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Identification and antibiotic sensitivity of bacteria occasionally isolated from differentiated and undifferentiated cultures of Sinai medicinal plants

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ABSTRACT

Plant cells, tissues and organ cultures initiated from twenty-one plants were examined for the presence of bacterial contamination. The plants were mostly medicinal plants from St. Katherine, Sinai, Egypt. The explants were mainly derived from sterile seedling parts. *In vitro* cultures initiated from Sinai's medicinal plants were not prone to bacterial contamination. Occasional occurrence of *Methylobacterium mesophilicum* (PPFM) on plates cultured with *Datura stramonium* was observed from slightly old cultures. Contamination with *Mycobacterium scrofulaceum* infrequently occured from explants derived from mature plants grown under green house conditions. Antibiotic sensitivity of *Methylobacterium mesophilicum* and *Mycobacterium scrofulaceum* were investigated against sixteen different antibiotics using the disc diffusion method. All antibiotic tested were inhibitory to *Mycobacterium scrofulaceum*, while only seven of the tested antibiotics inhibited the growth of *Methylobacterium mesophilicum*. Though the antibiotic sensitivity of those two bacteria against various antibiotics was variant, both bacteria showed the widest inhibition zone against Refampacin and Imipenem.

KEYWORDS: Antibiotics; bacterial contamination; *Methylobacterium mesophilicum*; *Mycobacterium scrofulaceum*; plant tissue cultures.

INTRODUCTION

The main prerequisites for plant cell and tissue culture initiation are appropriate explants, suitable nutrient medium, satisfactory growth conditions and sterile technique to maintain the purity of cultures (Caponetti 1996). Therefore, plant cell and tissue cultures are usually considered to be free of microorganisms. However, there have been cases where covert bacterial contamination has been present in tissue cultures without affecting growth or being macroscopically visible (Holland & Polacco 1994). Contamination could occur at any point in the tissue culture operation. The focus should be to keep the incidences to the absolute minimum. A diverse range of bacteria may contaminate plant tissue cultures including: plant pathogens, epiphytes, endophytes and accidental contamination from air or from human during handling (Stead *et al*. 1998; Nowak *et al*. 1998). Plant endophytes and pathogens may often cause more obvious symptoms than saprophytic and other contaminant not normally considered pathogens of plants (Fellner *et al*. 1996). A range of other organisms may also contaminate plant tissue cultures (Williamson *et al*. 1998). A substantial list of bacteria, fungi and insects that contaminate cell cultures was reported (Leifert & Woodward 1998). Many of these are regularly associated with plant tissues and are difficult to detect because they need the knowledge of appropriate culture media and culturing conditions.

In general, some plant/ microbe relationships are physiologically meaningful to the plant. For example, the symbiotic interaction of Soybean/ *Bradyrhizobium japonicum* in nitrogen fixing root nodules. Also, the genetic transformation of plant tissues by *Agrobacterium spp*. is beneficial to the plant. On the contrary, tissue cultures may experience

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unappreciated plant/ microbe relationships (Wildholm 1996). Covert bacterial contamination of plant tissue cultures may represent the unappreciated kind of interactions between plants and bacteria that take place routinely in lab benches and in culture vessels (Yang 1989).

Any possibility of microbial contamination in short- or long term cultures raise the fear of interference of microbial primary or secondary metabolism with plant metabolism. Microbial contamination may change the pH of culture media (Holland & Polacco 1994), diffuse microbial metabolites such as triterpenoids (Zundel & Rhomer 1985), produce plant growth regulators such as cytokinins (Holland 1997) or vitamin B12 (Basile *et al*. 1985). Methylotrophic PPFMs bacteria are known to interfere with plant metabolism through production of enzymes (Dunleavy 1990; Holland), interact with nitrogen metabolism (Holland *et al.* 1992), stimulate germination (Holland & Polacco 1994), enhance growth (Holland 1997) and produce cytokinins (Holland & Polacco 1994).

The present research study aims to a) examine the possible bacterial contamination of plant tissue cultures initiated from medicinal plants of Sinai, b) identify the bacterial species contaminating those plant cultures, c) determine the antibiotic sensitivity of those covert bacteria and d) suggest the best strategy to minimize loss of plant materials during short- and long-term cultures.

MATERIALS AND METHODS

Plant materials: Explants from twenty-one plant species belonging to nineteen genera and from twelve plant families were used to initiate various tissue and organ cultures (Table 1). Medicinal plant species were collected from Sinai, Egypt. Plant seeds and mature plants were gathered at the growing sites and stored under optimal conditions. At the time of collection, two pressed voucher herbarium specimens were prepared per species, of which one specimen was deposited as a reference in the Herbarium of St. Catherine Research Centre, Sinai, while the duplicate was retained in the Herbarium of the Department of Botany, Faculty of Science, Suez Canal University at Ismailia. Whenever possible, flowering or fruiting specimens were collected to facilitate taxonomic identification.

