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Identification of IAA-producing endophytic bacteria from micropropagated *Echinacea* plants using 16S rRNA sequencing

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Abstract

The presence of latent bacteria is a serious problem in plant tissue cultures. While endophytes are generally beneficial to plants *in situ*, they may affect culture growth under the modified conditions *in vitro*. The present study was undertaken to identify and characterize endophytic bacteria associated with the medicinal plant *Echinacea* in tissue culture. Based on classical microbiological tests and 16S rRNA analyses, it was found that endophytic bacteria associated with aseptically micropropagated *Echinacea* plantlets are representatives of several genera, *Acinetobacter*, *Bacillus*, *Pseudomonas*, *Wautersia* (*Ralstonia*) and *Stenotrophomonas*. Based on TLC and HPLC analyses, we found that *Pseudomonas stutzeri* P3 strain produces plant hormone, auxin (indole-3-acetic acid, IAA). Antibiotic resistance was also assessed as a virulence factor. The majority of endophytic bacteria were resistant to the antibiotic kanamycin, but susceptible to chloramphenicol. Recommendations for propagating *Echinacea in vitro* cultures involve the addition of chloramphenicol, tetracycline, and ampicillin, antibiotics that cause no side effects on these plant species.

Abbreviations: IAA – indole-3-acetic acid; MS – Murashige and Skoog medium; NA – nutrient agar; NB – nutrient broth; TSA – tryptic soy agar

Introduction

Echinacea is one of the most popular herbal supplements among the general public in the United States (the US market of *Echinacea* has reached $33 million per year), and the popularity of this remedy is due to its use in the treatment of infections and based on its immune-stimulatory and anti-inflammatory effects (Giles et al., 2000; Fintrac market survey 2001). Over the past 70 years, a number of components has been identified as having biological activity. It is widely accepted today that the active constituents are grouped into the unsaturated lipophilic compounds, caffeic acid phenols, and polysaccharides categories (Hall, 2003). The genus *Echinacea* is native to North America (McGregor, 1968) and according to a recent revision, *Echinacea* consists of two subgenera and four species (Binns et al., 2002). A repository of micropropagated commercially important *Echinacea* clones was created (Lata et al., 2003, 2004) to produce a reproducible source of standardized biomass for clinical studies. During the process of our plant selection, endophytic bacteria associated with *in vitro* shoot cultures of *Echinacea* were noticed after several

propagation cycles, over a nine months period. We have adopted the definition of endophytic bacteria as those microorganisms that do not visibly harm the host, live in the inner parts of the plants and can be isolated from tissues that have been previously surface disinfected (Hallmann et al., 1997). The identification of these bacteria is important for plant tissue culturists since these microorganisms gain entry and survive in microplantlets undetected (Thomas, 2004). While endophytes are generally not harmful to plants (Bacon et al., 2002), and being beneficial for the improvement of plantlet stress tolerance (Nowak et al., 1998), they may affect culture growth under the modified conditions *in vitro* and act as vitropathogens (Leifert and Cassells, 2001). Alternatively, they may induce hormone-mediated modification of *in vitro* response (Holland and Polacco, 1994), which puts into question the reproducibility of tissue culture protocols. *In vitro* propagation can also pose problems for the horticulture industry as some endophytic bacteria may overwhelm their host (Falkiner, 1990). Accordingly, increased attention has been directed to this problem by researchers seeking to characterize and identify plant-associated bacteria for purpose of developing anti-bacterial therapies for plant tissue culture (Leifert et al., 1991). The purpose of the present study was to identify and characterize endophytic bacteria associated with *Echinacea*, evaluate their resistance to antibiotic additives (antibiotic resistance patterns) during propagation, and to assess their ability to produce secondary metabolite IAA.

