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Efficacy and Utilization of Fungicides and Other Antibiotics for Aseptic Plant Cultures

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1. Introduction

1.1 *In vitro* culture

In vitro plant culture which encompasses cell, tissue, organ and also embryo culture has been a vital technique for mass multiplication of plants (propagules), elimination of plant diseases thorough meristematic tissue culture technique, plant conservation (through cryopreservation) and crop improvement through gene transfer (Pierik, 1987; George, 1993; Singh & Chand, 2003; Sarasan et al., 2011). *In vitro* culture techniques have been used to shorten breeding cycles of plants and to achieve genetic transformation (Singh & Chand, 2003). Despite the several merits of *in vitro* culture techniques to the modern world where plant diseases, flower/fruit abortions and low plant regeneration capacity are major challenges, many developing countries, especially in Africa do not utilize them to address the numerous challenges in crop production and improvements. This is mainly due to the high investment costs in equipment, chemicals and intensive and skilled labor demands (Sarasan et al., 2011).

The high costs for equipment, water, chemicals (such as plant growth hormones or regulators, surface sterilants, disinfectants etc.) and culture losses due to *in vitro* culture contamination among many others can cripple several *in vitro* plant culture techniques in many developing countries, especially in Africa. Possibly, this is the main reason we have limited or no interest to invest in tissue culture laboratories in Africa, except for the academic institutions for the sole purpose of learning the technique.

1.2 *In vitro* contaminants

The most common challenge for *in vitro* plant culture protocols in the tropics, especially stock plants derived from mature ortet has been elimination of culture contaminants. These culture contaminants have been problematic to the effect that there are instances where

propagation and mass multiplication of useful plants have failed. To a great extent, asepsis has always been a key factor towards successful *in vitro* plant culturing and mass multiplication. However, there have been limited research studies undertaken to document the success or failure of asepsis in many *in vitro* cultures of tropical plants.

There are several types of *in vitro* culture contaminants. They include various types of fungi, bacteria, viruses and other micro-organisms. Among these *in vitro* contaminants, fungi have been the most common and conspicuous microbes that cause many *in vitro* plant culture contaminations. In this chapter, we mainly outline a few common fungicides and other antibiotics (such as sterilants and/or disinfectants) and procedures employed to eliminate or reduce fungal *in vitro* culture contaminations to a manageable level as proliferation of fungi can cripple the whole plant culture process. We further highlight some common surface sterilants and disinfectants frequently utilized in plant culture laboratories to reduce or destroy fungal contaminants. In this respect, disinfectants and surface sterilants are chemicals used in plant culture laboratories to control or kill pathogens which may not necessarily be fungi. These surface sterilants or disinfectants may not be purely fungicides although they act against fungi. This chapter also presents the efficacy, handling, storage and the possible effects of fungicides on plant cultures and the roles of these fungicides in stock plant preconditioning to improve the efficacy of fungicides or other disinfectants. However, details of other forms of *in vitro* contaminants (such as bacteria, viruses and other micro-organisms) are outside the scope of this chapter.

2. *In vitro* culture pathogens

There are several pathogens (microbial contaminants) which have been a major threat to *in vitro* cultures due to their rapid proliferation characteristics (Enjalric et al., 1998). Axenic cultures are often mandatory throughout the entire *in vitro* culture process. The contaminated cultures are usually discarded at any stage of sub-culturing because many *in vitro* culture contaminants eventually cause plant death by exuding their phytotoxins (George, 1993).

Many fungal contaminants are visible right from the primary *in vitro* plant culture initiation except for the cryptic contaminants. Generally, these contaminants overgrow plant cultures and often outcompete them for resources in the nutrient media (Pierik, 1987). Consequently, *in vitro* contaminants often adversely interfere with culture growth and survival. Therefore, freedom of pathogens (asepsis) in plant cultures has been the ultimate goal in many *in vitro* protocols.

