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Surface sterilization of *Staurogyne repens* (Nees) Kuntze with hydrogen peroxide

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Abstract: Sterilization is the killing, destruction or removal of all forms of microorganisms with a substance or an object. In tissue culture studies, the environment and equipment are sterilized. Likewise, the plant to be produced must be sterilized. It uses many methods and chemicals for the surface sterilization of plants. In this study, surface sterilization of *Staurogyne repens* (Nees) Kuntze was investigated using hydrogen peroskite (H_2O_2) at different times (10-30 min) and concentrations (3.7- 7.4% v/v). Nodal explants were used in trials. No plant growth regulator was added to the culture medium. Contaminations in food media started to be observed after 5 days and all data were collected after four weeks. While bacterial contaminations were recorded, a few fungal contaminations were also observed. High levels of H_2O_2 negatively affected the regeneration capabilities of explants. Also, some explants died due to H_2O_2. Contamination percentages were recorded between 40-100% in H_2O_2 applied environments. The highest sterilization rate (25%) were obtained in explants exposed to 5.5% H_2O_2 for 20 min. It was then recorded on explants treated with 5.5% H_2O_2 for 30 minutes (20%). As a result, surface sterilization of *S. repens* was accomplished using H_2O_2. These results can be helpful for surface sterilization and tissue culture studies of *S.repens.*

Keywords: Contamination; nodal explan; sterilization; tissue culture

1 Introduction

Humans use various methods to make plants of nature more favourable. They continuously develop these methods depending on the needs and conditions. These methods are called "culture". Plant cultures can be divided into three main groups according to their historical development. The first is classical cultures, which include vegetative and generative reproduction (Boedeltje et al. 2008); the second is water and sand cultures (McCall 1915); the last one is the Plant Tissue Culture which is gaining importance today (Priyadarshan 2019; Phillips and Garda 2019; Rojsanga et al. 2020). Tissue culture is the cultivation of small tissue and organ parts in sterile and appropriate nutrient environments (Ahmad et al. 2020; Gupta et al. 2020).

Tissue culture enables the production of a large number of plants with the same characteristics, both phenotype and genotypic (Dogan 2018; Sax et al. 2019). In this technique, many parts (explants) such as shoot tips (Dogan 2019), node (Mishra et al. 2019) and internode (Koike et al. 2018), hypocotyl (Gutiérrez et al. 2019), leaves (Pathak et al. 2019) and roots (Carvalho et al. 2019) were used to create new plants.

Microorganisms are considered as contamination in tissue culture studies. The most important losses in plant culture are caused by contamination. These microorganisms can be viruses, bacteria, yeast, fungi. If the sterilization is insufficient, fungi, yeast and bacteria may occur (Oyebanji et al. 2009; Mandeh et al. 2012). The presence of microorganisms such as bacteria and fungi in tissue culture techniques is one of the factors preventing plant growth and development. Plants must be stored in sterile conditions to be reproduced *in vitro.* Sterile laboratory conditions are created to reduce the damaging effect of microorganisms. Although surface sterilization is applied, cultures are sometimes not free of bacteria and fungi. Generally, the presence of bacteria in the culture medium may be evident at the first stage. However, microorganism emergence in the nutrient medium can be observed later in the culture. Various chemicals are used in plant tissue cultures to minimize contamination or eliminate it completely. Some of these are ethanol, calcium or sodium hypochlorite, mercury chloride and hydrogen peroxide (Mahmoud and Al-Ani 2016; Orlikowska et al. 2017; Javed et al. 2017).

In this study, surface sterilization of *Staurogyne repens* (Nees) Kuntze was investigated using hydrogen peroxide (H_2O_2) at different times and concentrations. Thus, an important contribution was made to the production of this plant with tissue culture.

2 Materials and Method

Plant material was obtained from the aquarium store. In the studies, MS (Murashige and Skoog 1962) mineral salt and vitamins were used as nutrient media. No plant growth regulator was added to the culture medium. In addition, 3% sucrose (Duchefa) and 0.65% agar (Duchefa) were transferred to the food medium. The pH of the food medium was sterilized at 121°C for 20 min. under 1.2 atmospheric pressure after adjusting to 5.7 ± 1 using 1 N NaOH and 1 N HCl.

