Overcoming the challenges of endogenous contamination in micropropagation of fruit and nut trees©

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Abstract

Micropropagation has become a successful technique for fruit and nut tree rootstocks. However, success of a laboratory to produce a high rate of healthy shoots during multiplication stage depends on its ability to maintain cultures free from contamination. Although, all labs can easily start a clean culture from an explant, many labs report a flare-up of bacterial contamination after a few cycles of multiplication despite using their best laboratory practices. This flare-up is often blamed on endogenous bacteria within the micro shoots. This assumes that such bacteria were always present in the shoots, but were in quiescent stage and/or were non-culturable and suddenly they became active and grew on culture media. Such theory is believable since presence of endogenous bacteria in plants is well-known in the literature and only 1% of bacteria are culturable. To overcome this challenge of flare-up of contamination (endogenous or introduced), a laboratory should have a protocol in place to index their stock materials on a regular basis. For culturable bacteria, contamination can be detected by culturing samples of tissue in a nutrient broth for bacteria. For non-culturable bacteria, sections of stems can be eluted in water and the eluate observed under the microscope for bacteria. Bacteria-specific PCR tests are now available and are helpful. These procedures along with shoot tip cultures, occasional use of antibiotic and close visual observation have proved successful at Micro Paradox in maintaining our nuclear stock of walnuts, pistachio, and peach × almond hybrids free from contamination.

INTRODUCTION

Micropropagation is becoming a popular technique for commercial production of fruit and nut tree rootstocks. At present seven laboratories in California alone are producing rootstocks of walnuts, pistachio, and peach × almond hybrids. One of the challenge that laboratories face is to keep the cultures free of contamination. Almost every laboratory has a horror story to tell that they could not produce enough quantity of certain rootstocks due to flare up of bacterial contamination in their cultures. In some cases, contamination destroys all cultures and the laboratory has to start over from new explants from mother plants.

All laboratories are familiar with surface sterilization procedures and are able to establish cultures free of bacterial and fungal contamination. The mother plant is often indexed for common viruses and it is assumed that viruses and other fastidious organisms are absent in the mother plant and that one has to only worry about bacterial and fungal organisms. Fungi usually grow on tissue culture media and contaminated cultures can be easily discarded. Some of the culturable bacteria can also be easily seen on the tissue culture media and infected cultures are discarded. A challenge emerges when cultures start declining after a few generations of vigorous growth in the absence of any visible contamination. Often, it is blamed on endogenous bacterial contamination. It is assumed that some bacteria were always present in the cultures, but they were in a quiescent stage and suddenly they become actively growing in culture vessels and are responsible for the decline of cultures (Figure 1). This theory is credible as the presence of endogenous bacteria in plant

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tissue is well supported in the literature and the fact that only 1% of the bacteria are normally culturable. For commercial labs, this theory is easier to believe because they follow all good lab practices, believe that contamination is not introduced from environment and that it must be endogenous. However, a proof of introduced contamination is lacking. One should keep in mind that it is common to have 2-5% visible fungal contamination in growth rooms and that invisible bacterial introduction is highly possible.

Figure 1. Decline in pistachio plants in vitro due to endogenous bacterial contamination.

For this article, we have assumed that contamination could originate from endogenous bacteria in the stock or from introduction of non-culturable bacteria during handling and growing of plants in the lab. We have focused on detection of such contamination and management practices to keep bacteria away from tissue culture stock. We have successfully incorporated some of these techniques in our commercial program of walnut and pistachio rootstock production at Micro Paradox laboratory.

DETECTION TECHNIQUES

The following techniques are available. Each has some advantages and disadvantages.

Visual observation

This technique is the gold standard and is usually practiced in all tissue culture labs. The cultures are closely observed for visible contamination and any contaminated plants/ entire containers are discarded. A flash light can be helpful to see infections that are otherwise hard to see with the naked eye. A major limitation of this visual method is that non-culturable endogenous bacteria will go undetected.

Culture indexing

At Micro Paradox, this technique is routinely used. Samples of cultures are submitted to our in-house laboratory for bacterial counts. In this test, plants are crushed in sterile water to extract bacteria from the tissue and the extract plated onto general purpose bacterial media (KB, PDA, 523, etc). Recovery of bacteria from samples indicates infection by culturable bacteria. This test is very simple and effective. Multiple media should be used because each medium is selective for certain bacteria. Unculturable bacteria are not detected by this technique.

Bright field/phase contrast microscopy

In this method, stem tissue is sharply cut in a drop of water using a scalpel. Bacteria will ooze out from the tissue into the water within a few seconds. A slide (wet mount) is prepared and examined at 400X using a transmission microscope by a trained lab technician. At these magnifications several bacteria that vary in size from 1-3 micrometers can be seen as small particles. Many bacteria fall in this size and include *Pseudomonas*, *Xanthomonas*,

Erwinia, *Clavibacter*, *Bacillus*, and many others. Some highly motile bacteria are easily seen. If bacteria are suspected, then Gram staining can be done and examined further at 1000X magnifications. Microscopy works well if there is a large amount of bacteria in the tissue and enough will ooze out. Phase contrast microscopy is more effective (makes bacteria darker) than bright field microscopy.

Electron microscopy

Samples are submitted to a special facility equipped with an electron microscope. A specialist is needed to prepare and observe samples. Bacteria, phytoplasma, and viruses can be easily observed. The size and shape of the organisms also provides some clues on general identification of organisms. Species identification is not possible as several organisms have a similar shape. This technique is extremely helpful and is often used to identify unknown diseases in field samples. However, there are few facilities equipped with an electron microscope and testing is expensive for routine use.