2.1 Fungal contaminants

Fungal contaminants have posed a major threat at every stage of the *in vitro* plant culture process. These fungal contaminants are characterized by the presence of hyphae (thread-like) appearing on the explants and culture media. Colonization of fungi progresses with time and the plant cultures are eventually covered in fungal mycelia. Unlike other contaminants, fungal contaminants are visible in the culture media. Furthermore, the development and growth of fungal contaminants are faster than the growth of the plant cultures. However, the rapid proliferation of fungi in the plant cultures depends on the type of fungi colonizing the culture media.

The fungi colonizing the plants could be endogenous, cryptic or endophytic. Herman (1990) reported that endogenous or endophytic microbes are often difficult to decontaminate. It is known that many endophytes are beneficial for the growth of ortet plants in the wild, but they also become contaminants in culture media. Helander et al. (1996) reported that mutualism between endophytes and the host plant depends on the prevailing plant condition. Endophytes can be harmful to plant cultures although they are useful outside the plant culture laboratories. Examples of some trees that live and thrive with symbiotic microbes (mycorrhizae) include *Uapaca kirkiana* (Mwamba, 1995), but this mutualistic association also breaks once the host plant is stressed.

In the tropics, fungal contaminants are very common due to the prevailing favorable environmental conditions (especially the high temperatures and humidity) for the proliferation of fungal pathogens on the ortet plants. Furthermore, the conditions in the plant culture incubation chambers are favorable for the rapid growth of fungi and other *in vitro* contaminants. For instance, the range of temperatures (usually between 23 – 26 ^{o}C), high humidity and plant nutrients (available inside the culture vials or test-tubes) cannot deter or inhibit the proliferation of many fungi in the tubes placed in the culture incubation chambers. Moreover, some plants live and survive by mutual association with fungi in the wild. However, such fungi are likely to become '*vitropathogenic*' in the plant cultures.

2.1.1 Exogenous fungal contaminants

Generally, exogenous fungal pathogens are easy to eliminate from the stock plants using many fungicides. This is because such pathogens are found on the outer surface of the stock plants, and hence adequate contact between the fungicides and the pathogens and even the spores. However, insufficient surface disinfection might not dislodge exogenous pathogens and spores from the stock plants, and hence endogenous contaminants still pose a threat in plant culture protocols.

It is always difficult to detect the presence of either exogenous, endogenous or both pathogens on the stock plants. So it might be advisable to use systemic fungicides (e.g. Benomyl) before other disinfectants such as sodium hypochlorite are used. It is also a good practice to ensure that all possible culture contaminants are dealt with at the initial decontamination stage instead of re-disinfecting the explants at later stages (sub-culturing stages). Re-decontamination of explants might weaken the ensuing explants due to cell injury. This can easily cause death of explants.

2.1.2 Endogenous fungal contaminants

Symbiotic microbes which are beneficial to many plants may become contaminants in the culture media (Herman, 1990). It has been reported that endogenous or endophytic fungi become pathogenic to the host plants when the plants are stressed, for example, when the cell walls are weakened or under other unfavorable *in vitro* conditions (Darworth & Callan, 1996). The endogenous or endophytic microbes are often hard to decontaminate. According to Cassells (1991), culture asepsis is important in all plant culture protocols. However, many trees in the tropics live and survive in association with endogenous or cryptic microbes (fungi) in the wild (Darworth & Callan, 1996). It remains unclear whether this association may continue to be mutual or lethal to the growth of plant cultures. This is because some fungi may not be '*vitropathogenic*', despite being prolific in the cultures (Herman, 1990).

Fungal contaminants increase plant culture mortality as they compete for the same resources (nutrients and oxygen) in the culture media or produce phytotoxins to harm the plant cultures. Obuekwe & Osagie (1989) reported that fungi such as *Aspergillus niger* and *Aspergillus flavus* produce oxalate and aflatoxin poisons respectively that can cause death to plant cultures. To overcome the fungal contamination problem, commercial fungicides have been used to control *in vitro* fungal contaminations (Table 1).

