(PPM) PLANT PRESERVATIVE MIXTURE

Introduction

PPM™ is a heat stable preservative/biocide which can be used to effectively prevent or reduces microbial contamination in plant tissue culture.

At optimum doses, PPM™, which stands for Plant Preservative Mixture™, is an extremely effective Preservative/Biocide, yet does not impair in vitro seed germination, callus proliferation and callus regeneration.

Despite the most stringent use of sterile techniques, the contamination of plant cell and plant tissue cultures remain a persistent problem that can result in losses ranging from small number of cultures to the loss of whole batches.

PPM™ prevents the germination of both bacteria and fungi spores. It is heat stable and therefore can be autoclaved with the media.

PPM™ can be, and should be used as a standard ingredient in plant tissue culture media, and is also substantially less expensive than commonly used antibiotics.

While PPM™ was principally designed to inhibit airborne contamination, waterborne contamination and contamination introduced from human contact, it can also -- in many cases -- be used to reduce endogenous contamination.

The principal PCT scientist involved in the development of the PPM™ application is Dr. Assaf Guri. Dr. Assaf Guri holds degrees in genetics, applied genetics and plant breeding from the Hebrew University in Jerusalem and Michigan State University in the US. Before joining Plant Cell Technology, Inc., Assaf worked with the Volcani Agricultural Research Center in Israel, Michigan State University in East Lansing, Michigan and DNAP in New Jersey.

Mechanism of Action

PPM™ is a broad-spectrum preservative and biocide, which kills bacteria and fungi cells, prevents germination of spores, and in higher concentrations, can eliminate explants of endogenous contamination.

Previous research has shown that the active ingredients of PPM™ penetrate the fungus or the bacterium cell wall and inhibit the activity of key enzymes within the central metabolic cycles such as the citric acid cycle and the electron transport chain. Our data indicates that PPM™ may also inhibit the transport of monosaccharides and amino acids from the medium into the fungus or bacterium cells.

As in any biocide, a critical ratio of PPM™ molecules per microbial cell is needed to eliminate bacteria and fungi. Keep in mind that a given volume of PPM™ dose has a constant number of PPM™ molecules while the number of spores introduced to tissue culture via endogenous contamination is highly varied. Therefore, explants should not be "squeezed" into a beaker. There should be enough volume for free movement of the solution around the explant material.

ADVANTAGES OVER ANTIBIOTICS

- PPM™ is broad-based and effective against fungi.
- PPM™ is less expensive than antibiotics, making it affordable for wide and routine use.
- Since PPM™ targets and inhibits multiple enzymes, the formation of resistant mutants towards PPM™ is very unlikely.

- PPM™ is heat stable and in general can be autoclaved with media.

PROCEDURES

The procedures described below are generic. Slight modifications might be needed for your specific plant species. For assistance, contact Dr. Assaf Guri at guri1@erols.com

PPM™ significantly simplifies the tissue culture working procedures as follows:

1. Media containing PPM™ may be dispensed outside the laminar flow hood (LFH) exposed to the ambient air. The plates should be covered soon after agar solidification. In the event that media dispensing is done by a pump, we recommend passing autoclaved hot water through the hoses prior to and after media dispensing.

2. Heat sensitive or heat stable liquid media containing PPM™ does not need to be sterilized by Millipore filters or autoclaved provided that it will be stored in sterile containers and that the stock solutions are not contaminated. In rich media containing 200 mg/liter or more of amino acids or proteins, it is recommended to filter the media with the PPM™.

3. If working in the LFH the utensils (forceps or scalpels) do not need to be flamed. They should be periodically dipped in 70% alcohol. The LFH does not need to be certified and the work can be done as well outside the LFH on a clean surface for a period not exceeding 1 hour.

4. PPM™ is an acidic liquid solution (pH 3.8). PPM™ should be stored at 4°C. (see Safety Information below). At the recommended dose of 0.05 - 0.2% (v/v), PPM™ is added to the medium before or after autoclavation to prevent airborne and endogenous contamination at low inoculum densities.

5. PPM™ is less effective when exposed to high density of bacteria or fungi spores found regularly on seed's coat. For in vitro germination, seeds should be conventionally surface sterilized with EPA registered bleach. Therefore, in the presence of PPM™ (in the germination medium), the seeds can be rinsed under tap water in a non-sterile strainer and left to dry preferably in the LFH. If the utensil ends have touched active bacteria, fungi culture or otherwise suspected of being contaminated, they should be sterilized by autoclave or by use of an electric heating element.

6. **General Dosage levels:** With the exception of endogenous contamination, the recommended dose range is 0.05%-0.2%. (For callus proliferation, organogenesis and embryogenesis, the recommended range is 0.05-0.075%.) Add PPM to medium pre or post autoclavation to prevent airborne contamination and endogenous contamination at low inoculum densities or slow growing bacteria. To eliminate higher endogenous contamination densities, higher doses of PPM are needed (see paragraph 7 below).

7. **Endogenous Contamination:**

(a) For explants: gently and routinely shake / stir 1 cm. long explants (or shorter) for 4-12 hours in 4-5% v/v PPM™ solution supplemented as above with full MS strength basal salts without pH ing and without Tween 20. Without rinsing, insert into a medium supplemented with 0.05 - 0.1% PPM™ for herbaceous plants and 0.2% PPM™ for woody plants.

Note

Paragraphs 7(b) through 10 below are intended for ornamental plants, non-North American users only.