Materials and methods

Establishment of plant shoot cultures

The nomenclature used for *Echinacea* species is based on Binns et al. (2002) taxonomic classification. The North Central Regional Plant Introduction Station (NCRPIS), in Ames, Iowa provided seeds of *Echinacea* accessions – *E. pallida* var. *angustifolia* PI 312814 accession, *E. pallida* var. *pallida* PI 597603 accession, *E. purpurea* PI 23967 accession and *E. pallida* var. *tenneseensis* PI 631250 accession. Seeds were surface disinfected as follows: 1% NaOCl (20% v/v bleach) and 0.1% Tween 20 for 10 min, followed by washing thrice in sterile distilled water for 5 min prior to inoculation on the germination media. Aseptic seedlings were initiated on agar (0.8%) with solidified half strength MS medium without sucrose (Murashige and Skoog, 1962) using magenta jars with ventilated lids. Cultures were incubated at 25 ± 2 °C, 16 h photoperiod under fluorescent light with a photon flux of approximately 52 μmol m^{-2} s^{-1}. After 10 days, 0.5–1.0 cm long hypocotyls were taken as explants for the initiation of shoot cultures. Shoot cultures were obtained on half strength Murashige and Skoog salt medium containing 3% (w/v) sucrose, 0.8% (w/v) agar supplemented with 2.2 μM of benzyladenine (BA) per liter. The medium was adjusted to pH 5.7. The microplantlets were sub-cultured at an interval of 25 days.

Isolation, characterization, and identification of endophytic bacteria

Bacterial isolates were recovered from surface-sterilized shoot cultures of *Echinacea* propagated *in vitro* for 9 months. The isolation frequency of endophytes was calculated as the frequency of plant shoots exhibiting bacterial growth. Bacterial outgrowths from the plant surfaces were collected and sub-cultured onto tryptic soy agar (TSA). Petri plates were incubated 4–7 days at 30 °C in the dark. Isolates were purified and initially characterized using classical microbiological methods, as previously described (Halda-Alija, 2003, 2004). Media used for the characterization of bacterial isolates include MacConkey agar, Simon's Citrate agar, NA, and TSA. Seven different groups were distinguished on the basis of colony morphology, cell morphology, Gram reaction, oxidase test, and growth on various media and different temperatures (25, 30, 37, and 44.5 °C). Representative isolates, from each of the seven distinguished groups were selected and further analyzed by 16S rRNA sequencing.

16S rRNA sequencing

The 16S rRNA gene was PCR amplified from genomic DNA isolated from pure bacterial colonies (MO BIO Ultra Clean Microbial DNA Isolation Kit, MO BIO Laboratories, Solana Beach, CA, USA) following standard PCR protocols (Halda-Alija, 2003, 2004). Primers pF (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pR

(5′-AAG GAG GTG ATC CAG CCG CA-3′), a pair of highly conserved flanking sequences were used to amplify the 16S ribosomal genes. Cycle sequencing of the 16S rRNA amplification products was carried out as described by Halda-Alija (2003, 2004). Samples were separated by electrophoresis on a Beckman Coulter sequencer CEQ 8000 (Beckman Coulter Inc., Fullerton, CA) and Chromas software (2005) was used for primary edition of DNA sequences. Sequence analysis was performed by using the algorithms BLAST (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]. Multiple-sequence alignment methods were conducted using a freely available alignment program, Clustal X (version 1.81). Bacterial identifications were based on 16S rRNA gene sequence similarity (Halda-Alija, 2003, 2004). Neighbors joining phylogenetic trees (Saitou and Nei, 1987) were generated using the top 10 alignment matches. GenBank databases (Benson et al., 2002) were also used for the comparison.

The 16S rRNA sequences of endophytic bacteria reported in this paper have been deposited in the GenBank database under accession numbers: AY660543 (*Acinetobacter lwofii*) AY660544 (*Acinetobacter lwofii*), AY660542 (*Bacillus pumilus*) AY660546 (*Bacillus pumilus*), AY660545 (*Pseudomonas stutzeri*), AY660541 (*Wautersia paucula (Ralstonia pacula)*), AY660547 (*Stenotrophomonas maltophila*).

