AN ABSTRACT OF THE THESIS OF

Piyarak Tanprasert for the degree of Master of Science in Horticulture presented on May 13, 1996.

Title: Detection, Identification, and Antibiotic Treatment of Bacterial Contaminants from Micropropagated Strawberries

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Barbara M. Reed

Bacterial contamination is one of the most important problems both in plant tissue culture research and commercial laboratories. Early detection of contaminants saves time, effort, and money. Strawberry runner explants were screened for contaminants during June to August, 1994 and 1995. Most contaminants found were bacterial rather than fungal. Bacterial contaminants from 22 contaminated genotypes were isolated, purified, and identified to genus using standard biochemical tests such as Gram's stain, King's B medium, starch hydrolysis, and carbon source utilization (Biolog). From those 22 genotypes, 16 different bacterial strains were isolated. The majority were Gram-negative, motile, rod-shaped fluorescent pseudomonads. Xanthomonads, enterobacterium, and other unidentified Gram-negative and Gram-positive bacteria were also found. Minimal bactericidal concentrations (MBCs) of single (Timentin, streptomycin sulfate, dihydrostreptomycin,

and gentamicin) and combinations of two and three (Timentin, streptomycin sulfate, and gentamicin) were tested for inhibition of the bacterial isolates. Combinations of Timentin, streptomycin sulfate, and gentamicin killed all bacterial isolates tested. *Fragaria virginiana* subsp. *glauca* (S. Watson) Staudt and *F. x ananassa* Duch. Cv. Jucunda were inoculated with *Pseudomonas corrugata* or *Xanthomonas campestris* pv. *vesicatoria* then treated with a combination of Timentin, streptomycin sulfate, and gentamicin. *F. virginiana* subsp. *glauca* died after inoculation with either bacteria. Antibiotic treatments were 100% effective in eliminating *P. corrugata* from 'Jucunda', but only 23% of the plants inoculated with *X. campestris* pv. *vesicatoria* were freed of the bacteria.

Detection, Identification, and Antibiotic Treatments of Bacterial Contaminants from Micropropagated Strawberries

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Piyarak Tanprasert, Author

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I SAY LOVE CAN DO ANYTHING!"

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Dr. Barbara M. Reed participated the entire experimental designs, analyses, discussions, support, and critical editing.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF TABLES

LIST OF APPENDIX TABLES

Detection, Identification, and Antibiotic Treatments of Bacterial Contaminants from Micropropagated Strawberries

Chapter 1

Introduction and Literature Review

Microprogation of strawberry

Micropropagation of strawberry plants (*Fragaria* spp.) is widely used (Beech et al., 1988). The advantages of micropropagation are: 1) A large number of plants can be produced in a small space within a short time in any season. 2) Microprapagated plants produce more runners (Cameron and Hancock, 1986; Boxus, 1989). 3) Disease-free mother plants can be produced from meristem tip cultures (Boxus, 1989). 4) No mutations have been observed in field planting (Boxus, 1989). 5) Many different plant parts can be used such as meristem tips of runners (Adams, 1972; Eun et al., 1982; Broome and Zimmerman, 1984; Marcotrigiano et al., 1987; Beech et al, 1988; Simpson and Bell, 1989), buds of 1-2 mm in length taken from under scale leaves on stolons (Baker, 1980; Izsak and Izhar, 1983), anther (Eun et al., 1982), and axillary buds (Boxus, 1974).

Culture media. Many modifications of the media containing macronutrients, micronutrients, and vitamins have been used. Murashige and Skoog's (MS) (1962) medium without modification has

been used successfully by Beech et al. (1988). They cultured 'Cambridge Favourite' strawberry meristems excised from runners and found little "carry over" effect from the MS medium. Adams (1972) cultured strawberry meristems with medium containing inorganic salts and trace elements of Murashige and Skoog medium, supplemented with iron as NaFe EDTA at 40 mg/l, vitamin B mixture of Wetmore and Sorokin, and coconut milk. The success rates were 0-10% survival. Baker (1980) cultured 1-2 mm strawberry buds, taken from under scale leaves on stolons of healthy glasshouse grown plants. He found the best establishment and proliferation was with MS salts, 2% sucrose plus thiamine, pyridoxine, nicotinic acid, myo-inositol, adenine sulfate, and 1% agar. Eun et al. (1982) cultured the meristem tissues and anthers of strawberry using modified MS and Linsmaier and Skoog's (LS) (1965) media as a basal media to induce callus and differentiate organs. After five months of culture, 40-50 plantlets were produced. Simpson and Bell (1989) used MS mineral salts with 30 g/l sucrose, 0.1 mg/l inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 0.1 mg/l thiamine-HCl, and 7 g/l Oxoid purified agar at pH 5.6 to culture strawberry apical meristems excised from runner tips and to germinate seeds for synchronous seed germination. Izsak and Izhar (1983) used Boxus propagation medium for rapid propagation of individual strawberry seeds. They found germinating seeds proliferated rapidly and formed

tufts with many buds. Marcotrigiano et al. (1987) used Boxus medium but used 30 g/l sucrose rather than 40 g/l glucose to establish in vitro runner tips of 'Albo Marginata' plants. Only seven of 194 shoot tips grew into plants. Hunter et al. (1983) examined the influence of temperature and light intensity on micropropagation of strawberry plants (*Fragaria ananassa* Duch. cv. Cambridge Favourite). Adams medium was used to induce organogenesis and Boxus medium to induce rhizogenesis. The growth rates of the explants growth rates were influenced by temperature with maximum growth rates at 28 °C. The optimum light intensity for explant establishment and growth was at 4,000 lux; maximum propagule growth and development was at 6,000 lux; and best root initiation and development was at 7,000 lux. Damiano (1979) reported that potassium nitrate, between 2-14 mM, added to Boxus medium improved the growth rate of strawberry plants, cv. Aliso, ammonium at concentration over 3 mM depressed the growth rates of the plants. Boxus (1974) used medium containing undiluted Knop solution, the micro-elements of Murashige and Skoog, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 2 mg/l glycine, 0.1 g/l thiamine HCl, 100 mg/l meso-inositol, 40 g/l glucose, and 8 g/l agar at pH 5.6 to proliferate axillary buds of strawberry plants and to rejuvenate plants after culturing in media containing growth regulator for 1 to 1.5 year. Boxus (1976) used the macro-nutrients of Knop, micro-nutrients and vitamins of Murashige and

Skoog, 40 g/l glucose, and 8 g/l agar at pH 5.6-5.8 to proliferate virus-free strawberry cultures using meristem tips of strawberry plants.

Plant growth regulators. Plant growth regulators are important in micropropagation. To propagate plants, Adams (1972) used the mixture of 1 mg/l indolebutyric acid (IBA) and 0.1 mg/l benzylamino-purine (BAP) for strawberry meristem culture. They found two out of ten tips rooted and could be potted within five weeks, the remaining tips were green and healthy. Eun et al. (1982) grew meristems into plantlets by adding 0.2 mg/l kinetin to medium, but multiple shoots from a meristem were formed with 2 mg/l BAP or kinetin alone or 0.2 mg/l naphthalene acetic acid (NAA). Marcotrigiano et al. (1987) proliferated strawberry plants by using media containing 0.5 μmol IBA, 0.3 μmol gibberellic acid (GA_3) and benzyladenine sulfate (BA) at either 0.13, 4.4, or 13.2 μmol. They found slight increases in plant number in the absence of BA and good growth at 1.3 uM BA. Simpson and Bell (1989) stated that most strawberries will proliferate readily on growth medium containing 1.0 μM BAP, 0.3 μM GA_3, and 1.0 μM IBA, but their proliferation rate may vary two to three fold depending upon genotype.

Rooting. Boxus (1974; 1976) found that the development of strawberry axillary buds stops immediately after transfer to medium without cytokinin. Broome and Zimmerman (1984) also reported that strawberry roots can be easily formed only in the absence of cytokinins.

Baker (1980) stated that one-half strength MS medium without BA induced 405 rooted strawberry plantlets after one month. Izsak and Izhar (1982) induced roots by using Boxus rooting medium containing 1 ppm of β-IBA. In contrast to the above studies, Eun et al. (1982) induced roots from callus tissue or the base of shoots on media containing 0.2 mg/l 2,4-D and NAA plus 2-4 mg/l kinetin. The large shoots had roots after eight weeks of culture.

Acclimatization. Strawberry plants grown in vitro are anatomically different from pot-grown plants. They have poor cuticular wax formation, reduced number of stomata, smaller palisade cells, reduced trichome numbers, and poor root hair formation. These characteristics make the plants susceptible to desiccation when removed from culture, thus requiring careful acclimatization (Broome and Zimmerman, 1984). Boxus (1974) transplanted in vitro plants into a non-disinfected leaf-mould and maintained them for a few days under a plastic cover or under mist for acclimatization with high success. Mullin et al. (1974) transplanted plants into a mulch which had been partially sterilized by fumigation with chloropicrin; plants were placed under clear plastic wrap on the laboratory bench for 1-2 weeks. They gradually hardened off the plants and transferred them to a greenhouse. Boxus (1976) washed and placed the plantlets under plastic covers for 2-3 weeks to maintain a high relative humidity. Approximately one month

after transplanting, the plants could be planted in the field. Baker (1980) washed plants and planted them in plastic trays of peat : pumice (1 : 4) in a heated glasshouse; they were watered twice a day with Hoagland's nutrient solution, and the survival rates were 90-100%. Solid fertilizers gave poor results. After glasshouse establishment, plants could be transferred directly outside. Eun et al. (1982) transferred the hardened plantlets into pots with sterilized vermiculite. Broome and Zimmerman (1984) acclimatized plants by transplanting them to a porous artificial soil mix and grew them in a humid chamber at 65% relative humidity at 21-28 °C. After 1-2 weeks in the humid chamber the plants were successfully established in the greenhouse.

Bacterial contamination in micropropagated strawberry plants

Successful plant micropropagation requires the preventing, avoiding, and eliminating of microbial contaminants (Reed and Tanprasert, 1995). Microbial contaminants can cause major losses of plants in both research and commercial tissue culture laboratories (Cassells, 1991; Viss et al., 1991; Leifert and Waites, 1992). The best solution to prevent contamination is by using sanitary laboratory practices. These practices will exclude many potential contaminants (Reed and Tanprasert, 1995).

Sources of bacterial contamination. The sources of contaminants are often difficult to determine (Leifert and Waites, 1994). Bacteria which

contaminate plant cultures may originate from explants, operators, mites and thrips, or ineffective sterilization techniques.

Leifert et al. (1989; 1991) suggests that characterization of bacteria to species level is important possible methods for determining the source of contamination, and to eliminate and prevent microbial contamination. Leifert et al. (1991) found that different laboratories were contaminated with different organisms; therefore each laboratory should develop their own ways of preventing contamination.

