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Effects of Contamination Agent for Tissue Culture Applications of *Bacopa monnieri*

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Abstract: Biocides and plant protection products have been used in plant tissue culture sterilization procedures since they have broad spectrum, are inexpensive and resistant to autoclave process. This study aim was to determine the effects of two contamination agents on tissue culture conditions. MS medium was supplied with 0.5mg/L 6-benzylaminopurine and 0.1 mg/L indole-3-butyric acid as media. PPM (Plant Preservative Mixture) and Contaminacide and their three dosages (0, 3 and 6 ml/L) were used for maintaining tissue culture aseptic conditions. After plant explant were sow, vessel lids were remained open for three days under climate room conditions. Contamination rate and plant growth parameters were measured. Contamination did not occur in all dosages of Contaminacide and 6 ml/L PPM, although preservation-free and 3ml/L PPM-added media were contaminated. In conclusion 3ml/L Contaminacide added media were superior in terms of less preservation chemical cost, contamination rate, plant height, fresh and dry weight ($p \leq 0.05$).

Keywords: Sterilization, micropropagation, fungi, bacteria, PPM, Contaminacide

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1. INTRODUCTION

Sterilization processes are very important for tissue culture methods and reproduction procedures. Tissue culture methods and propagation stages are the most important problems of protection against fungal and bacterial infection (Reddy et al., 2021). Inhaled air contains significant microbial load due to humidity and temperature factors. These factors tend to multiply when suitable conditions are provided (Hanif, 2021). Rich sugar sources, high humidity and ideal growth conditions from the energy opening in the tissue culture medium cause a rapid proliferation of aerobic contamination factors.

Contamination losses are between %3 and %15 for each subculture stage even in strict sterile tissue culture laboratory (Boxus and Terzi, 1987). Plant tissue culture media are suitable for the growth of many fungi, yeasts and bacteria (Agrios, 1990). Because media for plant tissue culture are very similar to fungal media. Plant deaths occur due to some phytotoxic metabolites produced by fungi growth such as *Aspergillus*, *Alternaria* and *Penicillium* (Xu et.al., 2021). It

is also known that bacterial species with fermentative metabolism have pathogenicity mechanisms like fungi and yeasts (Leifert et al., 1989b).
Infected tissue culture vessels and in vitro plants die very quickly, especially since airborne microorganisms' reproduction and spread rates of occur faster than plants. If the infectious factors are not controlled, it is possible to quickly become infected with laminar flow, air conditioners and all plants in the laboratory (Kowalik and Grodek, 2002).

Sterilization and disinfection processes are applied to prevent contamination under tissue culture conditions. The purpose of using sterilization agent is to destroy the contaminant, not to prevent the life of the plant to which it is applied and not to cause somaclonal variation. The preferred sterilization method should be inexpensive, broad-spectrum, and the tissue should remain stable in different cultural

applications. The most used sterilization methods in tissue culture laboratories are heat, chemical sterilization and filtration methods. Steam sterilization method is widely used in the sterilization of nutrient media containers, nutrient media contents, metal equipment such as forceps, scalpels, and glassware. Hepafilters and injector/pipette tip hepafilters used in sterilization of sterile cabin and laboratory air are commonly used tissue culture sterilization methods. UV used in sterile cabinet sterilization and gamma sterilization ray sterilization methods used in sterilization of some disposable instruments can be given as examples. Bleach and ethanol are the most commonly chemicals in the surface sterilization of plant parts (Ahloowalia et. al., 2004). The reason why these two chemicals are more preferred in tissue culture applications is one of the most preferred methods due to the wide range of infection agents it prevents, being economical and common usage area.

The high price of antibiotics, the possibility of agents to develop resistance to antibiotics and their specific fields of action narrows the area of use. The high price of silver chloride or the negative effects of mercury chloride on the environment and human health also limit the use of these chemicals. Therefore, he use of plant protection products or biocides in tissue culture applications is becoming widespread. In plant tissue culture studies, contamination agents are added to prevent the proliferation of unwanted microorganisms such as bacteria and fungi from contamination by microorganisms. PPM (Plant preservative mixture), the most common contamination agent, is a solution developed to protect plant material from infection by microorganisms.