Bacterial strains sequenced were further subcultured using nutrient agar (NA) and nutrient broth (NB) for subsequent antibiotic resistance tests as given below.

Antibiotic resistance tests

Antibiotics representing different chemical families were selected and antibiotic susceptibility was determined by the plate agar and disk diffusion tests (Leboffe and Pierce, 2002). For both tests, Mueller Hinton Agar (Difco Inc.) was used. The results obtained by the plate agar method were confirmed with disk diffusion test (Leboffe and Pierce, 2002). Ten micrograms of Ampicillin, 30 μg chloramphenicol, 30 μg kanamycin, 10 μg streptomycin and 30 μg tetracycline were the antimicrobial agents that were tested individually using Kirby–Bauer disk method (Bauer et al., 1966). *Escherichia coli* ATCC 25922 was used as a control strain of known antibiotic susceptibility. The results from three replicates were averaged and statistical tests conducted as previously described (Halda-Alija, 2004).

Extraction, analysis, and quantification of indole-3-acetic acid (IAA)

To identify and quantify IAA production, 1 ml of 24 h old bacterial cultures were suspended in 100 ml nutrient broth. Bacterial cultures with and without L-tryptophan (100 mg L^{-1}) were individually grown for 48 h then centrifuged at 6000 rpm for 10 min. The supernatant was adjusted to pH 7–8 and then extracted with methylene chloride, CH_2Cl_2 (30 ml×3). The combined CH_2Cl_2 layers were discarded and the aqueous culture media was acidified with acetic acid to pH 2–3 and extracted again with CH_2Cl_2 (30 ml×3). The combined extracts were washed with H_2O (50 ml), dried sodium sulphate (Na_2SO_4), and evaporated to dryness. The residue was dissolved in 0.2 ml of methanol (MeOH) for TLC analysis, which was performed on silica gel sheets (Alugram® Sil G/UV_{254}, Macherey-Nagel, Germany) using the solvent system benzene–acetone–acetic acid (13:6:1) and reversed-phase plates (RP-18 F_{254S}, Merck, Germany) using the solvent system MeOH–H_2O (65:35). IAA was viewed under UV at 254 nm with R_f values of 0.53 and 0.54, respectively, in the above two systems.

Samples were then reconstituted in 1.0 ml of methanol for HPLC analysis. The HPLC instrument used was Waters LC Module I (Milford, MA, USA) equipped with a UV variable-wavelength spectrometer. A Discovery® C_{18} column (15× 4.6 mm, 5 μm) (Supelco, Bellefonte, PA, USA) and a mobile phase of MeOH–1% aqueous acetic acid (30:70) at a flow rate of 0.8 ml min^{-1} were employed. The UV detector was set at 254 nm. The retention time of IAA was detected at 17.06 min. IAA from Sigma Chemical Co., St. Louis, Mo., USA was used for identification and quantification purposes. The detection limit of the column was 0.3 μg ml^{-1}.

Results and discussion

Authenticity, plant genetic identity and genetic diversity within and among plant populations are