Explants Bacteria are associated as epiphytes or endophytes of plants (Sigee, 1993; Gunson and Spencer-Phillips, 1994). Many of the bacteria isolated from plant surfaces are saprophytes, but populations of certain phytopathogenic bacteria may be present as epiphytes on apparently healthy plants (Hirano and Upper, 1983). Tissue taken from plant parts at or below the soil level would very likely harbor soil-borne bacteria (Leifert et al., 1994b). The surface disinfection of the explant prior to its placement in tissue culture medium may not rid the tissue of microbial contamination. In some cases, bacteria associated with the explant may be protected from contact with disinfectants (Gunson and Spencer-Phillips, 1994; Leifert et al., 1994a).

Certain bacteria are reported to reside endophytically or be latent in vascular tissues of apparently healthy plants where they are protected from contact with disinfectants (Hayward, 1974). Some bacteria are in

substomatal cavities or intercellular spaces (Sztejnberg and Blakeman, 1973; Gunson and Spencer-Phillips, 1994). Endophytic bacterial contaminants enter through wounds and natural openings or through the root system (Cassells, 1991). Endophytes may actively grow in culture or may remain cryptic and become detectable only when placed on specialized media.

Explants from either field-grown plants, diseased specimens, or from parts are located close to or below the soil may be very difficult or impossible to disinfect (Leifert et al., 1994a). Contaminants from tissues of greenhouse-grown plants are mostly those associated with soil (Buckley et al., 1995) and may originate from irrigation water (Seabrook and Farrell, 1993).

Bacteria detected at initiation of tissue culture and in the first subculture of explant materials are usually Gram-negative, belonging to the Pseudomonaceae and Enterobacteriaceae (Leifert et al., 1994b). It is reasonable that members of the Pseudomonadaceae and Enterobacteriaceae are early contaminants of tissue culture, because these are often the dominant bacteria associated with aerial surfaces of plants grown under field conditions (Magnuson et al., 1990).

Operators Bacterial isolated from established cultures are often those associated with humans. Leifert et al. (1991) found *Staphylococcus*, *Micrococcus*, and *Lactobacillus*, which normally inhabit

human tissues, in plant cultures. Leifert et al. (1994a) detected contaminants among operators, suggesting poor aseptic technique. Contaminants were frequently Gram-positive bacteria such as *Staphylococcus*, *Micrococcus*, and *Bacillus* spp. (Leifert et al., 1989). Bacterial contaminants detected in older, previously-indexed cultures often grow well in indexing media, indicating a new infection rather than one carried from earlier cultures (Leifert et al., 1994a).

Mites and thrips Mites and thrips may carry bacterial or fungal contaminants in and on their bodies and move easily past loose caps from one vessel to another (Blake, 1988). Mites require a high humidity (above 70%) environment such as in plant tissue culture to reproduce and may complete their life cycle within 2-3 wks. Mites can also lay their eggs in cracks of growth room walls and the eggs remain viable for a long time. Thrips are another common pest of plants; it is crucial to make sure that only clean explants are taken. During incubation and storage of sterile culture vessels, media, and plant cultures, the laboratory should have strict hygiene especially to control mites and thrips.

Inefficient sterilization techniques Some bacteria such as *Bacillus* are very resistant to flaming and alcohol treatment (Boxus and Terzi, 1987). The temperature of an alcohol burner may be insufficient to kill spores of *Bacillus* and improper use of the Bacti-cinerator could place the tips of tools out of the effective heating zone. Insufficient reheat time

and a heavy organic load on the tools also reduces sterilization effectiveness (Singha et al., 1987). Inadequate autoclaving duration may allow survival of contaminants. Large volumes of liquids, fully-loaded autoclaves, and wrapped packages may require longer sterilization times (Burger, 1988).

Prevention of bacterial contaminants. Prevention of bacterial contamination can be accomplished at the stage of stock plants; explants; media preparation; subculturing; incubation; and storage of sterile culture vessels, media, and plant cultures. Leifert and Waites (1994) suggest that stock plants used for plant tissue cultures should be grown under protected conditions (glasshouses, growth chambers). Seabrook and Farrell (1993) observed a reduction of bacterial contaminants by irrigating stock plants with filtered water.

During media preparation, Leifert et al. (1994b) suggests using autoclave tape, physical temperature indicators, and biological methods that are based on the survival of *Bacillus* spores. Burger (1988) reports that preheating solutions before autoclaving reduces the overall time that liquids must be exposed to autoclave conditions; this is routinely accomplished during procedures to melt agar. He also reported that removing all air from the internal chamber of the autoclave will allow steam to reach all materials in the autoclave, and enclosing materials in sealed, autoclaving bags should be avoided. Leifert (1994a) recommends

a mechanized or semi-mechanized media pouring systems. Leifert et al. (1994a) prevented more than 50% of regular contamination of *Delphinium* cultures by acidifying the culture medium.

During subculturing of plant cultures, contaminants can be reduced by protecting the inside of the laboratory from the outside air, keeping the laboratory and growth rooms clean, and training of operators in aseptic technique (Leifert et al., 1994b). Leifert and Waites (1994) suggest routinely testing the laminar air flow cabinets for effectiveness.

Gunson and Spencer-Phillips (1994) found that pink pigmented, facultative methylotrophs produced methanol in plant cultures. This can be avoided by more frequent venting of tissue culture vessels, or including a protected aperture to allow free diffusion of gases. The internal atmosphere could be monitored for the accumulation of critical gases with special detectors (Gunson and Spencer-Phillips, 1994). Blake (1988) states that caps should allow reasonably free passage of gases and fit tightly over the culture vessels, but restrict the passage of water to prevent the medium from drying out.

Detection. Detection of bacterial contaminants is crucial, especially for those that are slow growing, are endophytes, or do not grow on plant tissue culture media (Kane, 1995; Leifert et al., 1989). Screening methods must be favorable to bacterial growth and easily used and interpreted (Reed et al., 1995). Screening procedures are available

for determination of many cultivable contaminants (Debergh and Vanderschaeghe, 1988; Leifert et al., 1992; Viss et al., 1991). Pathogen-free and contaminant-free cultures 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). Transferring contaminated cultures to the greenhouse rather than discarding them should be carefully reviewed, as the contaminants may cause difficulties at a later date. Contaminants which cause no visible harm to plant cultures have, in some instances, become pathogenic on potted plants (Kane, 1995).

Identification and characterization. Bacteria should be characterized and identified to tell the tentative sources (Leifert et al., 1991). Contaminants from indexing media must be purified before further identification. Bacterial contaminants can be identified by 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.

Bacterial contaminants can also be identified by newer identification techniques which provide results in 24-48 h. For example, the Biolog system detects carbon source utilization by the reduction of tetrazolium dye. Results are compared with a database of Gram-negative and positive bacteria, yeasts and lactic acid bacteria (Bouzar et al., 1993; 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 the presence of bacterial 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). Jones et al. (1993) and Verniere et al. (1993) suggested the use of more than one identification test for more accurate results.

Antibiotic treatments. Bacterial resistance to surface sterilization or endophytic bacteria lodged within the plant must be eliminated using antibiotic treatments; however, antibiotics should not replace careful aseptic technique (Mathias et al., 1987).

The ideal antibiotics should be soluble; be stable; be unaffected by pH, media, light, heat; produce minimal side effects; show a broad spectrum of activity; be bactericidal; be suitable to use in combination; have minimal chance of developing resistance; be inexpensive; and

nontoxic to plant cells and human health (Bastiaens et al., 1983; Pollock et al., 1983; Falkiner, 1988; 1990; Seckinger, 1995).

Antibiotics can be grouped by mode of action as inhibitors of bacterial cell wall synthesis, inhibitors of bacterial protein synthesis, or DNA replication blockers (Pollock et al.; 1983, Quesnel and Russell, 1983) and by chemical structure: aminoglycosides, quinolones, B-lactams, glycopeptides, polymyxins, marcolides, and lincosamides (Falkiner, 1990).

Antibiotics are incorporated into culture media or used as a brief treatment for specific contaminants (Leifert et al., 1992). Kneifel and Leonhardt (1992), and Leifert et al. (1992) recommend the use of short antibiotic treatments to prevent the development of antibiotic resistance in bacterial contaminants. It is also very important to determine whether antibiotics are bactericidal instead of bacteriostatic to avoid reoccurrence of bacteria (Mathias, 1987; Leifert et al., 1992). Combinations of antibiotics may be more effective in killing contaminants (Falkiner, 1988; Leifert et al., 1991; Kneifel and Leonhardt, 1992). If antibiotic combinations are synergistic, the effective concentration of each antibiotic can be reduced, and the reduced concentration of each antibiotic will produce fewer toxic side effects (Falkiner, 1988). Barrett and Cassells (1994) found that antibiotics lost some sensitivity in tissue culture media.

Cornu and Michel (1987) suggested that it is crucial to know the effect of antibiotic on both the bacteria and explant to be able to eliminate contaminants. Successful antibiotic treatment of infected plants requires determining the minimal bactericidal concentration (MBC) and its antibiotic phytotoxicity to plant materials before treatment begins (Barrett and Cassells, 1994). Antibiotics effective on isolated organisms may not be effective in contaminated plant cultures due to phytotoxicity or poor penetration into plant tissues (Bastiaens et al., 1983; Viss et al., 1991; Reed et al., 1995). Bacteria may reappear because of transient bacteriostatic activity (Bastiaens et al., 1983). Mathias et al. (1987) suggests the use of antibiotic treatments in liquid medium, because greater surface contact increases the uptake of antibiotics into internal tissues.

β-lactams have broad-spectrum activity by combining rifampicin with trimethoprim. Pollock et al. (1983) found that betalactam antibiotics such as penicillins, ampicillin, carbenicillin, and cephalosporins are the least toxic antibiotics to protoplast-derived cells of *Nicotiana plumbaginifolia.* They also found erythromycin and colistin useful. Aminoglycosides (streptomycin, neomycin, kanamycin, gentamicin, G418, amikacin, and tobramycin) are not recommended for protoplast culture of *Nicotiana plumbaginifolia* due to their toxicity to the ribosomes of chloroplasts and mitochondria. Aminoglycoside activity depends upon the pH of the treatment medium with better results at

neutral pH. Cornu and Michel (1987) treated contaminated *Prunus avium* L. cultures and found that aminoglycosides (tetracycline and rifampicin) have the broadest spectrum of activity against contaminants such as *Pseudomonas*, but at high concentrations induced chlorosis, necrosis, and death of explants. They also found great variability between clones of plants in susceptibility to all antibiotics.

Leifert et al. (1992) examined the phytotoxic effects of antibiotic treatment on micropropagated *Clematis*, *Delphinium*, *Hosta*, *Iris*, and *Photinia*. Antibiotic toxicity to plant growth was as follows: streptomycin > polymyxin > rifampicin > carbenicillin. Kneifel and Leonhardt (1992) found that mixtures of imipenem/ampicillin and imipenem/penicillin G at concentrations of 5 mg/l each were the most effective in inhibiting the growth of *Staphylococcus xylosus*, *S. aureus*, *S. cohnii*, *Bacillus* spp., *Corynebacterium* sp., and *Pseudomonas vesicularis*. Those antibiotic combinations also showed no phytotoxicity to *Drosera rotundifolia*, *Spatiphyllum* sp., *Syngonium* sp., white butterfly, and *Nephrolepis exaltata*. Buckley et al. (1995) worked with mint cultures, they found that the effectiveness of an antibiotic treatments depended on the antibiotic used and bacterial type. They also found that gentamicin, rifampicin, streptomycin sulfate, and Timentin were most effective at pH 6.5 and 7.5.