Table 1: Plant tissue and organ cultures initiated from medicinal plants of Sinai and tested for bacterial contamination

Plant species	Common name	Source of seeds	Explant origin	Culture
Umbelliferae *Daucus carota* L.	Carrot	Kew Gardens	Mature root/ Sterile seedling	Callus/ cell suspension/ hairy roots/ tumors
Zygophyllaceae *1. Peganum harmala* L.	Syrian rue	St. Kath. (Sinai)	Sterile seedling	Callus/ cell suspension/ regenerated plantlets hairy roots/ tumors
2. *Zygophylum* album L.f.*	White bean caper	Sinai	Mature plant	Callus/ root culture
3. *Zygophyllum coccineum* L. *	Scarlet bean caper	Sinai	Mature plant	Callus/ root culture
Solanaceae 1. *Nicotiana tabacum* L.	Tobacco	Kew Gardens	Sterile seedling	Callus/ somatic embryos
2. *Datura stramonium* L.*	Thorn-apple	S. El-Sheikh	Sterile seedling	Callus/ cell suspension/ root culture
3. *Hyoscyamus muticus* L.	Egyptian henbane	S. El-Sheikh	Sterile seedling	Callus/ cell suspension/ root culture
Leguminosae*: Acacia nilotica* (L.) Willd. Ex Del.	Gum-arabic tree	Botanical garden	Sterile seedling	Callus/ cell suspension
Chenopodiaceae 1.*Chenopodium murale* L.*	Wall goose-foot		Mature plant	callus
2. *Cornulaca monacantha*		St. Kath.		Callus
Capparaceae*: Cleome droserfolia* (Forssk.) Del.	Cleome	Sinai	Sterile seedling	Callus/ cell suspension
Euphorbiaceae *Ricinus communis* L.	Castor bean	Botanical garden	Sterile seedling	Callus/ cell suspension
Labiatea	Basil	S. El-Sheikh	Sterile seedling	Callus/ cell suspension

1. Ocimum basilicum L.*			& Mature stem	
2. Phlomis aurea Decne	Wick weed	St. Kath.	Sterile seedling	Callus/ cell suspension
Apocynaceae	Oleander	Botanical	Sterile seedling	Callus
1. *Nerium oleander* L.		garden		
2. *Vinca rosea* L.	Periwinkle	Botanical garden	Sterile seedling	Callus
Myrtacea	Blue gum	Botanical	Sterile seedling	Callus/ cell suspension
Eucalyptus globulus Labill.	tree	garden		
Compositae	White	St. Kath.	Sterile seedling	Callus
1. *Artemisia* herba-*alba* Asso	wormwood	(Sinai)		
2. *Artemisia* judaica L.	Judean wormwood	St. Kath. (Sinai)	Sterile seedling	Callus
Sonchus oleraceus L.*	Sow-thistle	Green house	Mature stem	Callus
Scrufulariaceae	Mullein	St. Kath.	Sterile seedling	Callus/ regenerated plantlets/ root
Verbascum siniaticum		(Sinai)		culture

Plant tissue and organ cultures: Plant tissue and organ cultures were initiated either from seedling parts of sterile seeds or from explants derived from mature plants (Figure 1). Established plant cultures maintained at our laboratory in Ismailia were analysed for the presence of bacterial contamination, especially *Methylobacterium mesophilicum* or *Mycobacterium scrofulaceum*. Cultures were initiated on Murashige and Skoog (MS) medium (Murashige & Skoog 1962) medium supplemented with 0.8 % Difico agar and 3 % sucrose.

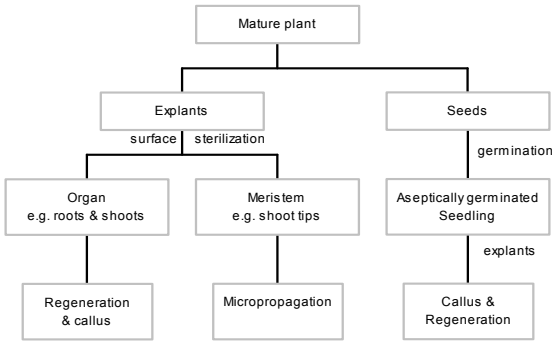

Figure 1: Schematic diagram describing common sources of explants derived either from mature plants or from aseptically germinated seedlings

The cultures were grown in the dark or in a 16 h photoperiod at 110 W/m^2 in order to test the susceptibility or resistant efficiency of plant cultures, grown at normal culture conditions, to bacterial contamination.