known to affect the quality of herbal products (Giles et al., 2000; Lindenmuth and Lindenmuth, 2000). In this study, we have characterized and identified endophytes that are propagated via planting material and are closely associated with *Echinacea*. Endophytic bacteria were observed in *Echinacea* micropropagated shoot cultures after 9 months *in vitro*. Thirty-nine isolates were selected based on colony morphology from four different species of *Echinacea* (20 isolates from *E. pallida* var. *angustifolia*, 4 from *E. pallida* var. *pallida*, 7 from *E. purpurea*, and 8 isolates from *E. pallida* var. *tenneseensis*). Representative isolates were chosen from each plant cultivar for further analysis (Table 1). The species level match was assigned to *Pseudomonas stutzeri, Acinetobacter lwofii*, *Bacillus pumilus, Wautersia paucula* (*Ralstonia paculua)*, and *Stenotrophomonas maltophila* (Table 1), using neighbors joining phylogenetic trees (Saitou and Nei, 1987). The endophytic bacterial community isolated from *Echinacea* included *Bacillus pumilus* (from *E. purpurea*), *Acinetobacter lwofii* and *Stenotrophomonas maltophila* (from *E. pallida* var. *angustifolia*), *Bacillus pumilus* (from *E. pallida* var. *pallida*), and *Pseudomonas stutzeri* and *Wautersia paucula* (from *E. pallida* var. *tenneseensis*). *W. paucula* was previously known as *Ralstonia paculua* (Vaneechoutte et al., 2004). *P. stutzeri*, a species known with exceptional genetic diversity (Rius et al., 2001), has been previously characterized as a rice endophyte (You et al., 1991; Rediers et al., 2003). *Bacillus pumilus* has been identified in citrus plants (Araujo et al., 2002), while *Stenotrophomonas maltophilia* has been reported to produce a range of anti-fungal metabolites (Minkwitz and Berg, 2001).

To test host specificity of endophytes, isolates were grown on MS medium without plants. None of the isolates exhibited growth on sucrose-free MS medium without the plants (H. Lata, and L. Halda-Alija, unpublished data, 2004); however, all isolates grew well on richer media (both TSA and NA) and on MS medium containing sucrose.

For the extraction, analysis, and quantification of indole-3-acetic acid (IAA), the representative isolates were chosen from each plant cultivar. IAA was clearly identified on TLC in the samples extracted from cultures of *Pseudomonas stutzeri* with and without L-tryptophan added to the culture media. The presence of IAA in *Pseudomonas stutzeri* was further confirmed and quantified by reversed phase high performance liquid chromatography (Figure 1). The yields of IAA with and without L-tryptophan are 188 ± 25 and 134 ± 10 $\mu g\ L^{-1}$, respectively (Figure 1). Our results corroborate with the findings of Halda-Alija (2003); *P. stutzeri* has produced IAA even when L-tryptophan, a precursor for IAA, was not added to the media either because bacteria synthesize L-tryptophan or IAA synthesis may occur independently of L-tryptophan (Halda-Alija, 2003).

While endophytes are generally beneficial to plants *in situ* (Bacon et al., 2002), they may affect culture growth under the *in vitro* conditions (Leifert and Cassells, 2001). *Azotobacter*, *Azospirillum*, *Pseudomonads*, *Acetobacter*, *Burkholderia, Enterobacter*, and *Bacilli* are growth promoting rhizobacteria (PGPR) known to be involved with production of phytohormones (Tang, 1994; Halda-Alija, 2003). However, detrimental effects of the phytohormones have also been observed as reported by Zelena et al. (1988) in corn where strong inhibition of root elongation was noticed.

Table 1. Sequence analysis of 16S rRNA of endophytic bacteria isolated from *in vitro* shoot cultures of *Echinacea*

Plant cultivar	Endophyte	Sequence analysis		
		Closest NCBI database match (accession no.)	No. of bases sequenced	% Identity
E. purpurea # 1	K3	*Bacillus pumilus* (AY660542)	1–821	97.0
E. pallida var. *angustifolia* #1	L4	*Acinetobacter lwofii* (AY660543)	1–807	98.0
E. pallida var. *angustifolia* #2	L5	*Acinetobacter lwofii* (AY660544)	1–827	97.0
E. pallida var. *angustifolia* #3	C2	*Stenotrophomonas maltophila* (AY660547)	1–797	97.0
E. pallida var. *pallida*	M2	*Bacillus pumilus* (AY660546)	1–811	96.0
E. pallida var. *tenneseensis* # 1	B2	*Ralstonia paucula* (AY660541)	1–816	96.0
E. pallida var. *tenneseensis* # 2	P3	*Pseudomonas stutzeri* (AY660545)	1–807	99.0

NCBI: National Center for Biotechnology Information.