The plant was kept under running water for 30 min in order to remove it from the wastes and reduce the microorganism density before surface sterilization. The upper body parts were cut (3-5 cm) and treated with H_2O_2 (35% - Merck Millipore) (3.7%, 5.5% and 7.4% v/v) at different duration (10, 15, 20 and 30 min). Then, rinsing was performed three times with sterile distilled water for five min. Nodal explants were isolated and transferred to test tubes. All values were taken for sterilization after four weeks.

3 Results and Discussion

Plant tissue culture is a modern biotechnological technique for the production of plants or herbal products. But the success of tissue culture depends on maintaining sterile production conditions. Contaminations from microorganisms cause extra time, effort and material spending in tissue culture studies. This is an important monetary problem. It is also the most serious factor responsible for losses in tissue culture. Bacterial or fungal contaminations can be environmentally sourced or endogenic. Many processes are carried out on plants to prevent microorganism pollution (Javed et al. 2017). In this study, H_2O_2 was treated with different time (10-30 min) and concentrations (3.7-7.4 v/v) for surface sterilization of *S. repens*. The nodal explants were used in sterilization studies. All values for sterilization were taken after four weeks (Table 1). Similarly, the use of H_2O_2 for surface sterilization was reported in *Stevia rebundiana* Bertoni (Halim et al. 2016), *Zantedeschia aethiopica* L. (Chen et al. 2017), Phoenix dactilefra L. (Metwaly et al. 2018), *Prunus persica* (L.) Batsch (Al Ghasheem et al. 2018) plants. In addition, the use of $HgCI_2$ in *Musa paradisiaca* L. (Shukla et al. 2019), Sugarcane (Singh and Gupta 2019) and *Catharanthus roseus* L. (Vandana et al. 2020) plants and the use of NaOH in *Phanera sirindhorniae* (Sirimat and Sakulsathaporn 2019) and *Cryptocoryne wendtii* (Klaocheed et al. 2020) plants have been reported for surface sterilization. This showed that different disinfectants can be used for surface sterilization of plants.

Table 1 Surface sterilization data of nodal explants treated with H_2O_2 at different concentrations and time

Hydrogen peroxide (H_2O_2)		Contamination rate (%)	Sterile and dyed explant rate (%)	Sterile and live explant rate (%)
%	Duration (min)			
3.7	10	100	-	-
3.7	15	100	-	-
3.7	20	85	10	5
3.7	30	75	20	5
5.5	10	100	-	-
5.5	15	85	5	10
5.5	20	55	20	25
5.5	30	50	30	20
7.4	10	90	-	10
7.4	15	70	20	10
7.4	20	50	35	15
7.4	30	40	50	5