Prospectus indicates that PPM contains 0.1305% 5-Chloro-2-methyl-3(2H)-isothiazolone and 0.0459% 2-methyl-3(2H)-isothiazolone. It is recommended that the storage must be at 4°C and usage dose of the product is 0.5-2.0 ml PPM per liter. It has been stated that the mechanism of action of PPM is both biocidal (>2ml/L medium) and biostatic (<2ml/L medium) and protection occurs by targeting the basic enzymes in the Krebs cycle and Electron Transport Chain. It is stated that the addition of 5-20 ml/L PPM to the growing medium is appropriate, and the effect of PPM will decrease when exposed to high concentrations of bacteria. It is stated in the user manual of Contaminacide that it is recommended to use 2-9 mL/L, and it contains non-ionic active substance, deionized water and Tween20.

B. monnieri plant extract widely contains different secondary metabolites, including saponins, alcohols, steroids, alkaloids, glycosides, sterol glycosides, phenylethanoid glycosides, sugars, some amino acids, flavonoids, and cucurbitaceous (Chakravarty et al., 2002; Rauf et al., 2013; Bhandari et al., 2007). Bacopacid, one of the bioactive components of *B. monnieri*, is known to protect the brain against oxidative damage and age-related cognitive deterioration (Saraf et al., 2011, Mukherjee et al., 2011). *B. monnieri* has a variety of bioactive compounds that are being investigated for therapeutic uses. *B. monnieri* extract is used to modulate neuropathological pathways involved in brain function and neuroprotection. Active ingredients from *B. monnieri* have been indicated to show possible therapeutic intervention against Parkinson's and Alzheimer's diseases. There are many studies investigating the effect of PPM, which is widely used in plant tissue culture (Compton and Koch, 2001; Digonzelli, et. al., 2005; Gu et. al., 2022; Miyazaki, et. al., 2010). However, there is no study has been found on literature with using Contaminacide on plant tissue culture investigation.

In this study, it was carried out to examine the effects of two contamination agents used in tissue culture applications on contamination formation and plant growth to prevent contamination factors during the propagation of *Bocopa monnieri* under tissue culture conditions.

2. MATERIAL AND METHOD

The study was carried out in tissue culture laboratory conditions at Çanakkale Onsekiz Mart University, Agriculture Faculty, Department of Field Crops. The plant species *Bacopa monnieri* used as plant material was obtained from the collection of Margeht Biotechnology Inc. As the growing conditions, 24 ºC 3.000 lux lighting and 16/8 hours light/dark photoperiod conditions were set. B5vitamin, 30g/L sucrose, 8g/L agar, 0.5mg/L 6-benzylaminopurine and 0.1mg/L indole-3-butyric acid were added Murashige and Skoog nutrient media. Contamination agents were added to the nutrient media before pH 5.7 adjusted with KOH and media autoclaved under 121ºC, 1.0 bar pressure and 20 minutes long. Study was carried out in three replications according to the randomized blocks experimental design, replication number set as 3 and 9 plants were placed in each container. Two different 3-6 mg/L application doses of two different contamination agents, PPM and Contaminacide, were applied as a treatment, and medium without the addition of contamination agent was used as the control group. PPM (Plant Preservative Mixture) and Contaminacide and their three dosages (0, 3 and 6 ml/L) were used for maintaining tissue culture aseptic conditions. *Bacopa monnieri* was used as plant material and contamination rate and plant growth parameters were measured. The PPM product of Plant Cell Technology company was supplied as cold chain through Turkey Distributor Bee Biotechnology. The Contaminacide was obtained from Margeht Biotechnology Inc.

After the solidification of the medium, apical shoots of *Bocopa monnieri* 1.5 cm long were placed in the nutrient medium under a sterile cabinet and the lids of the nutrient medium were left open for 3 days in 24ºC temperatures and 16/8h photoperiod climate room conditions. At the end of the 3rd day, the lids of the nutrient medium containers were closed. At the end of the 35th day following the explant sowing in the nutrient medium, the plant height, fresh and dry weight measurements were noted with the containers in which the infectious agent grew. The data were subjected to variance analysis with the GLM command in the SAS package program (SAS Institute, 1999).