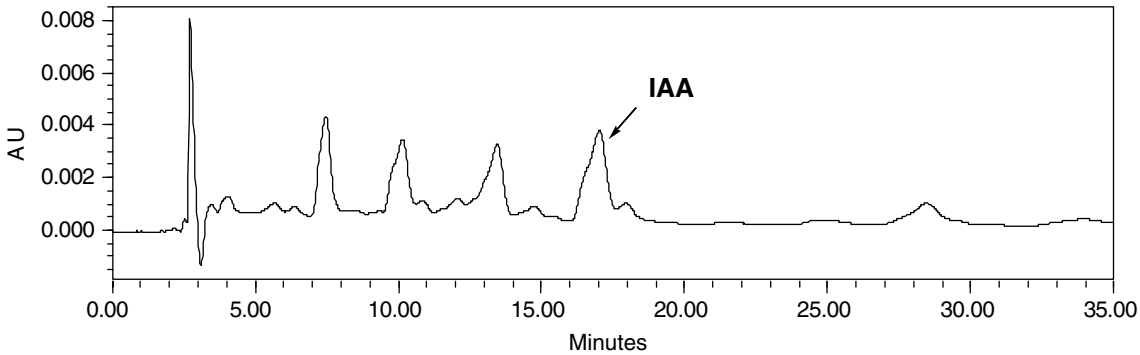

Figure 1. HPLC chromatogram for the production of indole-3-acetic acid (IAA) of endophytic bacteria *Pseudomonas stutzeri* (P3) isolated from *in vitro* shoot cultures of *Echinacea*.

In our study of these endophytes in shoot cultures of *Echinacea* the beneficial effects were not noticed. In fact, bacterial growth has surpassed plant growth causing detrimental effects to the host resulting in cessation of growth (Figure 2). Latent bacteria *in vitro* is highly undesirable as they can interfere with research conclusions and act as potential threat to micropropagation industry (Leifert and Woodward, 1998), *in vitro* gene banks (Van den Houwe and Swennen, 2000) and safe exchange of germplasm (Salih et al., 2001).

A range of antibiotics with different mechanisms of action were tested for their ability to suppress the growth of bacteria grown in pure culture (Table 2). Most isolates tested were sensitive to ampicillin, chloramphenicol, and tetracycline. It was possible to control the identified contaminants using antibiotics and autotrophic culture systems with elimination of sugar in the medium (Figure 2). These antibiotics were tested at lower concentrations to avoid side effects on plants, although long term effects on plants if any of antibiotic inclusions in culture media are unknown (Hennerty et al., 1988). Adding kanamycin to the media has caused significant tissue discoloration (R. Moraes and H. Lata, unpublished data, 2004).

This study constitutes the first report on endophytic bacteria from micropropagated shoot cultures of *Echinacea* (a medicinal plant used world-wide). At this point, it is not clear if latent endophytes have the potential to effect herbal products. However, IAA and melanin have trytophan as a common precursor. According to Pugh et al. (2005), *Echinacea* melanin is an active constituent responsible for immune stimulation of human monocytes. Thus, this study provides a baseline for assessing the mutualistic relationship between endophytic bacteria and medicinal plants. Further experiments are in progress to assess persistence and to determine the role of these bacteria in host–bacterium interactions.