Callus cultures were initiated from *Peganum harmala* (Khafagi *et al*. 1996b), *Datura stramonium, Hyoscyamus muticus, Nerium oleander, Ricinus communis and Acacia nolitica* (Khafagi 1998a) on MS medium supplemented with 1 µM 2,4-dichlorophenoxy acetic acid (2,4-D). Calli initiated from *Cleome* (Khafagi 1998b), *Eucalyptus* and *Ocimum* (Khafagi 1998a) were grown on MS medium with 1 µM 2,4-D, 1 µM naphthaleneacetic acid (NAA) and 0.5 µM Kinetin. *Phlomis* and *Verbascum* calli were initiated on MS medium with 1 µM 2,4-D and 0.5 µM Kinetin (Khafagi 1998a).

Regenerated plantlets were initiated from *P. harmala* callus cultures on regeneration medium supplemented with 3 µM benzyladenene (Khafagi *et al.* 1996b) and those from *Verbascum sinaiticum* were inoculated on MS medium supplemented with 3 µM kinetin, 0.05 µM naphthaleneacetic acid and 0.05 µM indoleacetic acid (Khafagi *et al*. 1996a). Hairy root cultures initiated from *P. harmala* and carrot tissues were initiated according to Khafagi *et al*. (1995) and Khafagi & El-Gazzar (2000).

Culture homogenization: To test bacterial contamination of plant culture materials, about 0.5 g fresh weight of tissue was homogenized in 5 ml of sterile saline solution in a sterile glass-Teflon homogenizer with the pestle driven by a drill press (Widholm, 1996). The homogenate was diluted 10-fold in sterile saline solution. About 0.5 ml of the diluted and undiluted samples was spread over the surface of either Ammonium Mineral Salts (AMS) plates (Corpe & Basile, 1982) or *Mycobacterium* Agar medium (Taber *et al.* 1991). The Petri

dishes were incubated at 28°C for 10 days to allow the phytopathogenic bacteria; *Methylobacterium* or *Mycobacterium* species to grow. Control plates with either bacterium isolated from *Datura* leaves or *Ocimum* shoots respectively, were included in each experiment. Pink or yellow colonies were visible within five days. Homogenates prepared from stale media discs punched out from Petri dishes of calli or micropropagated plantlets were also investigated for bacterial contamination by the same method as used for the callus itself.

Culture indexing and bacterial elimination: An alternative method is to index callus mass organs or stale agar discs by plating directly on either AMS or *Mycobacterium* Agar media. The culture indexing method for bacterial elimination tests various plant parts for the presence of phytopathogenic bacteria. If plant pathogenic bacteria are present in the plant part or culture tested, that plant or culture is either destroyed or subjected to various procedures designed to eliminate the pathogen. The method is adapted from Raju & Olson (1985) and Klopmeyer (1996). Regular check for bacterial contamination was scheduled twice a year using both culture homogenates and culture indexing methods in order to maintain healthy in vitro cultures of medicinal plants of Sinai.

Detection and elimination of PPFM from *Datura* leaves and seeds: Seeds and young mature leaves grown wild were collected and subjected to surface disinfestation. *Datura* leaves were cut into 2 cm wide strips that were placed in Clorox with detergent for surface sterilization. Seeds and leaves were surface sterilized separately using 50% commercial Clorox with 2 drops of Tween 20 with continuous shaking and rinsed in sterile distilled water. Seeds or leaves were pressed on AMS plates. The plates were incubated at 28°C for 10 days for evaluation of the presence or absence of PPFM. Colony forming units was counted for all cultures tested.

Identification of bacterial isolates: Identification of bacterial isolates was carried out based on the API 20 NE identification kit (BioMérieux, Marcy-l'Etoile, France) (Stead *et al*. 1998). In addition to this basic differentiation, further morphological and physiological tests, as outlined in Bergey's Manual of Systematic Bacteriology (Sneath *et al*. 1986) and Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) were necessary to identify the isolates. Pure bacterial isolates used for morphological and biochemical differentiation were obtained by streaking bacterial cultures on solid culture media, followed by incubation for 48 h at 30°C under aerobic conditions in the dark. Pure bacterial cultures were then maintained in suitable media.

Bacterial growth curve: Growth curves for either *Methylobacterium* or *Mycobacterium* were prepared in order to determine the exponential phase of each bacteria to inoculate plates for antibiotic sensitivity tests. Fresh and dry weights of bacterial growth were used to draw the growth curve pattern. AMS and *Mycobacterium* were inoculated separately with pink and yellow isolates respectively (Fig. 2). Flasks were incubated for six days at 28°C on a shaker. Three replicates of each bacterial container were harvested daily for four times.