(b) For tubers, bulbs and scales: shake / stir the entire tuber / bulb / scale in bleach. Rinse with water (can be done under non-sterile conditions). Slice the tuber / bulb / scale to thin slices. Shake / stir for 12-24 hours in 4 - 5% PPM™ solution supplemented with full strength basal salts without pH ing and Tween 20. Without rinsing, insert into a medium supplemented with 0.1 - 0.2% PPM™.

8. In cases where the above protocols do no yield satisfying results (especially thick explants, highly infested explants, seeds), we recommend the following:

(a) Shake / stir the explants in water (1hr for soft tissues and 2 hr for hard tissues).

(b) Shake / stir the explants in (50%) PPM™ supplemented with full strength MS basal salts (without pH ing and without Tween 20) for 5 -10 minutes.

(c) Without rinsing, insert the explants into the medium. In fungal contamination, the addition of PPM™ to the medium is optional. However, with bacterial or mixed contamination, the addition of 0.05 - 0.2% PPM™ to the medium in the first month is essential. Do not discard highly oxidized explants as approximately 50% of the explants will recover within 4 - 6 weeks.

Note

Refer to notes 2 and 3 below.

9. To decontaminate "in culture" contaminated plant material (rescue treatment):

(Note: The culture should not be left visibly contaminated longer than one week.)

(a) Clean the material mechanically using a soft tooth-brush under a stream of tap water. Shake / stir in a 50% PPM™ solution (diluted with sterile water) for 5 - 15 minutes. For bacterial or mixed contamination we recommend to lower the solution pH to the range of 2.8 - 3.2 by mixing 1:1 full strength PPM™ (100%) with 0.6 gr./liter Citric acid solution (use sterile water).

(b) Without rinsing insert into a medium with 0.05 - 0.2% PPM™ for at least one month. Keep the culture away from high light intensities for the first 10 days. As mentioned above, don't discard oxidized explants. Wait 4 - 6 weeks as approximately 50% should recover.

In some cases the fungal or the bacterial spores are located deep within the ex-plants beyond PPM's reach. In such cases, and after the water-soaking period, slice the ex-plants along and then stir/shake in 50% PPM for 5 -15 minutes.

THROUGH ALL THE ABOVE STERILIZATION PROCESSE(S), ENSURE THAT THE PPM PROFUSELY REACHES THE ENTIRE SURFACE OF THE EX-PLANT.

10. **To eliminate Agrobacterium:**

After co-cultivation, rinse the leaf discs with water. Dip (entirely) the transfected discs in a 100% PPM™ solution (supplemented with full strength basal salts) for approximately 2 minutes. Blot the discs between two sterile paper towels and place onto a medium supplemented with full-strength of the commonly used antibiotics. After 3 weeks, transfer to the medium with solely PPM at 0.05 0.075%

General Notes:

1. For the first transfer following the sterilization with PPM™, we recommend to insert the explants entirely into a semi-solid medium.

2. The 50% PPM™ solution can be reused approximately 10 times. The number of uses depends on the volume of the explants treated and the inoculum density. Keeping the 50% PPM™ solution stored at 4ºC prolongs its activity. If necessary, prepare two PPM™ solutions: one to disinfect endogenous contamination and the second, to disinfect "in-culture" contamination. The second solution should be filtered after each treatment, using 0.2 micrometer Millipore. The filtration process can be done in non-sterile atmosphere. A single filter can be used for the entire "lifespan" of the solution.

3. In cases where the treatment with 50% PPM™ is still insufficient, full strength PPM™ (100%) can be used. The treatment with 100% PPM™ is similar to the one described above for 50% PPM™, however, the exposure time should not exceed 10 minutes.

CONCLUSION

PPM™ most definitely will facilitate the work in any plant tissue laboratory and should significantly increase technician and laboratory productivity. However, conditions in each lab may vary which could have a bearing on the effectiveness of PPM™. It is advisable that staff follow the above guidelines and

thus find out for themselves how much "freedom" they achieve by using PPM™ and whether or not PPM™ works for their particular application. Test results are available.

When used as recommended, the test results prove that:

- PPM™ is effective against airborne contamination, waterborne contamination and contamination introduced from human contact.
- If used correctly, PPM™ will cure explants from endogenous contamination.
- At recommended doses (0.5 - 2ml/l), PPM™ does not impair in vitro seed germination, callus proliferation, callus regeneration, and axillary or adventitious buds' induction.

PATENT NO. 5,750,402 -- The formulation of PPM in tissue culture media at certain concentrations and the use of PPM in tissue culture at certain concentrations to prevent or eliminate microbial contamination is protected by US patent No. 5,750,402. Patents have been issued in Canada, New Zealand, Australia, the European Community, Israel and other countries. It is also patent pending in many other countries of the world.

SAFETY PROCEDURES

Safety Issues: PPM™ is non-toxic, however, inhalation and contact with skin and eyes should be avoided since PPM™ is an acid. PPM™ is non-toxic. It can be an irritant to skin, eyes, nose and throat. Precautions: We recommend that users wear gloves and splash goggles. Avoid contact with skin and eyes. Avoid inhalation. Use proper/adequate ventilation. The material should not be spray applied except with directed flow, positive pressure ventilation and protective equipment.

First aid measures: Ingestion If swallowed, give 2 glasses of water to drink. IMMEDIATELY see a physician. Never give anything by mouth to an unconscious person. Eye and Skin Contact IMMEDIATELY flush eyes with large amounts of water for at least 15 minutes. Wash affected skin areas thoroughly with soap and water. Remove and wash contaminated clothing thoroughly. Inhalation Move subject to fresh air.