Common chemicals	Type and some functions
Benomyl and Captain	fungicides - act as multiplication inhibitors
Formaldehyde (aldehyde)	fungicide - wide microbial activity (kills spores)
Sodium & calcium hypochlorite	oxidizing agents – kills wide range of pathogens
Teepol (detergent)	sterilant
Mercuric chloride	increases culture mortality
Copper oxychloride	both a fungicide and sterilant
Nystatin	antibiotic toxic to fungi and yeast

Table 1. Common fungicides and disinfectants used in plant culture laboratories

Establishing complete axenic cultures has not always been easy since some potential culture contaminants, especially endophytes mutually co-exist with plants. It is accepted that the success of achieving culture asepsis depends on the nature of stock plants used. This is because mature stock plants are highly loaded with fungal pathogens and spores, but mature stock plants are selected due to their fruiting precocity, especially for fruit trees. To achieve plant culture asepsis for such mature stock plants, there is need for pre-conditioning of ortet plants before collection of stock plants.

2.1.3 Antibiotics

Although the main focus of this chapter is on fungicides, antibiotics also play a vital role in achieving aseptic *in vitro* cultures. It has been observed that the use of fungicides alone can lead to proliferation of other *in vitro* contaminants such as yeast, bacteria, protozoa and other microbes (www.phytotechlab.com) hence broad-spectrum antibiotics are used in plant culture to control many *in vitro* contaminants. Other disinfectants and surface sterilants may not be necessarily fungicides but broad-spectrum antibiotics which are often used.

3. Fungicides for stock plant pre-conditioning

Fungicides are often used to reduce the pathogen loads from the stock plants to achieve culture asepsis in the culture laboratories. However, attention must be paid to the source of stock plants as many fungal contaminants are derived from the ortet plants. A few are then introduced during the culture initiation or at later stages of *in vitro* plant culturing. Leifert (1990) reported that sub-culturing processes are potential sources of contaminants and it is estimated that 5 - 15% of contaminants are introduced per sub-culture. This possibly suggests that a large number of fungal contaminants originate from the plants themselves. Other possible sources of fungal culture contamination have been due to poor handling of plant cultures during excision, initiation and sub-culturing and insufficient test tube sterilization and poor media preparation. These potential sources of fungal culture contaminations can be avoided with proper care when handling plant cultures.

Stock plants, especially from the wild are often difficult to decontaminate. For instance, *Uapaca kirkiana*, one of the wild trees of southern Africa, has been a difficult plant to eliminate *in vitro* fungal contaminants, especially when derived from mature ortet plants (Mng'omba et al., 2007). Success in culture asepsis of *U. kirkiana* has been achieved using seedling stock plants and 3.5% sodium hypochlorite (NaOCl) as a surface disinfectant (Maliro, 1997). However, they did not achieve any success in decontaminating adult *U. kirkiana* plant materials due to high fungal contamination at the initial stage.

Plants that live in association with endogenous fungi require preconditioning before collection. Application of Benomyl (Benlate), a systemic fungicide has been found effective on ortet plants before stock plant collection for *in vitro* culture. For instance, this method has been an effective way in reducing fungal load on *U. kirkiana* (Mng'omba et al., 2007). However, this process was time consuming as it involved isolation of grafted plants and placing in a screen or greenhouse where Benlate solution was regularly applied to eliminate fungi and the spores. The stock plants collected from such isolated plants are often free from heavy fungal infestation. It is advisable to avoid watering the whole ortet plants, but watering should be done to the polyethylene bags through drip irrigation or a hose pipe as most fungal pathogens and spores proliferate on wet plant surfaces.

To increase the chances of eradicating fungal pathogens and spores on the mother plants, pruning might be necessary to induce proliferation of new and rejuvenated lateral shoots which may not be highly loaded with fungal pathogens, and hence ease decontamination unlike the old plant shoots. Furthermore, the prevailing weather conditions during collection of stock plants play a vital role. Collecting stock plants from the forests or open field on a rainy day increases fungal load on the stock plants, and hence makes it difficult to eliminate the pathogens as this would require a rigorous decontamination process. It is, therefore, clear that fungicides and other disinfectants do play a vital role in the entire *in vitro* plant culture.