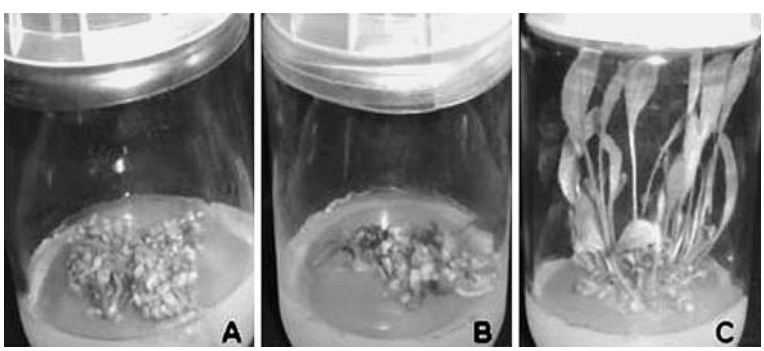

Figure 2. (A,B) Bacteria infected shoot cultures of *Echinacea*; (C) recovered shoot cultures using antibiotic ampicillin and autotrophic conditions.

Table 2. Evaluation of isolates susceptibility to well-known antibiotics

Isolate	Ampicillin		Chloramphenicol		Kanamycin		Streptomycin		Tetracycline	
	ZD	INT	ZD	INT	ZD	INT	ZD	INT	ZD	INT
K3	33.00 ± 0.00	S	18.33 ± 0.58	S	22.00 ± 0.00	S	21.33 ± 0.58	S	28.00 ± 0.00	S
L4	15.67 ± 0.58	I	25.00 ± 0.00	S	9.00 ± 0.00	R	16.67 ± 0.58	S	20.33 ± 0.00	S
L5	16.00 ± 0.00	I	23.33 ± 0.58	S	9.00 ± 0.00	R	15.33 ± 0.58	S	19.67 ± 0.00	S
C2	12.00 ± 0.00	R	17.67 ± 0.58	S	8.00 ± 0.54	R	4.00 ± 0.00	R	12.00 ± 0.00	R
M2	32.00 ± 0.00	I	17.67 ± 0.58	S	20.00 ± 0.00	S	15.67 ± 0.58	S	25.00 ± 0.00	S
B2	11.00 ± 0.69	R	10.00 ± 0.00	R	11.00 ± 0.69	R	5.00 ± 0.00	R	16.33 ± 0.58	I
P3	19.33 ± 0.58	S	17.00 ± 0.00	S	17.33 ± 0.58	S	12.00 ± 0.00	S	21.00 ± 0.00	S

ZD: Zone diameter (mm); INT: interpretation; I: intermediate; R: resistant; S: susceptible; interpretive standards: Ampicillin: ≤13 (R), 14–16 (I), ≥17 (S); Chloramphenicol: ≤12 (R), 13–17 (I), ≥18 (S); Kanamycin: ≤13 (R), 14–17(I), ≥18 (S); Stretomycin: 6 (R), 7–9 (I), ≥ 10 (S); Tetracyclin: ≤14 (R), 15–18 (I), ≥19 (S).

In conclusion, the results of this study demonstrated that (i) endophytes closely associated with *Echinacea* plantlets express phylogenetic diversity, (ii) *P. stutzeri* produced quantifiable amounts of IAA (iii) antibiotics were able to suppress growth of endophytes.

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References

Araujo WL, Marcon J, Maccheroni W Jr, Van Elsas JD, Van Vuurde JWL & Azevedol JL (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. Appl. Environ. Microbiol. 68: 4906–4914

Bacon CW, Glenn AE & Hinton DM (2002) Isolation, in planta detection and culture of endophytic bacteria and fungi. In: Hurst CJ (ed) Manual of Environmental Microbiology. 2nd edn (pp. 543–553). ASM Press, Washington, DC

Bauer AW, Kirby WM, Sherris JC & Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Path. 45: 493–496

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA & Wheeler DL (2002) GenBank. Nucleic Acids Res. 30: 17–20

Binns SE, Bernard RB & Arnason JT (2002) A taxonomic revision of *Echinacea* (Asteraceae: Heliantheae). Syst. Bot. 27: 610–632

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Falkiner FR (1990) The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. Int. Assoc. Plant Tiss. Cult. Newslett. 60: 14–21

Fintrac Market Survey: The US market for Medicinal herbs (2001) Rural Agriculture Incomes with a Sustainable Environment. March : 1–9