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Chapter 2

Detection and Identification of Bacterial Contaminants from Strawberry Runner Explants

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Abstract

Early detection of contaminants saves time, effort, and money for tissue culture laboratories. Microbial contaminants were successfully detected in runners partially submerged in one-half strength liquid MS medium. Contaminants detected during June to August, 1994 were from 45 of 70 strawberry genotypes and June to August, 1995 from 53 of 72 strawberry genotypes. More contaminants were bacterial than fungal. Bacterial contaminants from 22 contaminated genotypes were isolated, purified, and identified to genus using standard biochemical tests such as Gram's stain, motility, oxidase, and gelatinase; and carbon source utilization (Biolog) test. From those 22 genotypes, 16 different strains were found, the majority being fluorescent pseudomonads such as *Pseudomonas fluorescens* types A, F, and G. *P. corrugata*, *P. tolaasii*, and *P. paucimobilis*. *X. campestris*, *Xanthomonas* spp; and *Enterobacter cloacae* were also identified. Five Gram-negative and two Gram-positive contaminants could not be identified by the Biolog test. Biochemical tests were useful to confirm Biolog test results and characterize the bacteria. Bacteria found in this study were soil, water, and plant related, indicating that efforts to reduce explant contaminant levels should be centered on the care of stock plants or the sterility of the watering system rather than improved laboratory technique.

Introduction

Bacterial contamination is one of the most crucial problems of plant tissue culture, both in research and commercial production (Cassells, 1991; Leifert and Waites, 1992). Often bacterial contamination is difficult to detect (Debergh and Vanderschaeghe, 1988; Viss et al., 1991). Even healthy plants can contain several bacteria (Debergh and Vanderschaege, 1988; Leggatt et al., 1988), and some plant exudates may look similar to bacterial growth (Finer et al., 1991; Bastiaens, 1983). Contaminated plants may lack symptoms, or have reduced multiplication rates, reduced rooting rates, or may die (Leifert et al., 1989; 1992).

Contaminants in plant tissue cultures come from poor sterile technique (Cassells, 1991; Leifert and Waites, 1992), from bacteria that survive alcohol flaming, such as *Bacillus* spores (Boxus and Terzi, 1987; Singha et al., 1987), and from mites and thrips (Blake, 1988; Leifert et al., 1989). Contaminants also come from explants (Cassells, 1991; Kneifel and Leonhardt, 1992) such as epiphytic bacteria that are resistant to surface sterilization (Leggatt et al., 1988); and endophytic bacteria present in plants due to wounds, natural openings, or root systems (Cassells, 1991). Seabrook and Farrell (1993) reported bacterial contamination of explants caused by watering stock plants with dirty water. Skirvin et al. (1993) found that woody plant sticks forced in water prior to explant collection were quickly contaminated with a marker

bacterium added to the water. Endophytic bacteria may remain in plant tissues for a long time before bacterial growth or disease symptoms develop (Norman and Alvarez, 1994). Bacterial outbreaks cause major losses of time and resources (Seabrook and Farrell, 1993), thus rapid and dependable methods for early detection should be used to control and prevent bacterial spread (Norman and Alvarez, 1994; Reed et al., 1995). Kunneman and Faaij-Groenen (1988) note that the best bacterial detection can be achieved when the conditions for bacterial growth are optimal. Some contaminants are introduced during subcultures, thus plant cultures should be indexed at regular intervals and not only at the initiation of explants (Leifert et al., 1989, and Kane, 1995).

Several types of indexing media are used to detect bacteria: 523 medium (Viss et al., 1991; Reed et al., 1995); Murashige and Skoog (1962) medium alone or with additions such as yeast extract, peptone, or glucose (Boxus and Terzi, 1987; Leifert et al., 1989); culture medium with the addition of coconut water (Norman and Alvarez, 1994).

Many different bacteria are detected in plant cultures. Boxus and Terzi (1987) found *Torulopsis, Pseudomonas, Acinetobacter, Flavobacterium,* and *Corynebacterium.* Leggatt et al. (1988) detected yeasts, *Corynebacterium* spp., and *Pseudomanas* spp. Leifert et al. (1989) isolated *Bacillus, Enterobacter, Staphylococcus, Pseudomonas,* and *Lactobacillus.* Bacterial contaminants found in plants cultured for four weeks or less mostly were motile Gram-negative bacteria, while

contaminants from plants cultured for at least 12 months were more likely to be Gram-positive (Leifert et al., 1989). Kneifel and Leonhardt (1992) found that some plants contained mixtures of Gram-negative rods, Gram-positive rods, and cocci. Mint cultures were contaminated mostly with Gram-negative bacteria that are usually associated with plants and soils such as *Agrobacterium* and *Xanthomonas* (Buckley et al., 1995). Different types of bacteria are found on various plant species and at specific stages of plant cultures. Leifert et al. (1989) suggests that by determining bacterial identity, it may be possible to determine the source of contaminants.

Bacteria are identified and characterized by standard biochemical tests such as Gram stain, catalase, oxidase, and motility (Bradbury, 1988; Leifert et al., 1989); or by rapid identification methods such as the Biolog system (Jones, 1993; Lacroix et al., 1994) for carbon utilization, API test strips (Leifert et al., 1989; Lacroix et al., 1994), fatty acid profiles (FAP) (Jones et al., 1993; Buckley et al., 1995), DNA techniques (Jones et al., 1993), and protein electrophoresis (Lacroix et al., 1994). Jones et al. (1993) and Verniere et al. (1993) recommend the use of more than one test for more accurate identification.

The goals of this study were to devise an effective method for detecting bacterial contaminants in strawberry runner explants, and to isolate, characterize, and identify bacterial contaminants to taxonomic groups or more specific classifications.

Material and Methods

Plants material used. *In vitro* cultures were initiated from runners taken from pot-grown strawberry plants in a screenhouse at the USDA-ARS National Clonal Germplasm Repository, Corvallis, OR (Appendix 1). Runners were disinfested by immersion in 10% household bleach (5.25% sodium hypochlorite; Clorox, Oakland, CA) solution containing 1% Tween-20 (Sigma Chemical Co., St.Louis, MO) for 10 min, rinsed twice in sterile deionized water, and grown in individual 16x100 mm tubes containing one-half strength liquid Murashige and Skoog (MS) medium, pH 6.9 at 25 °C for 10 days with 16 h of light (25 uEm^2s^{-1}). After 10 days, runners were transferred to full strength MS medium with 170 mg/l NaH_2PO_4, 80 mg/l adenine sulfate, 1 mg/l benzyladenine (BA), 0.01 mg/l gibberellic acid (GA_3), 10 mg/l indoleacetic acid (IAA), 3 g/l agar, and 1.25 g/l gelrite at pH 5.7.

Plants response to submerged growth. Five runners of each of six *Fragaria* genotypes (*F. chiloensis* Lonquimay 02E, 04D, 04F, and 04G; *F. chiloensis* Maullin; and *F. chiloensis* Nashvelbuto Nat'l Park 01A) were submerged in one-half strength liquid MS medium. Another five runners each of the same *Fragaria* genotypes were partially submerged (the tips of runners were not submerged). Cultures were grown for 10 days in the

liquid medium and then transferred to solid medium to determine survival.

Detection of contaminants. Runners (2-12 from each genotypes) from 70 genotypes of *Fragaria* spp. were collected from June to August, 1994 and screened for contaminants by partially submerging them in liquid medium (above). Runners (2-12 from each genotypes) from 72 additional genotypes were collected from June to August, 1995, and screened as above but in medium with 265 mg/l peptone and 88 mg/l yeast extract. Bacterial growth appeared turbid within 10 days in liquid medium containing contaminated runners. Explants with no detectable contaminants after 10 days were transferred to solid medium and observed for visible contaminants at 21 days.

Isolation and purification of bacteria. Contaminants were transferred with a sterile cotton swab to 0.8% nutrient broth (Difco; Sigma) with 1% glucose and 0.1% yeast extract at pH 6.9 and incubated at 25 C. Bacteria were purified by repetitive streaking on nutrient agar (NA) plates.

Diagnostic tests for bacterial characteristics. Gram stain, KOH, motility, oxidase, starch hydrolysis, and gelatinase tests were performed according to the methods described by Klement et al. (1990).

Standard bacterial cultures. Cultures of standard bacterial strains were provided by Ms. Marilyn Canfield, Department of Botany and Plant Pathology, Oregon State University: *Xanthomonas campestris, Xanthomonas incanae,* and *Xanthomonas vesicatoria*; Dr. Joyce Loper, USDA-ARS, Horticultural Crops Laboratory, Corvallis, OR.: *Enterobacter cloacae* (EcCT-501), *Pseudomonas fluorescens* Pf-5, and *Pseudomonas putida* (A 12); and Dr. Patricia Buckley, USDA-ARS National Clonal Germplasm Repository, Corvallis, OR.: *Agrobacterium radiobacter.* These strains were used as reference cultures for comparison in the characterization and identification of bacterial strains isolated in this study.

Carbon substrate utilization. The ability of bacterial strains to oxidize 95 substrates was tested on Biolog Gram-negative and Gram-positive Microplates (Biolog, Inc., Hayward, Ca.). Bacteria were grown on tryptic soy agar (TSA) for 4-18 h at 30 °C then diluted in 0.85% saline solution at pH 5.5-7.0 to a standard optical density. The turbidity of bacterial solutions was read at 600 nm (spectrophotometer 20) to an absorbance of 0.21-0.26 for Gram-negative and 0.34-0.43 for Gram-positive bacteria. Bacterial solutions were aseptically transferred to Biolog Microplates (150 ul/wells) using a multichannel micropipette. The results were read visually at 4 and 24 h. The data were analyzed with

Biolog computer software leading to placement of the isolates into genera, species, or strains.

Results and Discussion

Plant response to submerged growth. Strawberry runners died when totally submerged in liquid medium, but partially submerged plants survived and grew (data not shown). Reed et al. (1995) submerged mint explants in one-half strength MS liquid medium for 6-10 days and all the explants survived. These results suggest that plant species perform differently in response to submerged growth in liquid medium. In the case of strawberry runners, shaking the cultures to allow air to circulate through the medium might produce better survival of totally submerged plants. Partial submersion provided high levels of contaminant detection and plant survival without agitation of the cultures.