4. Surface sterilants and disinfectants

Surface sterilants can be described as chemicals rendering plants free from any pathogens including fungal spores. Generally, surface sterilants act on the outside of the explants. There are several chemicals which have been used to free plants from pathogens before culture initiation onto the media (chemotherapy). They deter growth and proliferation of pathogens (fungi, bacteria and other types of microbes). In many cases, a combination of several surface sterilants, disinfectants and/or fungicides is used to improve the efficiency of subsequent fungicides in decontaminating stock plants. For instance, a few drops of Teepol (0.05%) are often applied to the water and this increases wettability of the plant surfaces. Also, penetration of fungicides into the outer plant cells might be enhanced by rinsing the stock plants in the water. Generally, Teepol (soapy water or detergent) has been one of those disinfectants or surface sterilants commonly used in many plant culture laboratories to enhance the removal of pathogens and/or fungal spores from the stock plants.

Another common disinfectant in plant culture laboratories has been ethanol (an alcohol derivative). Ethanol has been one of the commonest disinfectants used in plant tissue culture to eliminate pathogens and spores. It is used from the preparation room up to the laminar

air floor to kill pathogens and spores. This alcohol derivative has often been used to reduce culture contaminants as it kills fungal pathogens and spores on the stock plants.

The concentrations of sterilants and disinfectants widely used vary depending upon the nature of stock plants (soft vs. lignified plant surfaces) to be sterilized. A wide range of ethanol concentrations (20 – 100%) has been used, but high concentrations of ethanol (100% concentration) are rarely used, especially on soft stock plants because they can easily damage or injure the plant tissues. Generally, many epithytic contaminants (fungi) are eliminated, but not endophytes, and hence high concentrations of disinfectants are required to destroy these endophytic culture contaminants. Apart from the concentration of the disinfectants, exposure time must also be considered. Generally, the exposure time should be short (5 - 20 seconds), especially with the tender stock plants which are highly infested. Low concentrations of ethanol (≤50%) have often been used for tender and soft stock plants to avoid injuring the plant cells or tissues.

4.1 Improving decontamination process

For adequate plant sterilization (decontamination), stock plants are stirred in a beaker to ensure sufficient surface contact between stock plant and the fungicide. In this case, the exposure time, disinfectant concentration, and active ingredient (a.i.) are important factors determining the efficacy of the entire process of *in vitro* decontamination of stock plants. Rinsing the stock plants in the water assists in stopping the reaction between the chemicals (fungicide) and stock plants. The disinfection by fungicides and other surface sterilants in a sealed beaker (with aluminum foils) is followed by rinsing stock plants under running tap water for some time to remove the contaminants and also stop any reaction between the stock plants and the disinfectants.

5. Selection and efficacy of fungicides

Some fungicides commonly used to control plant culture contaminations during the *in vitro* culture include Benomyl, Captain and several others (Table 1). Utilization of these fungicides and other sterilants and disinfectants largely depends on their availability and the costs, especially in some Africa countries where these fungicides, sterilants and/or disinfectants are imported. Generally, they are not locally available in many African countries for use in plant culture laboratories.

The fungicides and disinfectants can be in the form of powder or liquid. Generally, the quantity of fungicides normally used per decontamination is very little. For instance, a low amount of Benomyl such as 0.14g per litre of water could be used to decontaminate some stock plants. Fungicides and disinfectants are harmful to human beings when swallowed or inhaled. Therefore, proper handling of all fungicides must be a priority.

5.1 Selecting fungicides

In many plant culture laboratories, systematic fungicides (such as Benomyl) are preferred in case of endogenous fungi. Selection of a particular fungicide depends on the nature of fungal contaminants as some are hard to eliminate (e.g. endogenous fungi). The use of systemic fungicides (such as Benomyl) has often been recommended. In some cases,

stronger disinfectants or fungicides have been used to remove culture contaminants, especially those which are hard to decontaminate. For instance, mercuric chloride ($HgCl_2$) has been effective in decontaminating pre-conditioned mature *U. kirkiana* stock plants (Mng'omba et al., 2007) where sodium hypochlorite (NaOCl) and Calcium hypochlorite ($Ca(OCl_2)_2$) have not been effective in decontaminating the above stock plants. There have been no aseptic cultures obtained regardless of the type and concentration of such disinfectants used on *U. kirkiana* stock plants. These fungi were suspected to be endogenous.

