimental Greenhouse of Floriculture Discipline. In order to induce direct organogenesis, two types of explant were used: apical buds and leaf fragments. Regarding callusogenesis, the biological material used to initiate callus cultures was represented by four types of explant: leaf fragments, flower stalk, receptacle and sepals. Because several types of biological material of different textures were used, there was a need to test the hypochlorite concentration and exposure time to obtain aseptic explants that would generate successful for *in vitro* culture.

Prior to disinfection with hypochlorite solution, the explants were washed in a continuous stream of water for 10 minutes to remove dust and other impurities. Then, the explants were kept in a 70% ethyl alcohol (EtOH) solution with 2-3 drops of Tween 20 for 1 minute and rinsed with distilled water. After immersing the explants in the disinfectant solution and passing the exposure time to hypochlorite, three or five successive rinses were performed with sterile water, for complete washing of the disinfectant. The explants were shaped to 10 mm, removing the affected parts of the sterilizer. In the case of explants taken from the flower the whole flower was disinfected, following to be sectioned in the laminary flow hood.

The experimental model of the asepsis stage of plant material was organized in the form of two trifactorial experiments, cumulatively analyzing both explants used for regeneration by direct organogenesis and for callusogenesis respectively indirect organogenesis, each species being treated

individually. Thus, according to Table 1, the study was 4x2x3 type, resulting in 36 experimental variants. Each treatment consisted of three repetitions, and each repetition included ten explants.

Table 1. The experimental model of the plant material asepsis stage

Abbreviation	**Factor A Explant type**	**Factor B NaOCl Concentration (%)**	**Factor C Exposure time (Minutes)**	**B x C interaction encoded (% x min)**
A	apical buds	0% (Control) 5% 10%	10 15 20	0-0 5-10 5-15 5-20 10-10 10-15 10-20
Ly	young leaves fragments			
Lm	mature leaves fragments			
F	flower			

RESULTS and DISCUSSION

Regarding the reaction of *P. caerulea* explants was registered an average contamination rate of 52.78%, lower than *P. quadrangularis* in which explants obtained an average contamination rate of 58.24%. The average browning rate of *P. caerulea* explants was 30.65%, exceeding the species *P. quadrangularis* in which 21.48% of the explants were browned following exposure to asepsis treatment. Finally, only 18.43% of *P. caerulea* explants and 21.20% of *P. quadrangularis* explants survived the asepsis stage being suitable to move to the next micropropagation stage depending on the purpose pursued for each type of explant (Figure 1).