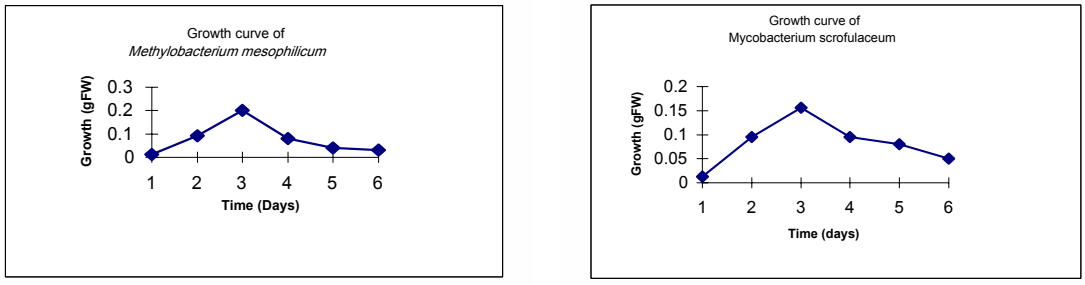

Fig.2: Growth curve of both *Methylobacterium mesophilicum* & *Mycobacterium scrofulaceum*. Bacterial fresh weight was determined for 6 days.

Antibiotic sensitivity of bacterial isolates: Growth inhibition of isolated bacteria by antibiotics was screened using commercially available antimicrobial susceptibility test discs (Oxoid, Unipath Limited, Basingstoke, Hampshire, UK) by the agar diffusion assay method. Sixteen antibiotics, having various antibacterial ranges of activity at various cellular sites in the bacterial cells, were selected for testing their inhibitory effects on those phytopathogenic bacteria. Six replicates of each antibiotic were tested for each culture. PPFM isolated from the surface of *D. stamonium* leaves and grown on AMS plates were suspended in saline solution and were added to cooled AMS medium before solidification. Medium containing PPFM was then poured into Petri dishes and allowed to harden. Antibiotic discs (6 mm diameter) were placed onto the surface of the AMS-PPFM plates. Antibiotic effectiveness against PPFM's was determined after 5 days incubation at 28C by measuring the diameter of the zone of inhibition around the discs where PPFM growth was inhibited. *Mycobacterium scrofulaceum* isolated from *Zygophyllum* shoots were maintained in *Mycobacterium* Agar medium and processed for antibiotic assay method as detailed for PPFM bacteria. Antibiotic sensitivity tests for the test bacteria were also tried on plant tissue culture media supplemented with either 0.5 % filter-sterilized methanol or 0.5 % glycerol grown at 28 C in the dark

Data analysis: The final data are reported as the mean of three replications for each antibiotic treatment. Means and the standard error of the means were calculated for the effect of sixteen antibiotics on both bacterial isolates contaminating plant tissue cultures. Analysis of variance and HSD Tukey's test (Zar 1984) were performed in order to determine the significance (P<0.05) of various antibiotics.

RESULTS

In vitro cultures initiated from medicinal plants of Sinai are free from covert bacterial contamination and can be maintained pure without fear of microbial contamination. Homogenates prepared from calli, organs or plantlets of plant cultures initiated and maintained for years in our laboratory are completely free from bacterial contamination, except for 2 % of senescent cultures initiated from *Datura stramonium* seedlings, which are contaminated with (8-15 cfu/ml) pink colonies. On the other hand, bacterial contamination with bright yellow colonies often occurs from insufficient surface sterilization of explants derived from mature *Zygophyllum album, Z. coccineum, Chenopodium murale, Ocimum basilicum and Sonchus oleraceus* plants. Count of the yellow colonies contamination insuffient surface sterilized explants were about 20-45 cfu/ml for most of the contaminating cultures tested.

Results listed in Table 1 indicate that only one plant derived from aseptically germinated seedling explants can be prone to bacterial contamination, which is *Datura stramonium.* Explants derived from outdoor plants are more prone to leaf-transmitted bacteria (Plants species with stars in Table 1). Pink colonies were identified as *Methylobacterium mesophilicum.* It is Gram-negative with non-acid fast rods which utilize methanol as the sole carbon source and gives pink to orange colonies. Yellow isolates were identified as *Mycobacterium scrofulaceum.* It is Gram-positive and acid-fast rods growing after three days on Mycobacterium Agar medium. Results of identification criteria for either bacterial isolates is documented in Table 2.