3.RESULTS

This study was carried out to keep the lids of two different contamination agents added to the nutrient medium under *in vitro* tissue culture conditions completely open for three days, to keep the nutrient medium sterile and to examine the effects on the growth of *Bocopa monnieri*. Deathly contamination occurred in the control and the medium with 3 ml/L PPM added groups at the end of the 35th day (Figure 1). The investigated parameters were plant height, fresh weight and dry weight, and the highest application was 2.43 cm, 54.45 mg and 25.14 mg in 3 ml/l Contaminacide added nutrient medium, respectively (Table 1). It was determined that there was no significant difference between 6 ml/L dose of PPM and Contaminacide contamination agents in terms of plant height and wet weight parameters, and 6 mL/L Contaminacide application was more effective than PPM in terms of dry weight.

Table 1. Results on the effects of PPM and Contaminacide contamination agents applied at two different doses on some parameters.

	Control	**Contaminacide**		**PPM**	
		3 ml/L	6ml/L	3ml/L	6ml/L
Plant length (cm)	0.00 C	2.43 A	1.64 B	0.00 C	1.65 B
Fresh weight (mg)	0.00 C	54.45A	49.99 AB	0.00 C	45.60 B
Dry weight (mg)	0.00 C	25.14A	11.00 B	0.00 C	10.22 C

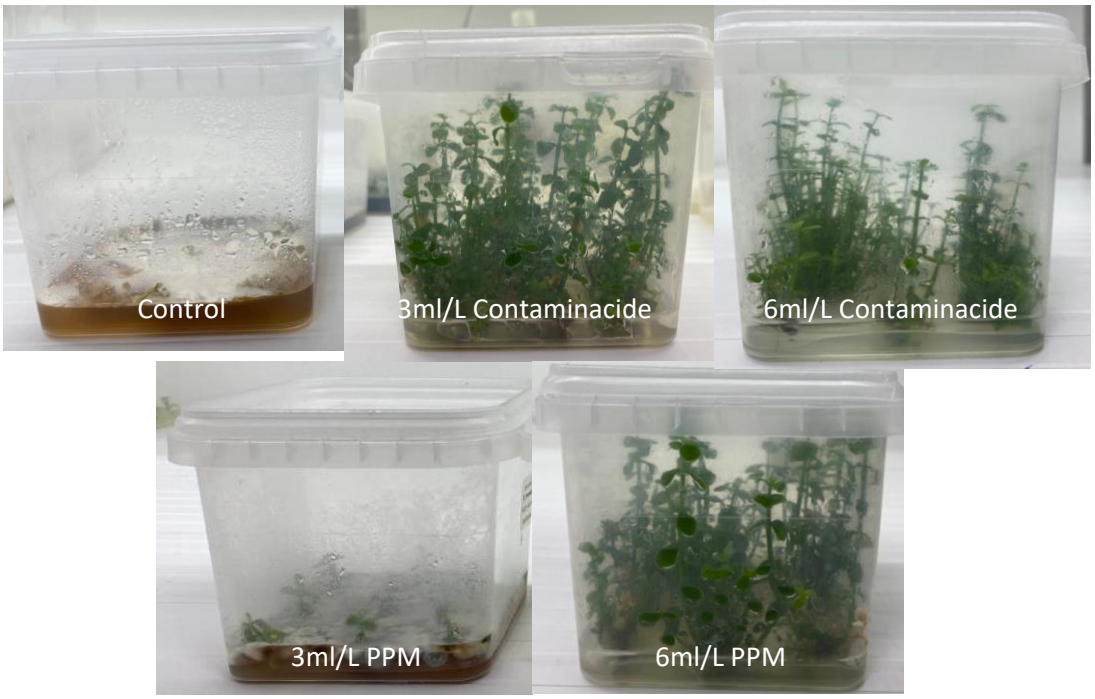

Figure 1. Control and two different contamination agent and dosages application results in *B. monnieri*

4. DISCUSSION

In related studies, PPM has been tested in various plant explants at various concentrations and it has been reported that some plants have a positive effect, while some have no positive effect (Compton and Koch, 2001; Digonzelli, et. al., 2005; Gu et. al., 2022; Miyazaki, et. al., 2010). It has been reported that PPM, which is used at different doses to control Pseudomonas contamination in sugar cane, inhibits bacterial growth, on the other hand, it does not have an inhibitory effect on length, fresh weight and cluster number (Digonzelli, et. al., 2005).

PPM applied at concentrations of 0.5 to 4.0 ml/L on chrysanthemum, European birch and rhododendron leaf explants had a very low effect on the percentage of shoot-forming explants and the number of shoots developed per explant in birch and rhododendron. It has been determined that it has a significant negative effect in terms of the number of shoots (George and Tripepi, 2001).