5.2 Efficacy of fungicides

Efficacy of fungicides depends on many factors including their active ingredient (a.i.), concentration (dosage), the type of stock plants (mature vs. new shoots), type of fungi (exogenous vs. endogenous), and the exposure time. Generally, rigorous decontamination is required for stock plants derived from mature ortet, especially when weaker disinfectants are used. This will also require longer exposure time. Generally, the use of concentrated (stronger) fungicides or disinfectants to eliminate contaminants from the stock plants requires a shorter exposure time.

Mercuric chloride as a culture disinfectant is stronger than sodium or calcium hypochlorite solutions for disinfecting stock plants and this could be the reason for its efficacy in decontaminating endogenous fungal contaminants. Danso et al. (2011) reported successful *in vitro* decontamination of sugarcane explants with mercuric chloride where NaOCl and $Ca(OCl_2)_2$ were less effective. Therefore, $HgCl_2$ is ideal for the control of endophytic fungal culture contaminants.

6. Effects of fungicides on plant cultures

6.1 Cell integrity

The use of stronger disinfectants and fungicides could weaken cell membrane and cell wall of the plants. This can lead to the discharge of cell sap (nutrients) which stimulates an outgrowth of endogenous fungi once placed onto the culture media. These endogenous fungi might become pathogenic to the explants under *in vitro* conditions (Darworth & Callan, 1996). For example, $HgCl_2$ might be strong to some plants and hence can easily damage their cells, especially after a long exposure (Mng'omba et al., 2007). Danso et al. (2011) reported a low survival of sugarcane plantlets when decontaminated with $HgCl_2$. Therefore, the application of fungicides should consider the right concentration (dosage) and exposure time in order to reduce injury to the cells of the explants.

The mortality of plant cultures could be high due to cell damage by the use of concentrated (strong) fungicides or disinfectants. Where possible, it is important to maintain cell integrity of the stock plants to avoid any undesirable effects on the plant cultures. However, this may be difficult with heavily fungal loaded stock plants. For instance, the use of disinfectants or surface sterilants such as NaOCl, $Ca(OCl_2)_2$ and many others for a heavy fungal loaded stock plants could result in the resurgence of endogenous contaminants at any stage after the culture initiation. Therefore, the utilization of strong disinfectants or surface sterilants is warranted for the highly fungal loaded stock plants such as those derived from the old plants.

6.2 Preconditioning mother plants

Application of fungicides to the mother plants in the field before collection of explants (preconditioning of the ortet plants) greatly contributes to aseptic cultures. This could reduce the need to use stronger disinfectants such as $HgCl_2$. Furthermore, it may also improve decontamination efficacy. For instance, the use of $HgCl_2$ alone was less effective on field collected *U. kirkiana* (Mng'omba et al., 2007). Thus, preconditioning of grafted *U. kirkiana* stock plants using Benomyl contributed to achieving culture asepsis. Therefore, preconditioning reduces the fungal loads on the stock plants.

6.3 Fungicide toxicity

The problem with the use of concentrated disinfectants and fungicides is that there is always a lengthy washing of stock plants in running tap water and distilled water to remove pathogens and, especially excess residues that may be lethal to the explants in the culture media. Our experience has indicated a possibility that there are some reactions between $HgCl_2$ and aluminum foil. This suggests a possible affinity for chloride (Cl^-) in that Al^{3+} could be replacing Hg^{2+}. We observed that the foil was completely eaten up as a result of this reaction. Since stock plants were covered with aluminum foil throughout the disinfection, rinsing and culture excision processes which also took time, this could mean accumulation of aluminum deposits in the cultures, and hence possible aluminum toxicity to plantlets.