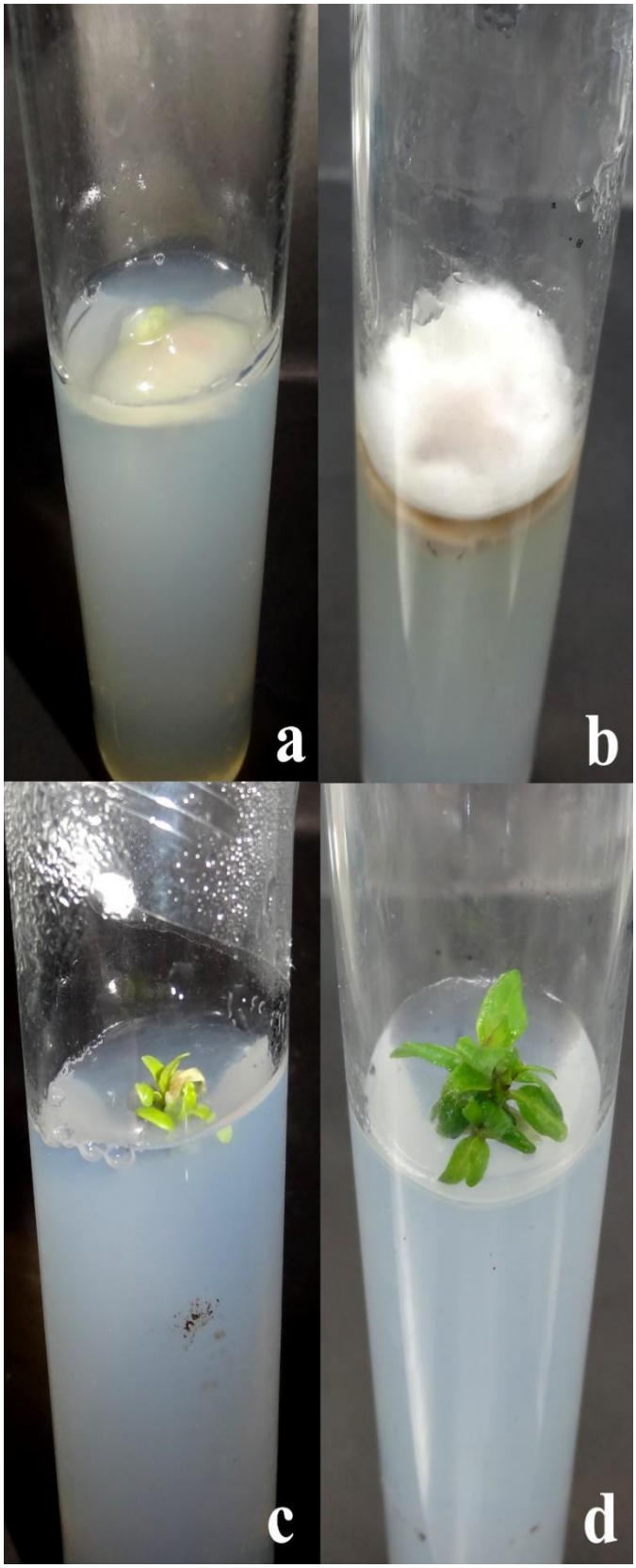

Fig 1 *S. repens* explants treated with H_2O_2 at different rates and periods. (a) bacterial contamination (b) and fungal contamination on the explant (c, d) sterile-strong shoots from the explant treated with 5.5% H_2O_2 for 20 min.

The first contaminations in the culture medium started to be observed on the 5th day. These contaminations became more evident after four weeks. The contaminations covered some explants and caused their death. In general, bacterial contaminations were observed (Fig 1a) and fungal contaminations were also detected in a small number of tubes (Fig 1b). As the H_2O_2 concentration and application time increased, the rate of the contaminations decreased. Some explants were damaged and died with high H_2O_2 concentration.

Contamination percentages were recorded between 40-100% in H_2O_2 applied environments. Maximum contamination levels (100%) were observed in explants treated with 3.7% H_2O_2 for 10 and 15 min. In general, the contamination level detected in cultures treated with high H_2O_2 was low. Minimal contamination (40%) was detected in explants exposed to 7.4% H_2O_2 for 30 min. Sereda et al. (2017) have followed a rather complicated path for surface sterilization of *S. repens.* Before sterilization, the plants were washed for 15 min with 0.01% tween-80 and then rinsed with running water. Plants were washed with sterile water after being treated with 70% ethanol. In addition, 5% chloramine B solution, 1% NaOCl and 0.1% $HgCl_2$ were applied in the sterilization process. The best sterile explants achieved 0.1% and 0.1% with $HgCl_2$ (5 min). In the current study, an easier way was proposed for surface sterilization of *S.repens* than Sereda et al. (2017).

In surface sterilization trials, sterile and live explant levels were determined between 5-25%. The highest sterilization rate (25%) were obtained in explants that interacted with 5.5% H_2O_2 for 20 min (Fig 1c and d). It was then recorded in explants treated with 5.5% H_2O_2 for 30 min (%20). Similarly, Al Ghasheem et al. (2018) applied 5% and 10% H_2O_2 for 10 and 20 min for the surface sterilization of the shoot tip and nodal explants of *P. persica* . They achieved the highest sterile and intact explants after 20 min treatment with 10% H_2O_2 for the shoot tip (25%) and 10 and 20 min treatment with 10% H_2O_2 for the nodal explants (20%).

5 Conclusion

Surface sterilization is an initial and important stage of tissue culture. After this stage, trials are established for reproduction. In this study, successful surface sterilization of *S. repens*, which is important in the aquarium industry, was explained using H_2O_2. The use of low levels of H_2O_2 was not successful for surface sterilization. In general, bacterial contamination has been detected in the food medium. The use of high levels of H_2O_2 also caused the explants to die, although it reduced the level of contamination. Although some explants were sterile, they lost their regeneration ability. It is very important to use disinfectant in optimum concentration. The best results were found in explants treated with 5.5% H_2O_2 for 20 min. These results can be helpful for surface sterilization and tissue culture studies of *S. repens*.

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