Polymerase chain reaction (PCR)

Recently polymerase chain reaction (PCR) tests have become available for several organisms. Universal primers that detect all bacteria are also available and amplify 1500 nucleotide base pairs. The samples of tissue culture material are submitted to a PCR lab. At Micro Paradox, we routinely submit samples to our in-house lab (CSP Labs) for universal PCR. Unfortunately, primers available today also react with chloroplast and mitochondria of some plants and result in a false PCR product. Therefore, if a PCR product is obtained from a sample, it must be sequenced. The sequence is then compared with GenBank database to confirm if it matches to a bacterium. A complete match of 1500 base pairs to a bacterium is highly reliable. A trained molecular biologist is required to conclude results of sequencing and to rule out any false results.

Next generation sequencing (future)

In this test, all DNA and RNA sequences in a sample are obtained and analyzed. The sequences that do not belong to plants are further analyzed if they match to microorganisms (bacteria, viruses, etc). By this technique, new pathogens in plants have been discovered. A classic example is discovery of red blotch virus in grapevines. At this time, only a few labs are capable of conducting this test and the test is very expensive. It is not routinely used today, but has great promise to detect and identify endogenous bacteria in the future.

MANAGEMENT OF ENDOGENOUS CONTAMINATION

There are several things that can be done to overcome issues of "so called" endogenous contamination in a commercial tissue culture lab.

Culture establishment

This is the most significant step to detect and exclude endogenous contamination. New cultures should be closely watched for contamination. Any cultures where bacterial streaking into the media from callus can be seen must be discarded. One must not attempt to save upper nodes and tips to save the cultures. Callus is most likely to have more concentration of bacteria if there is an infection. Callus samples can be tested by culture indexing. If cultures stay clean during a few transfers, they are likely to be free from any culturable bacteria. If vigor is high during these few transfers, they are likely to be free from non-culturable harmful bacteria. If cultures start declining after several generations, one should look at reasons other than endogenous bacteria arising from initial starting material.

Air flow in the tissue culture facility

Tissue culture laboratory design is important to keep contamination away from entering from outside. Clean areas (transfer hood area, media pouring area, and growth rooms) should be under positive pressure of HEPA-filtered air. Other rooms (worker entry, autoclave room, receiving area and any R&D areas should be under negative air pressure).

Air flow should be gentle that it does not create turbulence.

Good lab practices

Good lab practices are a part of continuous training of workers. Every lab has their own way to implement these practices and they work well if technicians and managers believe in the practices. These include dress code, hand sanitation, work surface sanitation, floor cleaning, and trash removal. Only authorized people should be allowed in clean work areas. Check lists can be used to audit compliance to good lab practices. Good lab practices will reduce workers introducing bacteria especially non-culturable bacteria that can go unnoticed.

On-site management

Many managers record a lot of data to associate general contamination to specific workers. Such contamination analysis requires a few weeks of observation and data analysis before pointing out to the technician for corrections. A proactive approach is to have strong, on-site management. A crew leader should closely supervise laminar flow hood workers and provide on-site corrections and certify technicians. More supervision is needed for the new workers during their first 2-3 weeks. At Micro Paradox, we have concluded that there is no significant relationship between a worker and degree of contamination if workers have been fully trained and supervised. Contamination should not be related to a worker if crew leader is happy with performance of the worker. If contamination rates remain higher in spite of good work by workers, a manager should look for reasons of such contamination other than workers.

Lab testing

Samples of tissue culture stock should be tested on a routine basis for contamination by methods such as culture index, microscopy and PCR. If contamination is detected, infected materials can be quarantined and not used in the multiplication program.

Nuclear program

Establishment of nuclear stock is very important. Establishment of new cultures from mother trees is considered nuclear stock. Since this stock is used for the multiplication phase, it must stay free from contamination. For maintenance, nuclear stock goes through unlimited generations until new materials are again introduced from the mother tree. Therefore, the chance of contamination from endogenous bacteria or introduced bacteria are most likely to be seen in the nuclear stock. For this reason, nuclear stock should be clearly marked, handled by most experienced technicians and frequently tested. It is also good idea to treat nuclear stock with an antibiotic on an annual basis as a preventive measure for contamination control. Finally, use of tips of cuttings to maintain nuclear stock avoids any contamination that could have been introduced recently and not have yet travelled to the tip of the cutting. By use of these practices, Micro Paradox has not seen any contamination for the last 5 years in its nuclear stock.

One-way multiplication system

Micropropagation when compared to conventional propagation provides a one-way system where propagation always starts from clean nuclear stock. Any infections are flushed out as materials are released from the laboratory. However, in practice, cuttings from the multiplication stage may be further multiplied for a few cycles. This can result in the spread of endogenous bacteria in the entire stock in the multiplication phase. To prevent this spread of endogenous bacteria, each generation in the multiplication should be tracked as it is moving to the next multiplication generation. For example, the first generation (M1) should be labeled as second generation (M2) upon transfer. By this method, any contamination will have limited distribution and not spread to the entire stock.

Removal of contaminated containers from the growth rooms

Some contamination (2-5%), usually fungal in nature, develops in growth rooms in spite of best practices followed. This contamination can increase significantly within a growth room if contaminated containers are not discarded quickly. Any contaminated containers should be carefully removed from the growth room, bagged and discarded as soon as possible. A common mistake done in the labs is to rescue uncontaminated plants in a container to another container. This involves opening a container in a laminar flow hood that increases chances for the contaminant to be airborne and contaminate other containers.

CONCLUSION

Controlling endogenous or introduced contamination can be a real challenge in a tissue culture laboratory. Above described detection and management techniques can be very helpful in overcoming this challenge. These techniques have worked well to eliminate or quarantine contamination at Micro Paradox.