There are several studies undertaken and illustrate the deleterious effect of heavy metals such as aluminum and mercury on plant growth in the fields. However, such studies have not been accomplished in plant culture laboratories. It is possible that the poor performance of some plant cultures or explants might be attributed to the presence of residues of the strong disinfectants and fungicides used during decontamination process and toxicity of the heavy metals. However, research studies are needed to confirm or establish whether the use of such disinfectants and heavy metals *in vitro* culture contributes to plant culture toxicity or mortality.

7. Storage and disposal of fungicides

7.1 Storage of fungicides

Fungicides, surface sterilants and disinfectants are obviously harmful to human beings, and hence proper handing and storage are always good and important practices in plant tissue culture laboratories. Poor storage of fungicides/disinfectants could lead to their fast deterioration. This can make them less efficient in decontaminating plant cultures. Poor storage can also accelerate the expiry of the fungicides and disinfectants as they may be exposed to moisture. Long exposure to moisture due to the poor storage could alter the chemical composition of the fungicides or disinfectants, and hence making them less efficient. Proper storage of fungicides should include observing its ideal storage temperature and good containers to avoid any possible damage or leakage.

7.2 Disposal of used fungicides

Proper disposal of fungicides and other disinfectants must be adhered to. In many plant culture laboratories, disinfectants, surface sterilants and fungicides are decanted through

water drainage system after rinsing the stock plants. Disinfectants such as $HgCl_2$ are known to be corrosive, and hence can damage the water drainage system. Proper disposal of such disinfectants must be taken into consideration. For instance, it may be advisable to dig a pit where $HgCl_2$ solutions must be disposed off and buried. This is because heavy metals are also not friendly to the environment, and hence proper disposal is needed.

8. Future research areas on fungicides

The effects of most fungicides and many other disinfectants on different stock plants and explants remain unanswered, especially for the strong fungicides or disinfectants used in decontaminating stock plants in the plant culture laboratories. To our knowledge there has been limited research in this area. Therefore, there is the need for more research studies in this area. For instance, the effects of the fungicide and disinfectant residuals on the growth and survival of plantlets in *in vivo* have not been thoroughly researched. Our experience has shown that the use of $HgCl_2$ may require several rinsing of stock plants. This is to get rid of the residues and we hypothesize that the residues could have deleterious effects on explants and later on the plantlets.

9. Conclusion

This chapter has outlined the significant roles that fungicides and other disinfectants play in plant culture laboratories in achieving aseptic *in vitro* plant cultures. This is a vital process for the success of *in vitro* plant culture protocols. However, selection of an appropriate fungicide or disinfectant is important since endogenous or endophytic pathogens require the use of systemic and strong fungicides to effectively disinfect stock plants. Proper handling of fungicides and disinfectants and also proper disposal of the used disinfectants in the plant culture laboratory are equally important as they may become corrosive to the water drainage systems, and hence dangerous to the environment. Many research studies are needed in order to increase our understanding with respect to various disinfectants used in the plant culture laboratories.

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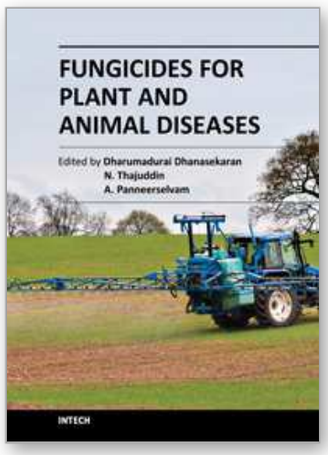

Fungicides for Plant and Animal Diseases

Edited by Dr. Dharumadurai Dhanasekaran

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A fungicide is a chemical pesticide compound that kills or inhibits the growth of fungi. In agriculture, fungicide is used to control fungi that threaten to destroy or compromise crops. Fungicides for Plant and Animal Diseases is a book that has been written to present the most significant advances in disciplines related to fungicides. This book comprises of 14 chapters considering the application of fungicides in the control and management of fungal diseases, which will be very helpful to the undergraduate and postgraduate students, researchers, teachers of microbiology, biotechnology, agriculture and horticulture.

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