Detection of contaminants. Contaminants were detected in 45 of 70 genotypes initiated in 1994 in basal medium (Table 2.1). Contaminants were 51% bacterial and yeast, 24.5% fungal, and 24.5% mixed contaminants. Only in four genotypes were all explants contaminated. Similar results were seen in 1995 in enriched medium with 53 of 72 genotypes contaminated; 60% bacterial and yeast, 23% fungal, and 17% mixed contaminants. Only in six genotypes were all explants

contaminated. The 10% increase in contaminants detected in 1995 might be due to the use of peptone and yeast extract in the medium, however this was only a screening process and these results need to be verified in a controlled experiment. This techniques did not distinguish between endophytic and epiphytic contaminants.

Table 2.1

Detection of contaminants in strawberry runner explants partially submerged in liquid medium for 10 days.

		Number of genotypes			
		Contaminated			
Year	Tested	Only Bacteria & yeasts	Only Fungi	Mixed[z]	Total
1994[x]	70	23	11	11	45
1995[y]	72	32	12	9	53

[x] 1/2 MS liquid medium
[y] 1/2 MS liquid medium with 265 mg/l peptone and 88 mg/l yeast extract
[z] Some explants of these genotypes were contaminated with bacteria and others with fungi.

Boxus and Terzi (1987) found that several bacterial and yeast contaminants could not be detected on proliferation or elongation medium, but were apparent and often lethal on rooting medium. Peptone and yeast extract added to the proliferation and elongation

medium allowed detection and elimination of all bacterial contaminants within two subcultures. Reed et al. (1995) found that liquid MS medium alone detected most contaminants from mint explants with few additional contaminants detected later. Additional experiments with contaminated mint explants found that medium with peptone and yeast extract produced more obvious and faster bacterial growth without causing toxicity to the plants (Tanprasert, unpublished data). Since the detection medium does not always give clearcut results, Debergh and Vanderschaege (1988) suggest that subtle symptoms such as brown spots on petioles of *Gerbera* and brown bracts at the base of *Marantha* provide additional aids to determine latent contaminants.

Characterization and identification of bacteria. Bacterial contaminants isolated from 22 strawberry genotypes included approximately 16 bacterial strains as determined by the Biolog database, standard cultures, and biochemical tests (Tables 2.2, 2.3, 2.4; Appendix 2). One to four bacterial strains were found per genotype. More than half of the genotypes tested contained more than one bacterial strain. Most of the contaminants were Gram-negative, rod shaped, motile, non-spore forming bacteria, and most were *P. fluorescens* types (Table 2.2; Appendix 2).

Pseudomonads are straight or slightly curved rods, motile by polar flagella, aerobic, have a strictly respiratory metabolism with oxygen; in

Table 2.2

Characterization of *Pseudomonas* species detected from strawberry runner explants.

Bacterial species	Number of contaminated genotypes	Motility	Gelatinase	Oxidase	King's B medium[z]	starch hydrolysis
P. corrugata	5	+	+	+	-	-
P. fluorescens type A	2	+	+	+	+	-
P. fluorescens type C	Standard culture	+	+	+	+	-
P. fluorescens type F (1)	10	+	+	+	+	-
P. fluorescens type F (2)	3	+	+	+	-	-
P. fluorescens type G	1	+	+	+	+	-
P. fluorescens type	2	+	+	+	+	-
P. paucimobilis A	1	+	+	+	-	-
P. tolaasii	2	+	+	+	-	-

[z] Fluorescent (+) on King's B medium under ultraviolet (UV) light.

Table 2.3

Characterization of *Xanthomonas* species and *Enterobacter cloacae* A isolated from strawberry runner explants.

Bacterial species	number of contaminated accessions	Motility	Gelatinase	Oxidase	Fluorescent pigment[z]	Starch hydrolysis
X. campestris pv.	Standard culture	+	+	-	-	+
X. incanae	Standard culture	+	+	-	-	+
X. vesicatoria	Standard culture	+	-	+	-	+
X. campestris pv. *vesicatoria*	1	-	-	w	-	+
Xanthomonas spp.	2	+	-	w	-	-
Enterobacter cloacae A	1	+	+	+	-	-

w weak reaction

[z] Fluorescent (+) on King's B medium under ultraviolet (UV) light.

Table 2.4

Characterization of unidentified Gram-negative and positive, rod-shaped bacteria isolated from strawberry runner explants.

	90	170	236	248D	978	148E	248E
Gram's stain	-	-	-	-	-	+	+
Pink pigment	-	+	-	+	-	-	-
Motility	+	+	+	+	+	+	+
Gelatinase	-	+	-	+	-	-	-
Oxidase	+	+	+	+	+	-	-
Fluorescent pigment[z]	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	+

[z] Fluorescent (+) on King's B medium under ultraviolet (UV) light.

some cases nitrate can be used. Some species are facultative chemolithotrophs (able to use H_2 or CO as energy sources). They are widely distributed in nature and some of them are plant pathogens. Pseudomonads may have no pigment, soluble pigments such as fluorescent (pyoverdin) and pyocyanin, or non-soluble pigments such as carotenoids (Krieg and Holt, 1984). Saprophytic fluorescent pseudomonads are very common in soil and plant rhizospheres. For example, Sands and Rovira (1971) found that *P. fluorescens* biovar G predominates in wheat rhizospheres. Plant pathogenic pseudomonads are normally isolated from lesions of plant hosts (Krieg and Holt, 1984). All *Pseudomonas* spp. isolated from strawberry runners were fawn (medium tan) in color except *P. paucimobilis* which was yellow. Many produced fluorescent pigment (fluoresced on King's B medium). *P. fluorescens* is separated into 7 different biotypes (A-G) (Stanier et al., 1966) or into 5 biovars (I - V) (Krieg and Holt, 1988) (Table 2.5). Each type can be further separated to many strains. Differentiation between biotypes requires a number of diagnostic tests and results are variable among strains of the same biovar. In this study, the most common bacteria found were *P. fluorescens* type F (13 isolates) and two strains could be distinguished based on the presence (10) or absence (3) of the fluorescent pigment. *P. fluorescens* type A (2 isolates) and G (1 isolate) were also found (Table 2.2).

Table 2.5
General Characteristics of *Pseudomonas fluorescens* biovars I - V
(from Bergey's Manual of Systematic bacteriology).

Characteristics	I	II	III	IV	V	*P. chlororaphis*	*P. aureofaciens*
	A*	B	C	F	G	D	E
Non fluorescent pigments:							
Blue, non diffusible	-	-	-	-	-	+	-
Levan formation from sucrose	+	+	-	+	-	+	+
Denitrification	-	+	+	+	-	+	d
Carbon sources used for growth:							
Adonitol	+	-	d	-	d	-	-
Butyrate	-	d	d	+	d	+	d
Ethanol	-	+	d	-	d	d	-
L-Arabinose	+	+	d	+	d	-	+
Propionate	+	+	d	+	+	+	+
Propylene glycol	-	+	d	-	d	-	-
Saccharate	+	+	d	+	d	+	+
Sorbitol	+	+	d	+	d	-	-
Sucrose	+	+	-	+	d	+	d

The meaning of the symbols are as follows:

+ 90% or more of strains are positive
- 90% or more of strains are negative
d 11-89% of strains are positive
* *P. fluorescens* biovars as designated by Stanier et al. (1966)

P. corrugata (5 isolates) had fawn-colored, wrinkled colonies and could hydrolyze gelatin, but not starch, as indicated in descriptions in Bergey's manual (Krieg and Holt, 1994). *P. tolaasii* (2 isolates) has a translucent colony and is pathogenic for cultivated mushrooms (Wong and Preece, 1979). *P. tolaasii* produces a white line on Pseudomonas Agar F (PAF) and pitts mushroom caps. *P. paucimobilis* (1 isolate) was motile, did not produce fluorescent pigment, and did not hydrolyze gelatin or starch. *P. paucimobilis* can be isolated from many sources such as tap water and plant surfaces (Holmes et al., 1977).

Xanthomonads are straight rods, motile by a single polar flagellum, obligately aerobic, and do not reduce nitrate. Colonies are usually yellow, smooth, and butyrous or viscid. All except one have xanthomonadin pigment produced from brominated aryl polyenes. They do not use asparagine as a sole carbon and nitrogen source. Growth of xanthomonads is inhibited by 0.1% triphenyltetrazolium chloride. Growth factors required usually include methionine, glutamic acid, nicotinic acid, or a combination of these. They are all plant pathogens (Krieg and Holt, 1984). *Xanthomonas* spp. found were yellow and oxidase negative or weakly positive (Table 2.3). Xanthomonads were isolated from three strawberry genotypes. One, *X. campestris* pv. *vesicatoria* is pathogenic to many plant species such as *Capsicum* spp. and *Datura stramonium*, but not strawberries (Krieg and Holt, 1984). The colonies were yellow and hydrolyzed starch. Two other *Xanthomonas* spp. had

yellow colonies, but did not hydrolyze starch. They could not be identified to species level by Biolog tests.

Enterobacteria are Gram-negative, straight rods which are oxidase negative. All except one species are motile by peritrichous flagella. They can grow in the presence or absence of oxygen. Enterobacteria are found in soil, water, fruits, vegetables, grains, flowering plants and trees, and can be plant pathogens (Krieg and Holt, 1984). *Enterobacter cloacae* A, found in strawberry runner explants in this study, was beige, moist, slimy, very motile and very fast growing.

Five other Gram-negative bacteria, two Gram-positive bacteria, and two yeasts were not identified in this study (Table 2.4). They are likely to be soil, water, or plant related microorganisms since runners of strawberry plants are closely associated with the soil and would easily be contaminated by soil-borne bacteria.

The mixtures of bacterial strains isolated varied from plant to plant. Mixtures were from different *Pseudomonas* species such as *P. corrugata* and *P. fluorescens* type F., or from different bacterial genotypes such as *P. tolaasii* and *X. campestris* pv. *vesicatoria*. Gram-negative and Gram-positive bacteria, fungi, and yeasts were sometimes isolated from the same genotypes (Appendix 1).

Standard bacterial cultures. Standard cultures were used together with other identification and characterization methods such as

biochemical and carbon source utilization (Biolog) tests. Standard cultures were very useful as controls in diagnostic tests and also for colony characterizations. Two strawberry runner bacterial isolates were the same as the standard cultures of *Enterobacter cloacae* (Fra 655) and *X. campestris* (Fra 557b).

Standard biochemical tests. Standard biochemical tests were useful for identifying many of the isolates. Gram staining was done immediately after purifying cultures in order to group bacteria. The Gram stain also helped to better visualize bacterial shapes. Bergey's Manual groups bacteria by using Gram-negative, Gram-positive, and bacterial shape catagories (Krieg and Holt, 1984). King's B medium was used for identifying fluorescent pseudomonads; it was especially useful in this study because many fluorescent pseudomonads were found. Starch hydrolysis together with xanthomonadins distinguished some xanthomonads; however, two xanthomonad isolates produced xanthomonadins, but did not hydrolyze starch.