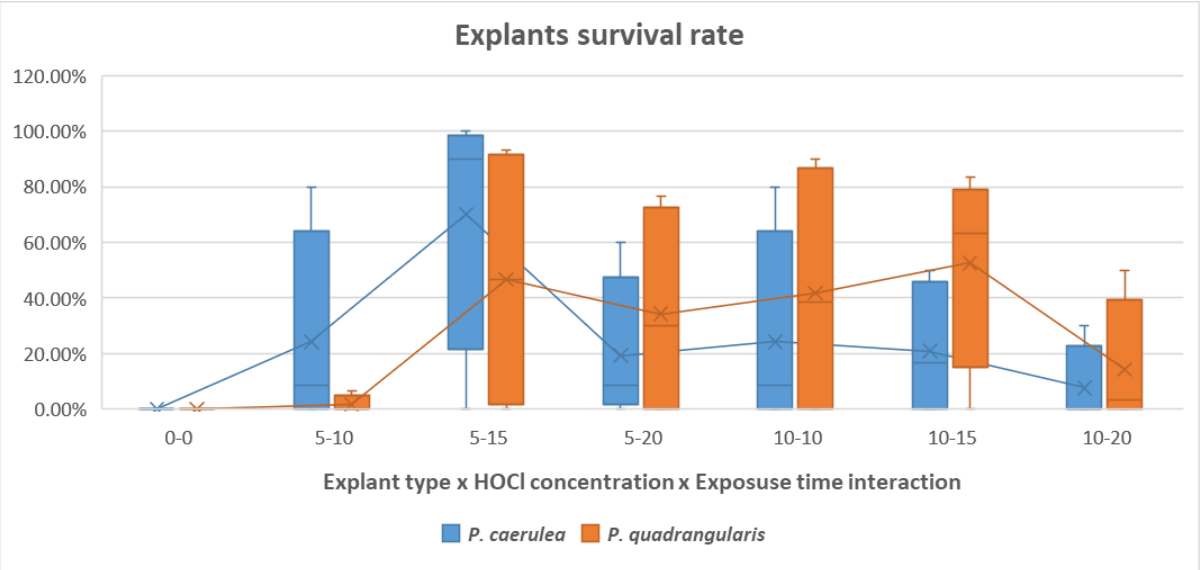

Figure 1. Interaction of factors on the survival rate of explants

Regarding the interaction of the three experimental factors, in the case of *P. caerulea* species on the treatment variant 5-10 (% NaOCl x min) survived 80% of mature leaf explants and 16.67% explants from young leaves. The apical and floral segments did not survive, registered 100% infections. For this treatment, the average survival rate of the explants was 24.17%. In contrast, the treatment variant 5-10 (% NaOCl x min) was not at all beneficial for *P. quadrangularis* explants, only 1.67% of them surviving. The treatment variant 5-15 (% NaOCl x min) obtained the best results regarding the survival rate of the explants, with average values of 70% for *P. caerulea* and 46.67% for *P. quadrangularis*. The treatment variant 10-20 (% NaOCl x min) was the most unfavorable for the disinfection of *P. caerulea* explants, the exposure time being too long for the herbaceous tissues of the species. For *P. quadrangularis*, the 10-15 treatment variant resulted in the highest average percentage of surviving explants (52.5%) because each type of explant supported the treatment variant.

Apex explants recorded a survival rate of 50% in response to disinfection treatment 10-10 (% HOC x min) for *P. caerulea* and 83.3% for *P. quadrangularis* (Figure 4). The contamination rate was total (100%) for treatment variants 5-10, 5-15, 5-20, 10-10 (% NaOCl x min) for both species. In the case of *P. caerulea*, a tissue necrosis rate of 67% was recorded due to the long exposure time to treatment 5-20 (Figure 2).

The explants represented by fragments of young leaves recorded a survival rate of 86.6% in response to asepsis treatment 5-15 and 80% in case of 10-10 (% HOC x min) for *P. caerulea* respectively 93.3% and 90% for *P. quadrangularis.* The browning rate was total (100%) in case of the 10-20 (% HOC x min) treatment option for both species (Figure 3).