Giles JR, Palat CT, Chien SH, Chang ZG & Kennedy DT (2000) Evaluation of *Echinacea* for treatment of common colds. Pharmacotherapy 20: 690–697

Halda-Alija L (2003) Identification of indole-3-acetic acid producing freshwater wetland rhizosphere bacteria associated with *Juncus effusus* L. Can. J. Microbiol. 49: 781–787

Halda-Alija L (2004) Incidence of antibiotic-resistant *Klebsiella pneumoniae* & *Enterobacter* species in freshwater wetlands. Lett. Appl. Microbiol. 39: 445–450

Hall C III (2003) Echinacea as a functional food ingredient. Adv. Food Nutr. Res. 47: 114–173

Hallmann J, Quadt-Hallmann A, Mahaffee WF & Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43: 895–914

Hennerty MJ, Upton ME, Harris DP, Eaton RA & James DJ (1988) Microbial contamination of *in vitro* cultures of apple stocks M26 and M9. Acta Hortic. 225: 129–137

Holland MA & Polacco JC (1994) PPFMs and other covert contaminants: is there more to plant physiology than just plant? Annu. Rev. Plant Physiol. Plant Mol. Biol. 45: 197–209

Leifert C & Cassells AC (2001) Microbial hazards in plant tissue and cell cultures. In Vitro Cell Dev. Biol. Plant 37: 133–138

Leifert C & Woodward S (1998) Laboratory contamination management: the requirement for microbiological quality assurance. Plant Cell Tiss. Org. Cult. 52: 83–88

Lindenmuth GF & Lindenmuth EB (2000) The efficacy of *Echinacea* compounds herbal tea preparation on the severity and duration of upper respiratory and flue symptoms: a randomized, double blind placebo-controlled study. J. Altern. Complem. Med. 6: 327–634

Lata H, Andrade Z, Schaneberg B, Bedir E, Khan I & Moraes RM (2003) Arbuscular mycorrhizal inoculation enhances survival rates and growth of micropropagated plantlets of *Echinacea pallida*. Planta Med. 69: 673–676

Lata H, Andrade Z, Bedir E & Moraes RM (2004) Mass Propagation of *Echinacea angustifolia*: a protocol refinement using shoot encapsulation and temporary immersion liquid system. Acta Hortic. 629: 409–414

Leboffe MJ & Pierce BE (2002) Microbiology Laboratory Theory and Application, Morton Publishing Company, Colorado

Leifert C, Ritchie JY & Waites WM (1991) Contaminants of plant tissue and cell cultures. World J. Microbiol. Biotechnol. 7: 452–469

McGreoger RL (1968) A new species and two new varieties of *Echinacea* (Compositae). Trans. Kansas Acad. Sci. 70: 366–370

Minkwitz A & Berg G (2001) Comparison of antifungal activities and 16S ribosomal DNA sequences of clinical and environmental isolates of *Stenotrophomonas maltophilia*. J. Clin. Microbiol. 39: 139–145

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Nowak J, Asiedu SK, Bensalim S, Richards J, Stewart A, Smith C, Stevens D & Sturz AV (1998) From laboratory to applications: challenges and progress with *in vitro* dual cultures of potato and beneficial bacteria. Plant Cell Tiss. Org. Cult. 52: 97–103

Pugh N, Balachandran P, Lata H, Dayan FE, Joshi V, Bedir E, Makino T, Duke SO, Moraes RM, Khan I & Pasco DS (2005) Melanin: dietary mucosal immune stimulant from *Echinacea* and other botanical supplements. Int. Immunopharmacol. 5: 637–647

Rediers H, Bonnecarrere V, Rainey PB, Hamonts K, Vanderleyden J & Mot RD (2003) Development and application of a dapB-based *in vivo* expression technology system to study colonization of rice by the endophytic nitrogen-fixing bacterium *Pseudomonas stutzeri* A15. