Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature

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Preventing or avoiding microbial contamination of plant tissue cultures is critical to successful micropropagation. Epiphytic and endophytic organisms can cause severe losses to micropropagated plants at each stage of growth (Cassells, 1991; Debergh and Vanderschaeghe, 1988; Leifert *et al.*, 1991). Bacterial contaminants are often difficult to detect because they remain mostly within the plant tissue (Debergh and Vanderschaeghe, 1988; De Fossard and De Fossard, 1988; Viss *et al.*, 1991). Contaminated plants may have no visible symptoms, reduced multiplication and rooting rates, or may die (Leifert *et al.*, 1989; 1992). Introduction of microorganisms due to poor aseptic technique or improperly sterilized equipment can be corrected with improvements in training or equipment handling, but eliminating internal contaminants is more problematic (Buckley *et al.*, 1995).

Procedures for producing aseptic cultures require attention to some or all of the following: 1) indexing explants and cultures for contaminants; 2) identifying the source of those contaminants; 3) identifying or characterizing the contaminants; and 4) eliminating the contaminating organism with improved cultural practices, antibiotics, or other chemical agents.

Sources and prevention of contaminants

The sources of contaminated cultures usually are difficult to determine (Leifert and Waites, 1994). Bacteria which contaminate plant cultures may originate from explants, laboratory environments, operators, mites and thrips, or ineffective sterilization techniques. Bacteria are associated with plants as epiphytes or endophytes (Sigee, 1993; Gunson and Spencer-Phillips, 1994). Explants from field-grown plants, diseased specimens, or from plant parts which are located close to or below the soil may be difficult or impossible to disinfect due to both endophytic and epiphytic microbes (Leifert *et al.*, 1994). Contaminants of greenhouse-grown plants are mostly those associated with soil (Buckley *et al.*, 1995) and may originate from irrigation water (Seabrook and Farrell, 1993).

Epiphytic bacteria may lodge in plant structures where disinfectants can not reach (Gunson and Spencer-Phillips, 1994; Leifert *et al*, 1994). Endophytic bacteria may be localized within the plant at cell junctions and the intercellular spaces of cortical parenchyma (Gunson and Spencer-Phillips, 1994). Contaminants found at explant initiation, present in explants from several collection dates and resistant to surface disinfestation are likely to be endophytic (Reed, *et al.*, 1995).

Every step of the plant tissue culture process should be considered in order to prevent contamination. These steps encompass handling of stock plants, type and handling of explants, media preparation, subculturing, incubation, and storage of sterile culture

vessels, media, and plant cultures. Leifert and Waites (1994) suggested that stock plants used for plant tissue cultures be grown under protected conditions (greenhouses, growth chambers) to decrease the populations of epiphytic organisms. Seabrook and Farrell (1993) showed that irrigating stock plants with filtered water, rather than the standard city water, reduced bacterial contamination. Old or diluted disinfectants may lose strength and should be discarded, with only fresh mixtures used for explant disinfestation (Leifert *et al.*, 1994). During subculturing of plant cultures, contaminants may be reduced by controlling the laboratory cleanliness and air source and by strict training of operators in aseptic technique (Leifert *et al.*, 1994).

Characterizing bacteria to determine the species provides important information about contamination sources, the amount of contamination from that source, and how to eliminate or prevent contaminants (Leifert *et al.*, 1989; 1991). Since laboratories are often contaminated with unique organisms, each should define methods to prevent or treat their specific contamination problems (Leifert *et al.*, 1991). Systems for analyzing procedures and processes may help in the design of systems to prevent or reduce microbial contamination (Leifert and Waites, 1994).

Indexing cultures

Detection of bacterial contaminants has traditionally been haphazard. Visual inspection of the medium at the base of the plant may provide evidence of some contaminants, but is not adequate for slow growing bacteria, endophytes, or those bacteria which do not grow on plant tissue culture media (Kane, 1995; Leifert *et al.*, 1989). Screening methods must be favorable to bacterial or fungal growth, and easily used and interpreted (Reed *et al.*, 1995). Screening procedures are available for identifying many contaminants (Debergh and Vanderschaeghe, 1988; Leifert *et al.*, 1992; Viss, *et al.*, 1991). Cultures free of cultivatable contaminants have been established as the result of screening procedures in both commercial and laboratory situations (Holland and Polacco, 1994; Kane, 1995; Reed *et al.*, 1995). Some bacteria, which are especially difficult to culture, require specialized media (George and Falkinham, 1986; Gunson and Spencer-Phillips, 1994), but most common contaminants can be detected with screening on two or three commercially available bacteriological media (Kane, 1995; Reed, *et al.*, 1995). A culture indexing system involving serial stem slices inoculated into liquid and agar-solidified yeast extract-glucose, Sabouraud-Glucose, and AC media and incubated for three weeks at 30°C detected most contaminants from more than 60 aquatic, marsh, and ornamental woody plant species. In most cases, a contaminant would grow on two of the three media (Kane, 1995). Initial growth of explants in a liquid culture system at pH 6.9 and later testing on 523 bacterial medium detected most contaminants from over 400 mint explants (Reed *et al.*, 1995). Contaminated cultures are sometimes rooted and transferred to the greenhouse instead of being discarded. This is a risky procedure, because contaminants which cause no visible harm to plant cultures may become pathogenic under greenhouse conditions (Kane, 1995).

Identification and characterization

Contaminants can be purified using standard bacteriological methods and characterization with biochemical tests such as Gram stain, motility, gelatinase, oxidase, and O/F (oxidation/fermentation) (Buckley *et al.*, 1995; Klement *et al.*, 1990). Bergey's Manual of Systematic Bacteriology contains descriptions of genera and species which are helpful

for identifying bacteria (Krieg and Holt, 1984). These traditional tests are labor-intensive and time consuming, but may be performed in any laboratory with common chemicals.

Identification techniques which provide results in 24–48 h are now available. The Biolog system detects carbon source utilization with the reduction of tetrazolium dye in response to cellular respiration. The results are compared with a response database of Gram negative and positive bacteria, yeasts and lactic acid bacteria (Bouzar *et al.*, 1995; Hildebrand *et al.*, 1993; Jones *et al.*, 1993). The API identification system is also a carbon source utilization test, but it relies on visual detection of the test bacterium (Leifert *et al.*, 1989; Verniere *et al.*, 1993). Fatty Acid Analysis Profiles (FAP) match fatty acid methyl esters with those of known organisms (Buckley *et al.*, 1995; Chase *et al.*, 1992; Stead *et al.*, 1992). DNA probes and 16S rRNA use PCR amplification and probes for known sequences (Klijn *et al.*, 1991). The reliability of these systems depends upon the number and diversity of bacteria in the databases. Many soil and plant bacteria have not been described or characterized, making these procedures less useful for plant biologists (Buckley *et al.*, 1995). Jones *et al.* (1993) and Verniere *et al.* (1993) suggest the use of more than one test for a more accurate identification.

Antibiotic treatments

Endophytic bacterial contamination is an important problem in plant tissue culture (Kneifel and Leonhardt, 1992) and can not be eliminated with any surface sterilization techniques, thus require antibiotic therapy (Mathias *et al.*, 1987). Ideal antibiotics should be soluble, stable, unaffected by pH, unaffected by media, lacking side effects, broadly active, bactericidal, suitable in combination, non-resistance inducing, inexpensive, and nontoxic to human health (Falkiner, 1990; 1988). Judicious use of antibiotics is especially important. An analysis of published research concludes that antibiotics are often incorporated as prophylactics in the tissue culture medium or are used to suppress or eliminate bacteria once a contaminant is detected (Leifert *et al.*, 1992). The continued use of antibiotics in the medium or repeated treatments with a single antibiotic may lead to bacterial resistance (Kneifel and Leonhardt, 1992; Leifert *et al.*, 1992). Care must be taken to insure that antibiotics are bactericidal rather than bacteriostatic, as is often the case, and that the cultures are monitored for recurrence of bacteria (Leifert *et al.*, 1992; Mathias *et al.*, 1987).

Many antibiotics exist that have not yet been evaluated on plants or their bacterial contaminants (Falkiner, 1990; Seckinger, 1995). Antibiotics are grouped by mode of action: inhibitors of bacterial cell wall synthesis, inhibitors of bacterial protein synthesis, and DNA replication blockers (Pollock *et al.*, 1983; Quesnel and Russell, 1983). Antibiotics can also be grouped by chemical structure: aminoglycosides, quinolones, B-lactams, glycopeptides, polymixins, macrolides, and lincosamides (Falkiner, 1990). The choice of antibiotic is dependent on the type of bacteria present (i.e. Gram negative or Gram positive), so initial characterization with Gram staining and some simple biochemical tests is essential (Buckley *et al.*, 1995). Carbenicillin, cephalothin, gentamicin, polymyxin, rifampicin, streptomycin, and Timentin have been used to treat plant tissue cultures (Buckley, *et al.*, 1995; Falkiner, 1988; Kneifel and Leonhardt, 1992).

Combinations of antibiotics may be advantageous where synergistic action occurs, but some are incompatible and may negate any positive effects of the individual drugs

(Falkiner, 1988; Kneifel and Leonhardt, 1992; Leifert *et al.*, 1991). Combinations of antibiotics at bactericidal concentrations are likely to be phytotoxic, but repeated use of single antibiotics may lead to bacterial resistance (Kneifel and Leonhardt, 1992; Leifert *et al.*, 1992). Barrett and Cassells (1994) found that antibiotic sensitivity is reduced in plant tissue culture media and emphasize the importance of determining the minimal bactericidal concentration (MBC) of antibiotics required for a particular bacterium. This can be accomplished with liquid culture of the bacteria with various concentrations of an antibiotic. If an effective concentration is found, then phytotoxicity to the plant material should be determined before treatment is attempted. Antibacterial effects of antibiotics can be enhanced at a more neutral pH (6.5–7.5) than is normal for plant culture media or nullified under acid conditions (Buckley *et al.*, 1995; Falkiner, 1988). Careful attention should be paid to environmental conditions because antibiotics may be inactivated by heat or light (Seckinger, 1995). Phytotoxicity varies greatly with plant type, so preliminary testing with plant cultures is important for successful treatment. Antibiotics effective on isolated organisms may be ineffective in treating contaminated plants, due to phytotoxicity or poor penetration into plant tissues (Reed *et al.*, 1995). Knowledge of the effect of antibiotics, on both bacteria and plants, is crucial for the elimination of contaminants and the recovery of healthy plants (Cornu and Michel, 1987).

Conclusions

Bacterial contamination remains a continuing threat to plant tissue culture, but techniques for reducing contamination are available. Laboratories must assess their situation, determine contamination sources, and change their laboratory operations to avoid or eliminate most of the contaminants. Several logical steps can be taken to greatly reduce bacterial contaminants in plant tissue cultures. Properly training operators in sterile technique and attending to the maintenance and use of autoclaves, laminar flow hoods and growth rooms are the first important steps toward avoiding environmental contaminants. Indexing cultures at the initiation stage and again throughout the culture cycle is a second step which can significantly reduce the number of explant–borne contaminants that escape detection. The third step is to identify persistent, possibly endophytic contaminants, and test them and the plant material with antibiotics to determine correct concentrations for effective treatment and minimal phytotoxicity.

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