Carbon substrate utilization (Biolog) test. Biolog was a rapid (24 h) and dependable method for identifying Gram-negative bacteria detected in this study, because most bacterial strains found were *Pseudomonas* spp. which were included in the database. Classification into strains was simplified with the use of the Biolog tests. The main difficulty in reading Biolog plates was from visual analysis of color change. Careful

observation was required because even a small misreading could cause the identification to shift from one bacterial strain to another. For example, accession 557b would be identified as *P. fluorescens* type A if the bacterium produced a weak response on maltose, D-pricose, alpha-keto valenic acid, and L-ornithine, but would be identified as *P. tolaasii* if the reactions were negative.

Because of the possibility of misleading results from test kits, Verniere et al. (1993) recommend using other biochemical tests to confirm results. Leifert et al. (1989) recommend using biochemical tests together with the API test for more reliable identification. API test strips were developed mainly for medical use and, thus, are less effective for plant and soil bacteria. The addition of carbon source utilization information to the database by an individual researcher can improve the usefulness of test kits. When Verniere et al. (1993) used Biolog's Microlog database, only 6.8% of their plant pathogenic bacteria were identified. When they added their own database to Microlog, up to 70% of the bacteria were identified.

After identification and characterization with Biolog and special biochemical tests, bacterial characteristics should also be confirmed with Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Some Gram negative and Gram-positive isolates could not be identified by Biolog. Biolog testing would have been more useful in this study if we

had built our own database and included more plant-related standard bacterial cultures.

Conclusions

Microbial contaminants were successfully detected in runners partially submerged in one-half strength liquid MS medium. Strawberry runners were very sensitive to being totally submerged in liquid medium. Contaminants were detected in 75% of genotypes tested in 1994 and 73% of genotypes tested in 1995. More contaminants were bacterial than fungal. Most genotypes had some contaminated explants.

Bacterial contaminants from 22 genotypes were purified, characterized, and grouped. Most of the bacteria found were Gram-negative, rod-shaped, motile, non-spore forming *Pseudomonas* species. *P. fluorescens* types A, F, and G were the most common contaminants found. *P. corrugata, P. tolaasii,* and *P. paucimobilis*; *Xanthomonas* spp., one *X. campestris*, two *Xanthomonas* spp; and *Enterobacter cloacae* were were also identified. Five Gram-negative and two Gram-positive contaminants could not be identified by the Biolog test. Biochemical tests were useful to confirm Biolog test results and characterize the bacteria.

Identification and characterization of bacterial contaminants provided information on the sources of contaminants. Bacteria found in this study were soil, water, and plant related, indicating that efforts to

reduce explant contaminant levels should be centered on the care of stock plants or the sterility of the watering system rather than improved laboratory technique.

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Chapter 3

Determination of Minimal Bactericidal and Effective Antibiotic Treatment Concentrations for Bacterial Contaminants from Micropropagated Strawberries

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Abstract

Minimal bactericidal concentrations (MBCs) were determined, for 16 bacterial strains isolated from strawberry runners. Bacteria were treated with single antibiotics: Timentin, streptomycin sulfate, gentamicin, and dihydrostreptomycin; and with combinations of two or three antibiotics: timentin, streptomycin sulfate, and gentamicin. Combinations of the three antibiotics (12 concentrations) were effective with all bacteria tested, thus they were used for plant treatments. Two strawberry genotypes with distinctive morphologies (narrow stem *F. virginiana* subsp. *glauca* (S. Watson) Staudt and wide stem *F. x ananassa* Duch. cv. Jucunda) were inoculated with *X. campestris* pv. *vesicatoria* and *P. corrugata,* grown for one week, then treated with combinations of Timentin, streptomycin, and gentamicin. *F. virginiana* subsp. *glauca* died after inoculation. Antibiotic treatments were 100% effective in eliminating *P. corrugata* from 'Jucunda', but only 23% of plants inoculated with *X. campestris* pv. *vesicatoria* were freed of the bacteria. No phytotoxicity was observed for any antibiotic treatment concentrations.

Introduction

Successful plant micropropagation requires the prevention, avoidance, or elimination of microbial contaminants (Reed and Tanprasert, 1995). Contaminants cause huge losses of plantlets in both research and commercial tissue culture laboratories (Cassells, 1991; Viss et al., 1991; Leifert and Waites, 1992). The best solution is employ suitable laboratory procedures and sanitary practices. These practices will exclude many potential contaminants (Reed and Tanprasert, 1995). Bacteria resistant to surface sterilization or endophytic bacteria lodged within the plant can only be eliminated using antibiotic treatments (Mathias et al., 1987).

Antibiotics are either incorporated into culture media or used as brief treatments for specific contaminants (Leifert et al., 1992). Kneifel and Leonhardt (1992), and Leifert et al. (1992) suggest the use of short term antibiotic treatments to prevent the development of antibiotic resistance in bacterial contaminants. It is also important to determine whether antibiotic treatments are bactericidal rather than bacteriostatic to avoid reoccurrence of bacteria (Mathias, 1987; Leifert et al., 1992). Combinations of antibiotics may be more effective in killing contaminants than single antibiotics (Falkiner, 1988; Leifert et al., 1991; Kneifel and Leonhardt, 1992). If antibiotic combinations are synergistic, the effective concentration of each antibiotic can be reduced; and the reduced

concentration of each antibiotic produces fewer phytotoxic side effects (Falkiner, 1988). Barrett and Cassells (1994) found that antibiotics lost some effectiveness in tissue culture media. Successful antibiotic treatment of infected plants requires the determination of the minimal bactericidal concentration (MBC) and the antibiotic phytotoxicity to plant materials before treatment begins (Barrett and Cassells, 1994). Antibiotics that are effective on isolated organisms may not be effective in contaminated plant cultures due to phytotoxicity or poor penetration into plant tissues (Bastiaens et al., 1983; Viss et al., 1991; Reed et al., 1995). Bacteria may reappear because of transient bacteriostatic activity (Bastiaens et al., 1983). Mathias et al. (1987) suggest using antibiotic treatments in liquid medium, because greater surface contact increases the uptake of antibiotics into internal tissues.

The goals of this study were to 1. determine minimal bactericidal concentrations of antibiotics for the control of bacterial contaminants detected from strawberry runner explants, 2. to determine effective treatments for contaminated plants, and 3. to determine an effective medium to detect bacterial contaminants after antibiotic treatments.

Materials and methods

Bacterial isolates used. Bacteria used were those isolated from strawberry runner explants (Ch. 2): *P. corrugata, P. fluorescens* types A and F (1 and 2), *P. tolaasii, P. paucimobilis, X. campestris* pv. *vesicatoria, Xanthomonas* spp., *Enterobacter cloacae* A, three unknown species, and standard cultures (see material and methods, Ch.2)

Minimal bactericidal concentrations (MBCs). MBCs were tested for sensitivity to single antibiotics (Timentin, gentamicin, streptomycin sulfate, and dihydrostreptomycin); combinations of two antibiotics (Timentin + gentamicin, Timentin + streptomycin sulfate, and gentamicin + streptomycin sulfate); and Timentin, streptomycin sulfate, and gentamicin combined. MBCs of the antibiotics were estimated by inoculating 50 µl of 48 h old cultures of bacterial isolates to a series of antibiotics tube dilutions in one-half strength MS medium, at pH 6.9. The tube dilutions contained doubling serial dilutions of antibiotic to a final volume of 2 ml per tube. After 5 days incubation at 25 °C, each dilution was transferred to nutrient agar and tryptic soy agar plates with a sterile applicator stick and incubated at 25 °C for 4-7 d to determine bacterial growth. The lowest concentration of antibiotic that showed no bacterial growth was considered to be the minimal bactericidal concentration. Antibiotic concentrations were chosen from earlier published reports (Falkiner, 1988; Buckley et al., 1995).

Single antibiotics. Timentin (T), streptomycin sulfate (S), and dihydrostreptomycin (D) (µg/ml): 62.5, 125, 250, 500, and 1000; and gentamicin (G): 3, 6.25, 12.5, 25, and 50 were tested.

Combinations of two antibiotics. All combinations of Timentin (125 and 250 µg/ml) and gentamicin (6.25 and 12.5 µg/ml), Timentin (125 and 250 µg/ml) and streptomycin sulfate (250 and 500 µg/ml), and streptomycin sulfate (250 and 500 µg/ml) and gentamicin (6.25 and 12.5 µg/ml) were tested.

Combinations of three antibiotics. All possible combinations of Timentin (125 and 250 µg/ml), streptomycin sulfate (250 and 500 µ g/ml), and gentamicin (6.25, 12.5, and 25 µg/ml) were tested.

Phytotoxicity. Fragaria x *ananassa* Duch. cvs. Florida Belle, Sierra, Headliner, Fortune, and Jucunda; *F. virginiana* subsp. *glauca* (S. Watson) Staudt; and *F. virginiana* subsp. v*irginiana* Duch. were treated with single antibiotics (µg/ml): 1000 (T), 1000 (S), 1000 (D), and 50 (G); combinations of two antibiotics: 250 (T) + 12.5 (G), 250 µg/ml (T) + 500 (S), and 500 (S) + 12.5 (G); and combinations of three antibiotics: 250 (T) + 500 (S) + 25 (G).

Plant treatments. 'Jucunda' and *F. virginiana* subsp. *glauca* were inoculated with *P. corrugata* and *X. campestris* pv. *vesicatoria* and grown

for seven days. Contaminated plants were treated with the combinations of three antibiotics (Timentin, streptomycin sulfate, and gentamicin) solutions at (μg/ml) 500 (T) + 250 (S) + 25 (G), 1000 (T) + 250 (S) + 25 (G), and 1000 (T) + 500 (S) + 25 (G). After 10 d of treatment, bases of plants were touched to 523 medium (Viss et al., 1991) then transferred to multiplication medium containing 265 mg/l peptone and 88 mg/l yeast extract (Boxus and Terzi, 1987). Bacterial contaminations were recorded seven days after each three week subculture.

Statistical analysis. Analysis of variance (ANOVA) was conducted for the plant treatments in a completely randomized design using 'StatGraphic 7.0' (Statistical Graphics Corp., Rockville, MD). Multiple range test for difference of means was performed by LSD.

Results and discussion

Single antibiotic treatments. Timentin was most effective against xanthomonads and some unknown strains. Pseudomonads, Enterobacteria, and other unknown species required higher concentrations (up to more than 1,000 μg/ml) (Table 3.1, 3.2).

Gentamicin was bactericidal for most isolates at 3 μg/ml for pseudomodads, 6.25 μg/ml for xanthomonads, 12.5 μg/ml for Enterobacteria, and at various concentrations up to more than 50 μg/ml for unidentified bacteria (Table 3.1, 3.2).

Effective dihydrostreptomycin concentrations varied among the different pseudomonad and xanthomonad strains. The concentrations required to kill pseudomonads and xanthomonads varied from less than 62.5 μg/ml to more than 1,000 μg/ml. Dihydrostreptomycin was bactericidal at approximately 62.5 μg/ml for Enterobacteria and other unidentified isolates (Table 3.1, 3.2).

Streptomycin was bactericidal at 62.5 μg/ml for most pseudomonads, except one strain of *P. fluorescens* type F and *P. paucimobilis*. Results were varied for xanthomonads and the unidentified isolates. *Enterobacteria cloacae* A was killed at concentrations greater than 125 μg/ml (Table 3.1, 3.2).

Combinations of two antibiotics. Combinations of gentamicin + streptomycin, and Timentin + streptomycin were effective in killing the bacteria. The combination of gentamicin + streptomycin at 6.25 + 250 μ g/ml killed all pseudomonads, *X. campestris* pv. *vesicatoria*, one unidentified *Xanthomonas* spp, and unidentified isolates. *P. paucimobilis* was killed only at concentrations as high as 12.5 + 500 μg/ml (Table 3.5). Similar results were found with Timentin and streptomycin combined,

Table 3.1

Minimal bactericidal concentrations in µg/ml determined at pH 6.9 for four antibiotics against pseudomonads isolated from strawberry runner explants.

Bacterial strain	Isolate	Timentin	Gentamicin	Dihydrostreptomycin	Streptomycin sulfate
P. corrugata	68d	>500	<3	<125	<62.5
	131	>500	>3	<125	<62.5
P. fluorescens type A	90a	>1000	<3	<62.5	<62.5
P. fluorescens type F (1)	19b	>62.5	<3	>250	<62.5
	20	>500	>3	>1000	<62.5
	212	>500	<3	>1000	>1000
	215	>500	<3	<62.5	<62.5
P. fluorescens type F (2)	19e	>1000	<3	<62.5	<62.5
	624	>1000	<3	>62.5	<62.5
P. tolaasii	557b	>1000	<3	<62.5	<62.5
P. paucimobilis	180	>125	<3	>1000	>1000

(1) = Fluorescent on King's B medium
(2) = Non-fluorescent on King's B medium

Table 3.2

Minimal bactericidal concentrations in μg/ml determined at pH 6.9 for four antibiotics against two xanthomonads, one enterobacterium, and four unidentified species isolated from strawberry runner explants.

Bacterial strain	Isolate	Timentin	Gentamicin	Dihydrostreptomycin	Streptomycin sulfate
X. camestris pv *vesicatoria*	557a	>500	>6.25	<62.5	<62.5
Xanthomonas spp	270(6)	>250	>6.25	>1000	>1000
	270(c)	<62.5	>6.25	>1000	>1000
	978				
Enterobacter cloacae A	655	>1000	>12.5	<62.5	>500
unknown species	90b	>125	<3	<62.5	<62.5
	170a, 248d	>500	>50	<62.5	<500
	248e	>1000	>50	>125	<62.5

Table 3.3

Growth of pseudomonads treated with antibiotic combinations (μg/ml) of streptomycin sulfate and gentamicin.

Bacterial strain	Isolate	500+12.5	250+12.5	500+6.25	250+6.25	Untreated
P. corrugata	68d	-	-	-	-	+
	131	-	-	-	-	+
P. fluorescens type A	25	-	-	-	-	+
P. fluorescens type F(1)	19b	-	-	-	-	+
P. fluorescens type F(2)	624	+	+	+	+	+
	320e	-	-	-	-	+
P. fluorescens type G	82b	-	+	+	+	+
P. tolaasii	557b	+	+	+	+	+
P. paucimobilis	180	-	-	-	+	+

(1) = Fluorescent on King's B medium
(2) = Non-fluorescent on King's B medium

Table 3.4

Growth of xanthomonads, enterobacterium, and other unidentified bacteria treated with antibiotic combinations (μg/ml) of Timentin and gentamicin.

Bacterial strains	Isolate	250 + 12.5	250 + 6.25	125 + 12.5	125 + 6.25	Untreated
X. campestris pv. *vesicatoria*	557a	-	-	-	-	+
Xanthomonas spp.	270(6)	-	-	-	+	+
	270c	-	-	+	+	+
	978	-	-	-	-	+
Enterobacter cloacae A	655	-	-	-	+	+
Unknown species	90b	-	-	-	-	+
	170a, 248d	-	-	+	+	+
	248e	+	+	+	+	+

Table 3.5

Growth of *P. paucimobilis*, xanthomonads, enterobacterium, and other unidentified bacteria treated with antibiotic combinations (μg/ml) of Timentin and gentamicin.

Bacterial strains	Isolate	250 + 12.5	250 + 6.25	125 + 12.5	125 + 6.25	Untreated
P. paucimobilis	180	-	+	+	+	+
X. campestris pv. *vesicatoria*	557a	-	-	-	-	+
Xanthomonas spp.	270(6)	-	-	-	-	+
	270c	-	-	-	-	+
	978	-	-	-	-	+
Enterobacter cloacae A	655	-	-	-	-	+
Unknown species	90b	-	-	-	-	+
	170a, 248d	+	+	+	+	+
	248e	+	+	+	+	+

Table 3.6

Growth of *P. paucimobilis*, xanthomonads, enterobacterium, and other unidentified bacteria treated with antibiotic combinations (μg/ml) of Timentin and streptomycin sulfate.

Bacterial strains	Isolate	250 + 500	250 + 250	125 + 500	125 + 250	Untreated
P. paucimobilis	180	-	-	+	+	+
X. campestris pv. *vesicatoria*	557a	-	-	-	-	+
Xanthomonas spp.	270(6)	-	-	+	+	+
	270c	-	-	+	+	+
	978	-	-	-	-	+
Enterobacter cloacae A	655	-	-	-	-	+
Unknown species	90b	-	-	-	-	+
	170a, 248d	-	-	-	-	+
	248e	-	-	-	-	+

which at 125 + 250 μg/ml, killed all pseudomonads, *X. campestris* pv. *vesicatoria*, one unidentified *Xanthomonas* spp., and the unidentified bacteria at 125 + 250 μg/ml. *P. paucimobilis* was killed at 250 + 250 μ g/ml (Table 3.6).

Results of the combination of timentin + gentamicin were unsatisfactory. Many pseudomonads, xanthomonads, and unknown strains were only killed at very high concentrations (Table 3.3, 3.4).

Combinations of three antibiotics. All bacterial strains tested were killed at all concentrations tested when a combination of all three antibiotics were used.

Minimal bactericidal concentrations (MBCs). MBCs should be tested to use as reference concentrations for determining plant treatments (Leifert et al., 1991). The concentrations used to treat plants usually are two to four times greater than MBCs (Leifert et al., 1991). Plants in this study were treated with combinations of antibiotics, since those were the only treatments effective on all isolates. 'Jucunda', inoculated with *X. campestris* pv. *vesicatoria,* required eight times the Timentin, two times the streptomycin, and four times the gentamicin over the lowest MBC of the three antibiotics in combination to produce 23% bacteria-free plants. But 'Jucunda' inoculated with *P. corrugata* was 100% clean when treated with a combination of the three antibiotics at concentrations four times

the Timentin, one times the streptomycin, and four times the gentamicin over the lowest MBC.

Phytotoxicity tests. Buckley et al. (1995) found that several types of antibiotic phytotoxicities effect mint cultures. These included stunting, yellowing, curling, bleaching of leaves or death, depending upon the antibiotic and its concentration. None of seven strawberry genotypes treated with single and two- and three-antibiotic combinations showed toxicity to the antibiotics tested. 'Jucunda' exhibited some leaf bleaching at antibiotic concentrations higher than those tested for phytotoxicity, but recovered when removed from treatment solutions.

Plant treatments. Both bacterial strains were lethal to *F. virginiana* subsp. *glauca*, so it was not treated with antibiotics. Ten percent of 'Jucunda' plants were bacteria-free after treatment with 500 (T) + 250 (S) + 25 (G), and 23.33% were uncontaminated when treated with 1000 (T) + 500 (S) + 25 (G) or 1000 (T) + 250 (S) + 25 (G). 'Jucunda' inoculated with *P. corrugata* were 100% bacteria-free after all treatments.

Detection of bacteria from treated plants. When 523 medium was used to detect bacteria after antibiotic treatments, only 3% of 'Jucunda' appeared contaminated with *X. campestris* pv. *vesicatoria* (data not shown). Contamination detected in medium with peptone and yeast extract was 90% for the antibiotic combination of (μg/ml): 500 (T) + 250

(S) + 25 (G), and 77% for both antibiotic combinations (μg/ml): 1000 (T) + 250 (S) + 25 (G) and 1000 (T) + 500 (S) + 25 (G). The results showed that the number of plants remaining contaminated with bacteria after treatment with (μg/ml): 500 (T) + 250 (S) + 25 (G) was significantly worse (P-value = 0.02) than those treated with either (μg/ml): 1000 (T) + 250 (S) + 25 (G) or 1000 (T) + 500 (S) + 25 (G). Carry over of antibiotics probably interfered with bacterial growth on 523 medium. These results suggested 523 medium would be useful as an indexing medium only after at least one subculture following treatment.

Conclusions

Determination of minimal bactericidal concentrations (MBCs) helped determine proper treatment concentrations. Pseudomonads, *X. campestris* pv. *vesicatoria*, and some unknown strains were easily killed by gentamicin, streptomycin, and dihydrostreptomycin but some fluorescent pseudomonads, some xanthomonads, and *P. paucimobilis* were not. Timentin was not effective against most pseudomonads, *X. campestris* pv. *vesicatoria*, enterobacteria, and others, but was effective against bacteria resistant to gentamicin, streptomycin, and dihydrostreptomycin. The combinations of Timentin, streptomycin, and gentamicin showed promising results against all the bacteria. All bacterial strains were killed even at the lowest concentrations: μg/ml 125 (T) + 250 (S) + 6.25 (G). Phytotoxicity was not a problem with these

antibiotics. The three antibiotics combined produced different results depending on the plant and bacterial genotypes. *P. corrugata* inoculated into 'Jucunda' was killed, but *X. campestris* pv. *vesicatoria* was killed in only 10-23% of the plants treated.

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Appendices

Appendix 1

Strawberry genotypes screened for contaminants.

LOCAL ID	PLANT NAME	TAXON	AUTHORITY	BACTERIA FOUND	
379.001	OF 34	*Fragaria chiloensis*	(L.) Duchesne		
366.001	CA 1466	*Fragaria chiloensis* subsp. *lucid*	(Vilm.) Staudt		
34.001	Redwood Creek Park -37	*Fragaria chiloensis* subsp. *lucid*	(Vilm.) Staudt		
408.001	Yaquina A	*Fragaria chiloensis* subsp. *pacifica*	Staudt		
151.001	Profumata de Tortina	*Fragaria moschata*	Duchesne	*P. fluorescens* type F (1)	*P. fluorescens* type
157.001	*Fragaria moschata*	*Fragaria moschata*	Duchesne		
96.001	UC-06	*Fragaria vesca* subsp. *bracteata*	(A. A. Heller) Staudt		
556.001	*Fragaria vesca* subsp. *bracteata*	*Fragaria vesca* subsp. *bracteata*	(A. A. Heller) Staudt		
557.0.01	*Fragaria vesca* subsp. *bracteata*	*Fragaria vesca* subsp. *bracteata*	(A. A. Heller) Staudt	*X. campestris* pv. *vesicatoria*	*P. tolaasii*
558.001	*Fragaria vesca* subsp. *bracteata*	*Fragaria vesca* subsp. *bracteata*	(A. A. Heller) Staudt	*P. fluorescens* type F (1)	*P. corrugata*
95.001	UC-05	*Fragaria vesca* subsp. *californica*	(Cham. & Schldl.) Staudt		

LOCAL ID	PLANT NAME	TAXON	AUTHORITY	BACTERIA FOUND
573.001	Frost King	*Fragaria vesca* subsp. *vesca*		
75.001	UC-04	*Fragaria vesca* subsp. *vesca*		
338.001	CA 1226	*Fragaria virginiana* subsp. *glauca*	(S. Watson) Staudt	
276.001	*Fragaria virginiana* subsp. *glauca*	*Fragaria virginiana* subsp. *glauca*	(S. Watson) Staudt	
549.001	*Fragaria virginiana* subsp. *glauca*	*Fragaria virginiana* subsp. *glauca*	(S. Watson) Staudt	
280.001	*Fragaria virginiana* subsp. *platy*	*Fragaria virginiana* subsp. *platypetala*	(Rydb.) Staudt	
76.001	UC-10	*Fragaria virginiana* subsp. *virginiana*		
74.001	UC-11	*Fragaria virginiana* subsp. *virginiana*		
77.001	UC-12	*Fragaria virginiana* subsp. *virginiana*		

LOCAL ID	PLANT NAME	BACTERIA FOUND		
Cultivar	*Fragaria x ananassa* Duchesne			
401.001	Aberdeen			
68.001	Aiko	*P. fluorescens* type F (1)	*P. corrugata*	
964.001	Annapolis			
414.001	Anneliese			
170.001	Armore	Gram - unidentified		
7.001	Badgerbelle			
270.001	Badgerglo	*Xanthomonas* spp		
148.001	Beaver	*P. fluorescens* type F (1)	Gram + unidentified	*P. corrugata*
505.001	Beaver Sweet			
115.001	Blakemore			
526.001	Bountiful			
320.001	CA 64.28-18	*P. fluorescens* type F (2)		
331.001	CA 70.27-103			
570.001	Califour			
166.001	Cardinal			
3.001	Catskill			
27.001	Cavalier			
90.001	Cheam	*P. fluorescens* type A	*P. fluorescens* type F (1)	
1201.001	Clare			
403.001	Columbia			

LOCAL ID	PLANT NAME	BACTERIA FOUND	
Cultivar	*Fragaria x anana* Duchesne		
13.001	Cyclone		
211.001	Dabreak		
212.001	Delite	*P. fluorescens* type F (1)	
260.001	Deutsch Evern		
71.001	Douglas		
494.001	Dunlap		
1.001	Earliglow		
534.001	EarliMiss		
171.001	Early Midway		
624.001	EB 185	*P. fluorescens* type F (2)	
138.001	Fairfax		
633.001	Florida 70-D-34		
4.001	Florida Belle		
271.001	Fou Chu		
147.001	Garnet		
236.001	Grenadier	*P. fluorescens* type F (1)	Gram - unidentified
25.001	Guardian	*P. fluorescens* type A	
215.001	Honeoye	*P. fluorescens* type F (1)	
82.001	Hood	*P. fluorescens* type G	*P. fluorescens* type

LOCAL ID	PLANT NAME	BACTERIA FOUND		
Cultivar	*Fragaria x ananass* Duchesne			
221.001	Howard 17 (Premier			
200.001	Hsing Yu			
128.001	Big Joe			
217.001	Komsomalka			
120.001	Koralovaya			
496.001	Korona			
199.001	Kurume			
79.001	Linn			
248.001	Litessa	*P. corrugata*	Gram + unidentified	Gram unidentified (2)
14.001	Marlate			
659.001	Mars			
511.001	Marshall			
219.001	Marsyalakaya			
445.001	MDUS 3022			
649.001	MDUS 4774			
652.001	MDUS 5012			
653.001	MDUS 5097			
654.001	MDUS 5120			
655.001	MDUS 5130	*Enterobacter cloacae* A		

LOCAL ID	PLANT NAME	BACTERIA FOUND
Cultivar	*Fragaria x ananassa* Duchesne	
657.001	MDUS 5189	
164.001	Midway	
406.001	Molalla	
132.001	Morioka 17	
119.001	Narcissa	
660.001	NC 3892	
220.001	Northland	
78.001	Northwest	
84.001	Olympus	
113.001	Orland	
940.001	ORUS 3727 ORUSM 264	
943.001	ORUS 4916 UCM 731	
939.001	ORUS 740-7	
267.001	Pantagruella	
152.001	Prelude	
116.001	Primella	
509.001	Protem	
86.001	Puget Beauty	

LOCAL ID	PLANT NAME	BACTERIA FOUND	
Cultivar	*Fragaria x ananassa* Duchesne		
579.001	Red Cross		
396.001	Red Giant		
225.001	Redcoat		
529.001	Redcrest		
978.001	Redgem	*Xanthomaonas* spp.	Gram - unidentified
184.001	Rosanne		
1168.001	Seneca		
153.001	Sentinel		
29.001	Sequoia		
185.001	Sivetta		
973.001	Solprins		
183.001	Sparkle		
154.001	Stoplight		
235.001	Stoplight seedling		
19.001	Sumner	*P. fluorescens* type F(1)	
180.001	Superbe Remontant Delbard	*P. paucimobilis*	
443.001	Suwannee		
229.001	Tago		
254.001	Tenira		

LOCAL ID	PLANT NAME	BACTERIA FOUND	
131.001	Tennessee Beauty	*P. fluorescens* type F(1)	*P. corrugata*
6.001	Titan		
81.001	Totem		
664.001	Trumpeter		
232.001	Tyee		
20.001	Vermilion	*P. fluorescens* type F(1)	

Appendix 2.1

Carbon source utilization of *Pseudomonas fluorescens*.

Carbon Sources[y]	Isolates[x]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Water	-	-	-	-	-	-	-	-	-	-	-	-	-
α-cyclodextrin	-	-	-	-	-	-	-	-	+	-	-	-	-
Dextrin	-	-	-	-	-	-	-	-	+	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	+	-	-	-	-
Tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-galactosamine	-	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-D-glucosamine	+	+	-	-	-	+	+	+	+	-	-	+	+
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-arabitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-
i-Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-fucose	-	-	-	-	-	+	/	-	+	-	+	-	-
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-	-	-	-	-	-	-
α-D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
m-Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-lactose	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactulose	-	-	-	-	-	-	-	-	-	-	-	-	-
Maltose	+	/	+	-	-	-	-	/	-	-	+	-	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mellibiose	-	-	-	-	-	-	-	-	-	-	-	-	-
β-methyl D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-
D-psicose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-
D-sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+
Turanose	-	-		-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+
Mono-methyl succinate	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
cis-Aconitic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Citric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Formic acid	+	/	+	+	+	+	+	+	+	+	+	+	+
D-galactonic acid lactone	+	+	+	+	+	+	+	+	+	+	+	+	+
D-galacturonic acid	+	+	+	+	+	+	+	+	+	+	+	+	+

Carbon Sources	Isolates												
	1	2	3	4	5	6	7	8	9	10	11	12	13
D-gluconic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucosaminic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucuronic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-hydroxybutyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
β-hydroxybutyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
γ-hydroxybutyric acid	-	-	-	-	-	-	-	-	-	-	-	-	-
p-hydroxyphenylacetic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Itaconic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
α-keto butyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-keto glutanic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
α-keto valeric acid	+	/	+	-	+	-	-	-	+	-	+	+	+
D,L lactic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Malonic acid	+	+	+	+	+	+	+	+	+	+	/	+	+
Propionic acid	+	+	+	+	-	+	+	+	+	+	+	+	+
Quinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
D-saccharic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Sebacic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Bromo succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucuronamide	+	+	+	+	+	+	+	+	+	+	+	+	+
Alaninamide	+	+	+	+	+	+	-	+	+	+	+	+	+
D-alanine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-alanine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-alaryl-glucine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-aspatic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
L-glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycyl-L-aspartic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycyl-L-glutamic acid	+	+	+	+	+	+	+	+	+	-	+	+	+
L-histidine	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydroxy L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+
L-leucine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-omithine	+	+	+	+	-	+	+	+	+	+	+	+	+

Carbon Sources	Isolates												
	1	2	3	4	5	6	7	8	9	10	11	12	13
L-phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-
L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+
L-pyroglutamic acid	+	-	+	-	+	+	-	+	+	+	+	+	+
D-serine	+	+	+	+	+	+	+	+	+	+	+	+	/
L-serine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-theronine	+	+	+	+	+	+	+	+	+	+	+	+	+
D,L-camitine	+	+	+	+	-	+	+	+	+	+	+	+	+
γ-amino butyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Urocanic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Inosine	+	+	+	+	+	+	+	+	+	+	+	+	+
Uridine	+	+	+	+	+	+	+	+	+	+	+	+	+
Thymidine	-	+	-	-	-	-	-	-	-	-	-	-	-
Phenyl ethylamine	-	-	-	-	-	-	-	-	-	-	-	-	-
Putrescine	-	-	-	-	/	-	-	+	-	-	-	-	-
2-Amino ethanol	+	+	+	+	+	+	+	+	+	+	+	+	+
2,3-Butanediol	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
D,L-α-glycerol phosphate	+	+	+	-	/	+	+	-	+	-	-	-	+
Glucose-1-phosephate	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose-6-phosephate	-	-	-	-	-	-	-	-	-	-	-	-	-

x	Isolates	Similarity index
	1. 19	0.942
	2. 19	0.866
	3. 20	0.942
	4. 68	0.729
	5. 131	0.816
	6. 148	0.759
	7. 148	0.501
	8. 151	0.607
	9. 212	0.715
	10. 320	0.811
	11. 320	0.801
	12. 558	0.835
	13. 624	0.939

y

- \- Negative (No color)
- \+ Positive (Purple)
- / Weak positive (Faint purple)

Appendix 2.2

Carbon source utilization of *Pseudomonas* spp.

Carbon Sources[y]	Isolates[x]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Water	-	-	-	-	-	-	-	-	-	-	-	-	-
α-cyclodextrin	-	-	-	-	-	-	-	-	-	-	+	-	-
Dextrin	-	-	/	-	-	-	-	-	-	-	+	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	+	-	-
Tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80	+	+	+	+	+	+	+	+	+	+	+	+	+
N-acetyl-D-galactosamine	-	-	-	-	-	-	-	-	-	-	-	-	-
N-acetyl-D-glucosamine	-	-	+	+	+	-	+	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-	+	+	+	-	-	+	+
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-arabitol	+	+	+	+	+	+	+	+	+	+	-	+	+
Cellobiose	-	+	-	-	-	-	-	-	-	-	+	-	-
i-Erythritol	-	-	-	+	-	-	-	-	-	-	-	-	-
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
L-fucose	-	-	-	-	-	-	+	+	+	-	/	-	-
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Gentiobiose	-	-	-	-	-	-	-	-	-	-	+	-	-
α-D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
m-Inositol	+	+	+	+	+	+	+	+	+	+	-	+	+
α-D-lactose	-	-	-	-	-	-	-	-	-	-	+	-	-
Lactulose	-	-	-	-	-	-	-	-	-	-	+	-	-
Maltose	/	-	-	-	-	/	+	-	-	/	+	-	-
Mannitol	+	+	+	+	+	+	+	+	+	+	-	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
D-mellibiose	-	-	-	-	-	-	-	-	-	-	+	-	-
β-methyl D-glucoside	-	-	-	-	-	-	-	-	-	-	+	-	-
D-psicose	+	+	/	+	-	/	+	+	+	+	+	+	-
D-raffinose	-	-	-	+	-	-	-	-	-	-	+	-	-
L-rhamnose	-	-	-	-	-	-	+	+	+	-	-	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+	-	+	+
Sucrose	+	+	+	+	+	+	-	-	-	+	+	-	-
D-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+
Turanose	-	-	-	-	-	-	-	-	/	-	+	-	-
Xylitol	-	-	-	-	-	-	+	-	/	-	-	-	-
Methyl pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+
Mono-methyl succinate	+	+	+	+	-	+	+	+	+	+	+	+	+
Acetic acid	+	+	+	+	/	+	+	+	+	+	+	/	/
cis-Aconitic acid	+	+	+	+	+	+	+	+	+	+	/	+	+
Citric acid	+	+	+	+	+	+	+	+	+	+	-	+	+
Formic acid	+	+	/	+	+	+	+	+	+	+	/	+	/
D-galactonic acid lactone	+	+	+	+	+	+	+	+	+	+	-	+	+
D-galacturonic acid	+	+	+	+	+	+	+	+	+	+	-	+	+

Carbon Sources	Isolates												
	1	2	3	4	5	6	7	8	9	10	11	12	13
D-gluconic acid	+	+	+	+	+	+	+	+	+	+	-	+	+
D-glucosaminic acid	+	+	+	+	+	+	+	+	+	+	-	+	+
D-glucuronic acid	+	+	+	+	+	+	+	+	+	+	-	+	+
α-hydroxybutyric acid	/	/	/	-	-	+	+	+	+	+	+	-	/
β-hydroxybutyric acid	+	+	+	+	+	+	+	+	+	+	+	+	+
γ-hydroxybutyric acid	-	-	-	-	-	-	-	+	-	-	-	-	-
p-hydroxyphenylacetic acid	-	-	-	+	-	-	-	-	-	-	-	-	-
Itaconic acid	-	-	-	+	-	-	+	+	+	+	/	+	+
α-keto butyric acid	-	/	-	+	-	+	+	+	+	+	+	-	/
α-keto glutanic acid	+	+	+	+	+	+	+	-	+	+	+	+	+
α-keto valeric acid	/	-	-	-	-	+	/	+	+	+	/	-	-
D,L lactic acid	+	+	+	+	+	+	+	+	+	+	/	+	+
Malonic acid	+	+	+	-	+	+	+	+	+	+	-	+	+
Propionic acid	+	+	+	-	-	+	+	+	+	+	/	-	/
Quinic acid	+	+	+	+	+	+	+	+	+	+	-	+	+
D-saccharic acid	+	+	+	+	+	+	+	+	+	+	-	+	+
Sebacic acid	-	-	-	-	-	-	-	-	-	-	-	-	-
Succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Bromo succinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucuronamide	+	/	/	+	+	+	+	+	+	+	-	+	/
Alaninamide	/	/	/	+	+	+	+	+	+	+	+	-	/
D-alanine	+	+	+	+	+	-	+	+	+	+	-	+	+
L-alanine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-alaryl-glucine	+	+	/	+	+	+	+	+	+	+	+	+	+
L-asparagine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-aspatic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
L-glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycyl-L-aspartic acid	-	-	-	-	-	-	-	-	-	-	/	-	-
Glycyl-L-glutamic acid	/	-	/	-	/	/	+	+	+	+	+	/	/
L-histidine	+	+	+	+	+	+	+	+	+	+	-	+	+
Hydroxy L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+
L-leucine	/	+	/	-	+	+	+	+	+	+	-	+	/
L-omithine	/	/	/	-	+	+	+	+	+	+	/	+	-

Carbon Sources	Isolates												
	1	2	3	4	5	6	7	8	9	10	11	12	13
L-phenylalanine	-	-	+	-	-	/	-	-	+	-	-	-	-
L-proline	+	+	+	+	+	+	+	+	+	+	+	+	+
L-pyroglutamic acid	+	+	/	+	+	+	+	+	+	/	-	+	+
D-serine	/	+	+	-	-	/	+	+	+	+	-	-	-
L-serine	+	+	+	+	+	+	+	+	+	+	+	+	+
L-theronine	+	+	+	-	+	+	+	+	+	+	+	-	/
D,L-camitine	+	+	-	+	+	+	+	+	+	+	-	+	+
γ-amino butyric acid	+	+	/	+	+	+	+	+	+	+	-	+	+
Urocanic acid	+	+	+	+	+	+	+	+	+	+	-	+	+
Inosine	+	+	+	+	+	+	+	+	+	+	-	+	+
Uridine	+	+	+	-	+	+	+	+	+	+	-	+	+
Thymidine	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenyl ethylamine	-	-	-	-	-	-	-	-	-	-	-	-	-
Putrescine	-	-	/	-	+	-	+	+	+	+	-	/	-
2-Amino ethanol	+	+	+	+	+	+	+	+	+	+	-	/	+
2,3-Butanediol	-	-	-	-	-	-	-	-	+	-	-	-	-
Glycerol	+	+	+	+	+	+	+	+	+	+	-	+	+
D,L-α-glycerol phosphate	-	+	/	-	+	-	+	+	+	+	-	-	/
Glucose-1-phosephate	-	-	-	-	-	-	-	-	-	-	+	-	-
Glucose-6-phosephate	+	-	-	-	-	-	-	+	+	-	+	-	-

x **Isolates** **Similarity index**

1. 68 (*P. corrugata*) 0.714
2. 131 (*P. corrugata*) 0.708
3. 148 (*P. corrugata*) 0.906
4. 248 (*P. corrugata*) 0.507
5. 558 (*P. corrugata*) 0.750
6. 25 (*P. fluorescens* A) 0.611
7. 90 (*P. fluorescens* A) 0.620
8. 82 (*P. fluorescens* G) 0.532
9. 82 (*P. fluorescens* type)
10. 151 (*P. fluoresnces* type)
11. 180 (*P. paucimobilis* A) 0.747
12. 80 (*P. tolaasii*) 0.708
13. 557 (*P. tolaasii*) 0.690

y
- \- Negative (No color)
- \+ Positive (Purple)
- / Weak positive (Faint purple)

Appendix 2.3

Carbon source utilization of xanthomonads and enterobacterium.

Carbon Sources[z]	Isolates[x] 1	2	3	Carbon Sources	Isolates 1	2	3	Carbon Sources	Isolates 1	2	3
Water	-	-	-	Turanose	-	-	+	D-alanine	+	+	+
α-cyclodextrin	-	-	-	Xylitol	-	-	-	L-alanine	+	+	+
Dextrin	+	+	+	Methyl pyruvate	+	+	+	L-alaryl-glucine	+	+	+
Glycogen	+	+	+	Mono-methyl succinate	+	+	+	L-asparagine	-	+	+
Tween 40	-	/	+	Acetic acid	+	+	+	L-aspatic acid	+	+	+
Tween 80	-	/	+	cis-Aconitic acid	+	-	+	L-glutamic acid	+	+	+
N-acetyl-D-galactosamine	-	-	+	Citric acid	-	+	+	Glycyl-L-aspartic acid	-	+	+
N-acetyl-D-glucosamine	+	+	+	Formic acid	-	-	+	Glycyl-L-glutamic acid	+	+	+
Adonitol	-	-	-	D-galactonic acid lactone	-	-	+	L-histidine	-	-	+
L-arabinose	-	+	+	D-galacturonic acid	-	-	+	Hydroxy L-proline	-	+	-
D-arabitol	-	-	-	D-gluconic acid	-	-	+	L-leucine	-	-	-
Cellobiose	+	+	+	D-glucosaminic acid	-	-	-	L-omithine	-	-	+
i-Erythritol	-	-	-	D-glucuronic acid	-	-	+	L-phenylalanine	-	-	-
D-fructose	+	+	+	α-hydroxybutyric acid	+	+	/	L-proline	+	/	+
L-fucose	+	+	-	β-hydroxybutyric acid	-	+	+	L-pyroglutamic acid	-	-	-
D-galactose	+	+	+	γ-hydroxybutyric acid	-	-	-	D-serine	-	-	+
Gentiobiose	+	/	+	p-hydroxyphenyl acetic acid	-	-	+	L-serine	+	+	+
α-D-glucose	+	+	+	Itaconic acid	-	-	-	L-theronine	-	-	+
m-Inositol	-	-	+	α-keto butyric acid	+	-	-	D,L-camitine	-	-	-
α-D-lactose	-	/	+	α-keto glutanic acid	+	-	+	γ-amino butyric acid	-	-	/
Lactulose	+	/	+	α-keto valeric acid	-	-	-	Urocanic acid	-	-	+
Maltose	+	+	+	D,L lactic acid	+	+	+	Inosine	-	-	+
mannitol	-	-	+	Malonic acid	+	+	+	Uridine	-	-	+
mannose	+	+	+	Propionic acid	-	/	/	Thymidine	-	-	+
D-mellibiose	+	-	+	Quinic acid	-	-	/	Phenyl ethylamine	-	-	-

	Isolates				Isolates				Isolates		
Carbon Sources	**1**	**2**	**3**	**Carbon Sources**	**1**	**2**	**3**	**Carbon Sources**	**1**	**2**	**3**
β-methyl D-glucoside	-	-	+	D-saccharic acid	-	-	+	Putrescine	-	-	+
D-psicose	+	+	+	Sebacic acid	-	-	-	2-Amino ethanol	-	-	-
D-raffinose	-	-	+	Succinic acid	+	+	+	2,3-Butanediol	-	-	-
L-rhamnose	-	-	+	Bromo succinic acid	+	+	+	Glycerol	+	+	+
D-sorbitol	-	-	+	Succinamic acid	+	+	/	D,L-α-glycerol phosphate	-	-	+
Sucrose	+	+	+	Glucuronamide	-	-	+	Glucose-1-phosephate	-	-	+
D-trehalose	+	+	+	Alaninamide	+	+	+	Glucose-6-phosephate	-	-	+

x **Isolate** **Similarity index**

1. 557 (*Xanthomonas campestris* pv. *vesicatoria*) 0.937
2. 978 (*Xanthomonas* spp.)
3. 655 (*Enterobacter* cloacae A) 0.960

y
- \- Negative (No color)
- \+ Positive (Purple)
- / Weak positive (Faint purple)