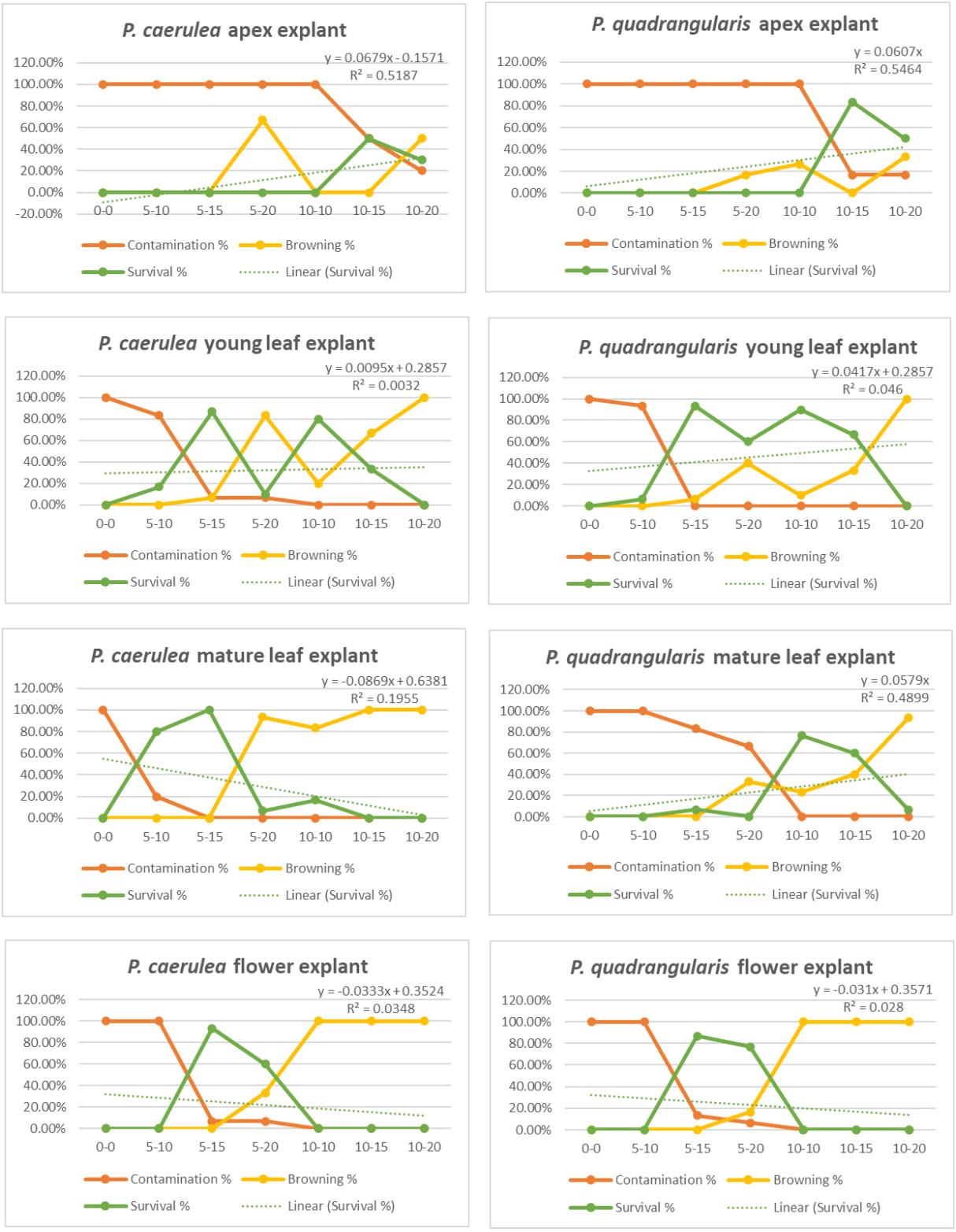

Figure 2. Explant reaction to asepsis treatment

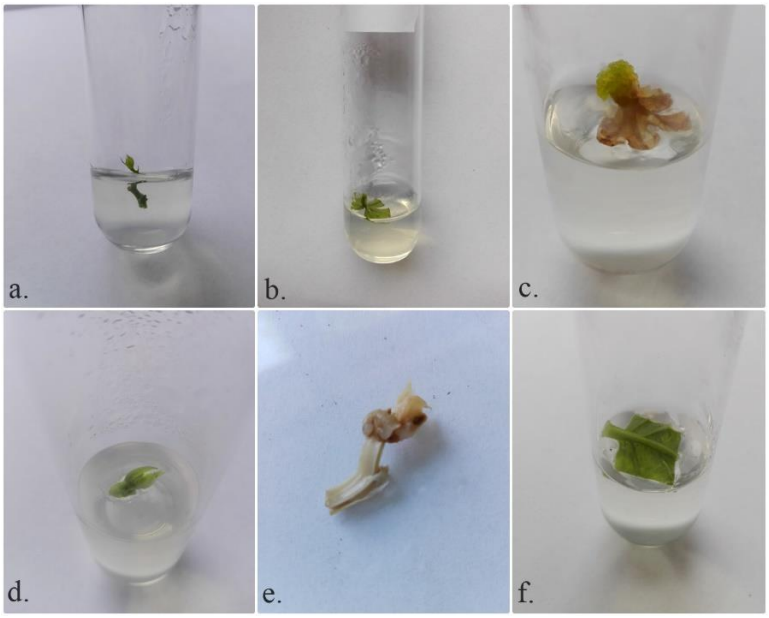

Figure 3. *P. caerulea*: a. aseptic apex explant, b. aseptic leaf explant, c. browning leaf explant callused; *P. quadrangularis*: d. aseptic apex explant, e. browning apex explant, f. aseptic leaf explant.

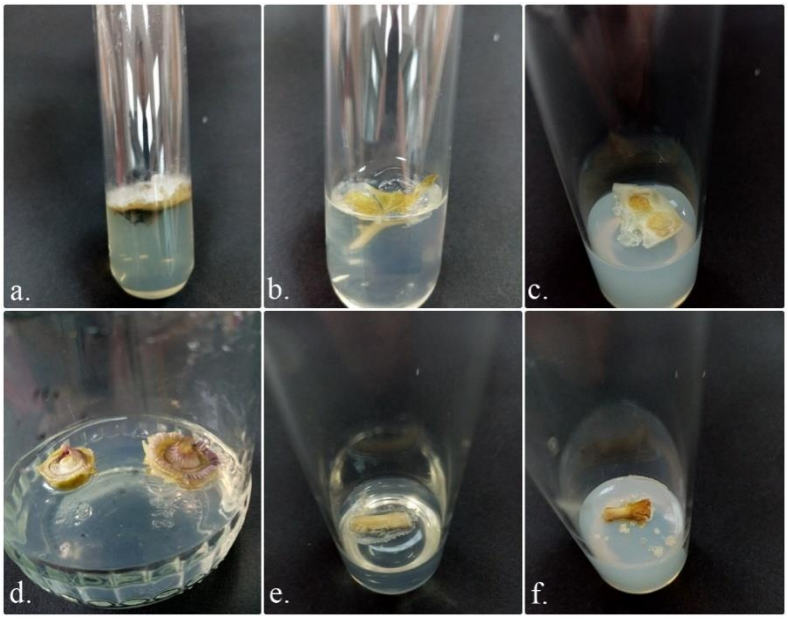

Figure 4. *P. caerulea*: a. infected apex explant, b. infected + browned leaf explant, c. browning sepals callusing; d. browning receptacele, e. browned + infected peduncle; f. *P. quadrangularis* browned + infected peduncle.

In his research, Jafari et al. (2016) obtained the lowest rate of *P. caerulea* seeds contamination, 0% in the case of treatment with 15% sodium hypochlorite at an immersion time of 10 and 15 minutes and at 20 % and 25% hypochlorite for immersion for 5, 10 and 15 minutes.

Oyebanji et al. (2009) applied to *Salcia chinensis* explants a surface sterilization protocol with 70% ethanol for 1 min followed by 1% sodium hypochlorite (NaOCI) (+ 2-3 drops of Tween 20) for 15 min proved whitch was most effective for maximum survival percentage (99%) in leaf explants whilst 70% ethanol washing for 2 min, followed by 0.1% mercuric chloride ($HgCl_2$) for 5 min proved to be more effective for maximum survival percentage (96%) in nodal explants.

Conclusion

The average contamination rate was very high for both species due to the presence of strongly proliferated endogenous contaminants in the mother plant. Therefore, it is recommended that the biological material used to tissue cultures initiation be derived from plants resulting from the micropropagation process, or if it possible from explants from *in vitro* seed germination. In the absence of alternative sources of biological material, an attempt was made to establish an effective protocol for asepsis of explants following the interaction between the concentration of the hypochlorite solution and the exposure time.

The average browning rate in *P. caerulea* was higher than in *P. quadrangularis* because from a phenotypic point of view, the plant has less woody shoots and the leaves are not leathery as in *P. quadrangularis* case, but the treatments to which the explants were subjected have were the same.

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