Comparison of culture homogenization and culture indexing methods reveals that culture indexing is a direct, simple and inexpensive test that does not require sophisticated laboratory equipment and supplies, while preparation of homogenates can be time-consuming. On the other hand, culture indexing was a useful and fast method for observing insufficiently sterilized seeds to be discarded before inoculation on plant tissue culture media. It can be also guide the selection of an appropriate sterilization method. However, calli

suspected of bacterial contamination need the exact number of colony forming units for evaluation of the contamination level, which can only be obtained from a homogenous layer of tissue spread over the surface of specific bacterial-formulated media.

To avoid bacterial contamination during culture of plant cells, we established the following practice in order to maintain our cultures absolutely free from bacterial contamination. It is important to: 1) check the efficiency of the surface sterilization method for seed preparation prior to seedling growth using culture indexing, 2) dip for one minute in ethanol, prior to surface sterilization is so important to minimize the microbial load of seeds, 3) sterilize seeds in small patches is important to minimize seed loss during surface sterilization, 4) carefully check for explants originated from mature plants by culture indexing, 5) transfer some callus cultures periodically into specific bacterial-formulated media for checking, 6) application of culture indexing method should be done using both solid and liquid specific bacterial-formulated media and 7) Turbidity of liquid cultures and appearance of bacterial colonies indicating positive culture contamination and should be followed with culture homogenization in order to count the contaminating colonies and know the degree of bacterial contamination of the specific culture. Plantlets initiated on the micropropagated media from *P. harmala* and *V. sinaiticum* were also investigated for any sign of contamination upon growth on specific media for either investigated bacterial species. Though *in vitro* propagation cultures were usually maintained in 100% humidity in the light, all plantlets initiated and maintained in our laboratory were healthy and completely free from bacterial contamination as recorded from both culture homogenates and culture indexing methods.

Table 2: Basic identification results of both pink and yellow bacterial isolates isolated from plant tissue cultures of medicinal plants of Sinai

	Bacteria	
Criteria	Pink isolate [a]	Yellow isolate [b]
Gram reaction	-	(+)
Acid fastness	-	+
Morphology of bacterial cells	rods, occurring in rosettes, branched and pleomorphic	short to long rods or filaments
Endospores	-	-
Motility	+	-
Pigmentation	insoluble pink-orange pigment	non-diffusing yellow pigment
Casein hydrolysis	+	n.d. [c]
Catalase activity	(+)	+
Oxidase activity	(+)	-
Aesculinase activity	(+)	n.d.
Haemolytic activity	-	-
Urease	-	+
β-Galactosidase	+	-
Nitrate reduction	+	-
Acid phosphatase	n.d.	-
α-Esterase	n.d.	(+)
Tween hydrolysis	n.d.	-
Grows at 25°C	+	+
Grows at 45°C	-	-
NaCl Tolerance	-	-
Carbon source		
Methanol	+	-
Glucose	+	+
Fructose	+	+
Mannose	+	n.d.
Mannit	+	n.d.
Maltose	-	n.d.
Malat	+	n.d.
Citrat	+	n.d.
Xylose	+	n.d.
Arabinose	+	n.d.
Aspartate	+	n.d.
Glutamate	+	n.d.
Result	*Methylobacterium meophilicum* (Austine & Goodfellow 1979) Green & Bousfield 1983	*Mycobacterium scrofulaceum* Prissick & Masson 1956

[a] Pink bacterial isolate was isolated from callus cultures of *Datura stramonium* initiated from aspetically germinated seedlings. [b] Yellow colonies were isolated from callus cultures initated from leaf discs of *Ocimum basilicum* plants grown in the greenhouse of the Department Botanical Garden. + = Positive reaction, (+) = weakly positive reaction, - = negative reaction, [c] n.d. = not determined.

Transformed cultures induced through *Agrobacterium*- mediated gene transfer system, such as tumors or hairy root cultures were also investigated for the presence of any secondary infection. Elimination of *Agrobacterium tumefaciens* and *A. rhizogens* themselves for the transformation plates and culture is easy using exogenously applied antibiotics supplement to plant tissue culture medium. Carbencillin (0.5 mg/ml) is effective in the elimination of *Agrobacterium tumefaciens* and Carbencillin (0.5 mg/ml), cefotaxime (o.3 mg/l) and vancomycin (0.2 mg/l) are useful antibiotic additive to get rid completely of *A. rhizogens.* Apart from those bacterial-transformation tools, no secondary infection was recorded either from transformed culture homogenate or culture indexing using whole transformed plant material.

Antibiotic sensitivity tests of *Methylobacterium mesophilicum* or *Mycobacterium scrofulaceum* reveals that refampacin, imipenem and oxytetracycline strongly inhibited the growth of both bacteria. Moderate to weak antibacterial activities was produced from choloramphenicol, streptomycin and vancomycin towards of *M. mesophilicum.* On the other hand, moderate antibacterial activity towards *M. scrofulaceum* was produced from several antibiotics like penicillin, semisynthetic penicillin, beta-lactams and weak activity from, cefotaxime and nitrofurans antibiotics (Table 3). Antibiotic sensitivity tests towards combination of antibiotic mixture reveals that few antibiotics give antagonistic reactions such as refampacin. Also, few antibiotic mixtures produce additive interaction towards either bacterial isolate. Synergistic reactions usually occur from closely related antibiotics, which inhibit the same mechanism of action in the prokaryotic cell, for example, Penicillin and Amoxycillin. The analysis of variance results show that refampacin and imipenem are the most significantly effective antibiotics for both bacterial organisms (Table 3).

Table 3: Antibiotic sensitivity of two bacterial species occasionally contaminating plant tissue cultures initiated from Sinai medicinal plants (diameter of inhibition zone in cm)

Antimicrobial Agent	Disc con-tent (µg)	*Meth. mesophilicum*	*Myco. scrofulaceum*
Aminoglycosides			
Gentamycin [GM][1] (Garamycin)[2]	10	0	2.13± 0.15 c
Streptomycin [S] (Streptomycin)	10	1.38± 0.06 d	2.47± 0.20 c
Rifamycins			
Refampacin [RA] (Rematan)	30	4.95± 0.03 a	3.28± 0.04 b
β-Lactams			
Penicillin [P] (Penicillin)	10	0	1.85± 0.54 c
Amoxycillin [AMX] (Amoxil)	25	0	2.92± 0.04 b
Sulbactam / Ampicillin [SAM] (Unasyn)	30	0	1.9± 0.06 c
(Cefotaxime) [CTX] (Cefotaxime)	30	0	1.12± 0.04 d
Cephazolin [KZ] (Totacef)	30	0	2.33± 0.23 c
Chloramphenicol [C] (Chloramphenicol)	30	1.9 ± 0.35 c	2.22± 0.16 c
Macrolides			
Erythromycin [E] (Erythromycin)	15	1.13 ± 0.19 d	1.9± 0.06 c
Tetracyclines			
Oxytetracycline [OT] Oxytetracycline	30	3.73± 0.18 b	3.2± 0.12 b
Lincomycins			
Clindamycin (DA) (Lincocin)	30	0	2.37± 0.12 c
Glycopeptides			
Vancomycin [VA] (Vancocin)	30	1.25± 0.03 d	2.3± 0.12 c
Carboxypenems			
Imipenem [IPM] (Tienam)	10	4.1± 0.06 a	3.6± 0.12 b
Nitrofurans			
(Nitrofurantoin) [F] (Colifuran)	200	0	0.97± 0.03 d
Norfloxacin [NOR] (Noroxin)	10	0	1.67± 0.19 d

[1] Abbreviation, [2] Trade name,
Values represent the means of replicates ± SE (n=6). Values followed by different letters are significantly different at the P> 0.05 level based on the mea comparison range using Tukey's HSD test.

The antibiotics tested illustrate a wide range from weak to strong activity for both bacteria, although, PPFM was resistance to most antibiotics. Various antibiotic combinations were investigated for maximum growth inhibition of covert bacterial contamination. Results in Table 4 list the additive, synergistic and antagonistic interaction between different combination mixtures of antibiotics. Additive and synergistic combination may be used but antagonistic action of any antibiotic hampers its application in any further combination.

Application of the selected antibiotic in its effective dose should be used for plant tissue culture remediation. The actual zones of inhibition of five selected antibiotics determined for bacterial contamination of both *Datura stramonium* and *Ocimum basilicum* callus cultures (Table 5) revealed slightly smaller than that recorded for the same bacteria in specially formulated bacteriological media (Table 3). Investigation of the phytotoxicity of those selected antibiotics on both callus cultures revealed safe usage of refampacin, impenem and vancomycin, while oxytetracycline and streptomycin produced some signs of growth inhibition to the explants.

Table 4: Antibiotic mixtures applied against *Methylobacterium* or *Mycobacterium* for bacterial growth inhibition tests in a specially formulated nutrient media. [a] Full list of the tested antibiotics and their abbrevations is indicated in Table 2; [b] n.d. = Bacteria tested is resistant to one of the antibiotics in the antibiotic-mixture.

Antibiotic mixutre	Concentration µg/disc	*Meth. mesophilicum*	*Myco. scrofulaceum*
RA/C [a]	30/30	Additive	Additive
RA/OT	30/30	Synergistic	Synergistic
RA/S	30/10	Antagonistic	Antagonistic
RA/VA	30/30	Antagonistic	Antagonistic
RA/IPM	30/10	Antagonistic	Antagonistic
IPM/VA	10/30	Synergistic	Synergistic
KZ/C	30/30	n.d. [b]	Additive
DA/SAM	30/30	n.d.	Antagonistic
P/OT	10/30	n.d.	Synergistic
P/AMX	10/25	n.d.	Synergistic
P/VA	10/30	n.d.	Synergistic
VA/AMX	30/25	n.d.	Synergistic

Table 5: Effect of five selected antibiotics supplemented to plant tissue culture medium on both callus culture and bacterial growth. [a] Phytotoxicity of antibiotics to callus culture of both plants was evaluated as any observable change of callus growth (C), decrease of chlorophyll content (Ch), growth inhibition (GI), or no phytotoxiicty (-); [b] Diameter of zones of inhibitions determined for the effect of five selected antibiotics on either *Methylobacterium mesophilicum* or *Mycobacterium scrofulaceum* inoculated to Plant tissue culture medium [c] Values represent the means of replicates (n= 6 ± SE).

		Datura stramonium		*Ocimum bascilicum*	
Antibiotic	Concentration µg/disc	Phytotoxicity[a]	Diameter of inhibition zone (cm)[b]	Phytotoxicity	Diameter of inhibition zone (cm)
Refampacin	30	-	2.5 ± 0.03 [c]	-	2.7 ± 0.12
Oxytetracycline	30	C	2.1 ± 0.06	C	2.4 ± 0.23
Imipenem	10	-	2.3 ± 0.30	-	2.8 ± 0.18
Vancomycin	30	-	1.2 ± 0.35	-	1.9 ± 0.15
Streptomycin	10	Ch	1.1 ± 0.12	Ch	2.1 ± 0.13

DISCUSSION

The scope of this study was to examine the frequently of bacterial contamination of plant tissue cultures initiated in our laboratory in Ismailia and to adapt a simple, convenient and efficient microbiological method(s) for quality control assurance. Also, to isolate, identify and determine the extent of bacterial contamination of long-term cultures of *Datura, Sonchus, Chenopodium, Ocimum* and *Zygophyllum* species. We usually initiate plant, cell, tissue and organ cultures from wild medicinal plants growing in Sinai Peninsula for either secondary

metabolite production (Khafagi 1997, 1998; Khafagi *et al*. 1995) or in vitro propagation (Khafagi 1997; Khafagi *et al*. 1996 a & b). Both biotechnological applications need the absolute insurance of microbial-free cultures.

Isolation and identification of covert bacterial contamination from *Datura stramonium* revealed the presence of pink pigmented facultative methylotrophic bacteria (PPFM), which identified as *Methylobacterium mesophilicum* (Austin & Goodfellow 1979) Green & Bousfield 1983. Similarly, *Methylobacterium mesophilicum* occurred occasionally in few plant tissue culture systems, especially callus cultures of *Datura innoxia* (Widholm 1996).

Generally, PPFM bacteria make up a large percentage of the aerobic, heterotrophic microbial populations found on leaf surfaces. It is seed-transmitted and is regularly associated with mature plants (Dunleavy 1990). PPFMs have been isolated from the leaf surfaces of many plant species (Corpe & Basile 1982). These gram-negative rod bacteria (*Methylobacterium mesophilicum*) can utilize methanol as a carbon source, but their growth on multi-carbon media can be much slower than that of other leaf heterotrophs. The utilization of methanol from leaf surfaces apparently allows PPFMs to compete with other faster growing bacteria. Corpe & Rheem (1989) suggested that degradation of methylated pectin is one likely source of methanol in the plant. Methanol production by plants in the field and in culture as well as a stimulatory effect of small quantities of methanol on plants (Corpe & Basile 1982) has been discussed.

Identification of yellow colonies isolated from few explants derived from outdoor plants unveiled the presence of *Mycobacterium scrofulaceum.* Likewise, *M. scrofulaceum* was isolated as yellow colonies contaminate the medium and plant tissue of many *in vitro* propagated ornamentales (Taber *et al.* 1991). Also, the isolation of a pigment-producing *Mycobacterium* isolate form senescent suspension and protoplast cultures derived from mature *Pinus sylvestris* L. trees was reported recently (Laukkanen *et al*. 2000).

Remediation of bacterial contamination of plant tissue cultures using exogenous antibiotics is gaining recent acceptance (Fellner *et al*. 1996; Kneifel & Leonhardt 1992). Endogenous levels of natural antimicrobials produced from *in vitro* cultures of medicinal plants play an important role in long-term defense against covert bacterial contamination (Khafagi 1998a). Sinai medicinal plants are known to contain about 75% antimicrobial activity (Khafagi & Dewedar 2000), this is also true for medicinal plants generally. About 85% of Sinai medicinal plants produce antimicrobial activity from their in vitro cultures (Khafagi 1997 & 1998a). We usually depend on this endogenous trait of antimicrobial production to guard plant tissue cultures from possible contamination. The results of this study admit that endogenous levels of antimicrobials known to produce from *in vitro* cultures of *Datura stramonium* or *Ocimum basilicum* (Khafagi 1998a) is not enough to protect cultures from seed- or leaf-transmitted bacterial contamination.