To evaluate the phytotoxicity of PPM in the meristematic tissues of epiphytic orchids, *Dendrobium thyrsiflorum* Rchb.f. (1875) in a study using seeds and seedlings, it was reported that when 0.1% PPM was added to the medium, it did not show an observable growth inhibitory effect on protocorm, shoot or root development. However, it has been reported that PPM supplementation above 0.2% has a negative effect on *D. thyrsiflorum* explants, and that at high

PPM concentrations, root tissues of young in vitro seedlings were damaged (Chanh et. al., 2023).

The effect of PPM was investigated in four different (*Juglans regia* L.) walnut cultivars grown in tissue culture medium. One-year-old shoots of walnut cultivars were taken into DKW medium with and without PPM. Adding 0.2% v/v PPM to the starting medium reduced the number of contaminated explants from an average of 67.8% to 37.3% and increased the percentage of green shoots from 2.2% to 12.6% for all cultivars. After rinsing the explants in 5.0% v/v PPM and then inoculating in medium containing 0.2% v/v PPM, 21.7% of clean shoots were obtained. These shoots were indexed on 523 detection media and 87.5% were reported to be bacteria-free (Kushnarenko, 2022).

Carica papaya cv. Three treatments were performed on PPM taken from posterior Prabhath explants for endogenous contaminant control. Axillary shoot tips (1.0-1.5 cm) were treated with 5% (T1) PPM for 4 hours, 50% (T2) for 10 minutes, and 100% (T3) for 10 minutes. It was observed that no application yielded positive results because explants succumbed to microbial contamination (80% in T1) or phytotoxicity effect/contamination (90% in T2 and 95% in T3) at the end of 4-6 weeks. Another experiment using a multi-step surface sterilization treatment (carbendazim-cetrimide-HgCl2) followed by culturing in papaya growth medium supplemented with 0.05% PPM showed 35% significant bacterial contamination compared to 40% in the control. The results indicated the prevalence of several PPM-tolerant endophytic bacteria in papaya and most of them survive in the MS-based environment, and this should be considered when using PPM for contamination management (Thomas et. al., 2017).

Two different immersion sterilization procedures were applied of the *Miscanthus* spp. genotype under plant tissue culture conditions with and without PPM. In the PPM study, explants obtained from two adult plants with accession numbers PI 668371 and PI 668375 on contamination were placed in MS medium with 1 mL/L PPM. At the end of the study, aseptic applications showed no difference in the percentage of bacterial and fungal contamination and the percentage of explant survival. It was observed that there was less bacterial contamination (28%) with the addition of PPM compared to its absence. For percentage of fungal contamination, it was stated that PPM had no significant effect on plant PI 668371 and a lower percentage of fungal contamination (16%) was found in PI 668375 accession. It has been emphasized that PPM may be an effective agent to prevent bacterial contamination in in vitro cultures of Miscanthus spp. and fungal contamination in the accession of PI 668375 (Ledo et. al., 2019).

5. CONCLUSION

In plant tissue culture applications, sugar added to the nutrient medium as a carbon source makes the nutrient medium particularly susceptible to bacterial and fungal contamination. The risk of contamination of the tissue culture medium is very high because the bacterial spores or fungal hyphae are invisible even if effort is made. Some contaminants can be stored in the vascular bundles for very long time due to their endophyte properties and their emergence time may occur even after a few subcultures. In this case, systemic contamination agents play a very important role against vascular microorganisms.

In this study, it was carried out to determine the effects of 3 and 6 ml/L PPM and Contaminacide added to the MS medium and the effects of the containers without preservatives on the protection from contamination and plant growth due to the exposure time for three days. While all the media without preservatives and 3 ml/L PPM added were contaminated, there was no contamination in the environment containing the product named Contaminacide with 3 ml/L addition, on the other hand, it was concluded that in vitro plantlets were superior to other applications in terms of plant height, fresh and dry weight ($p<0.05$).

This study is the first study on the contamination agent named Contaminacide, and it is predicted that it would be beneficial to apply the product at different doses and test it on different plants.

Ethics Committee Approval
N/A

Peer-review
Externally peer-reviewed.

Conflict of Interest
The authors have no conflicts of interest to declare.

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