It has been reported that covert bacteria obviously can be found endogenously in the *in vitro* plants rather than on the surrounding plant tissue culture medium (Podwyszynska & Hempel 1987). In order to inhibit bacterial growth, antibiotics can be incorporated into the plant culture medium. However, it has to be taken into consideration the phytotoxicity of some antibiotics (Santos & Salema 1989) and that the repeated use of single antibiotics may cause resistance development by the bacteria. Thus antibiotics should be restricted to a very limited culture stages. According to our findings (Khafagi *et al.* 1999) and also to (Kneifel & Leonhardt 1992) only a limited number of antibiotics, such as cefotaxime, can be applied successfully without producing phytotoxicity. We demonstrated that a bacteriostatic effect could be obtained with oxytetracycline at 10µg/plate but some phytotoxicity of that antibiotic was observed on callus cultures of both *D. stramonium* and *O. basilicum.* Therefore, only those antibiotics should be applied which enable sufficient bacterial inhibition at a concentration level that dose not harm the plant tissues.

The present study has shown that the safe bacteriostatic goal can be achieved by using either 30 μg/plate refampacin or 10μg/plate imipenem towards *Methylobacterium mesophilicum*. Rifampicin interferes with mRNA formation by binding to prokaryotic RNA polymerase. Though effective, rifampicin has been used only for short periods because of rapid emergence of bacterial resistance (Falkiner 1990). Imipenem usually produce marked activity against bacterial species producing Beta-lactamases. The mechanism of action of imipenem is directed towards bacterial cell wall peptidoglycan synthesis. On the other hand, cefotaxime, the cephalosporine antibiotic, which inhibit cell wall synthesis of bacteria appears to be non-toxic to plant cells at concentration up to 100 μg/ml (Khafagi *et al.* 1999; Pollock *et al.* 1983). Cefotaxime could be used in combination with common sterilization procedures to eliminate *Mycobacterium scrofulaceum* from *in vitro* cultures of some plants.

In conclusion, *Methylobacterium mesophilicum* and *Mycobacterium scrofluaceum* should not be regarded as serious problems in most plant tissue cultures. Addition of exogenous antibiotics should be restricted to the necessary culture stages only. Regular check of bacterial contamination of both short- and long-term cultures is fundamental.

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الملخص العربى

تعريف البكتيريا المعزولة من مزارع الأنسجة المتعضية والغير متعضية لنباتات سيناء الطبية وتأثير المضادات الحيوية عليها

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تم دراسة مدى تواجد الملوثات البكتيرية فى مزارع الأنسجة، والخلايا والأعضاء المستزرعة من 21 نباتا من نباتات سيناء الطبية (سانت كاثرين- سيناء- مصر). معظم المزارع النباتية مستزرعة من أجزاء البذور المستنبتة تحت ظروف معقمة. أثبتت الدراسة أن مزارع الأنسجة المستزرعة من نباتات سيناء ليست ملوثة بالبكتيريا فيما عدا مزارع نبات الداتوره، خاصة المخزنة لبعض الوقت. يصيب مزارع نبات الداتوره النامية لفترة طويلة، نوع من البكتريا التي تتطلب وجود الكحول الميثيلى كمصدر كربونى وهى *مثيلوبكتريم ميزوفيليكم*. كما وجد أن قليل من المزارع المستزرعة من بعض أجزاء النباتات الكاملة التي تنمو فى الهواء الطلق قد تصاب بنوع من البكتريا المخاطية تسمى *ميكوبكتريم سكروفيليكم*.

تم دراسة مدى حساسية هذين النوعين من البكتريا للمضادات الحيوية المختلفة، حيث تم تجربة ستة عشر نوعاً من المضادات الحيوية التى تنتمى إلى العديد من الأقسام ولها تأثير متنوع على وظائف الخلية الميكروبية. وقد وجد أن بكتيريا *مثيلوبكتريم ميزوفيليكم* تتأثر بسبعة فقط من المضادات الحيوية بينما يتأثر النوع الثانى من البكتريا بكل المضادات المستخدمة. وبالرغم من اختلاف حساسية هذين النوعين من البكتيريا للأصناف المختلفة من المضادات البكترية إلا أنهما يتفقان فى أن الريمفابسين والأبيانيم يسببان أشد تأثير مضاد لنموهما.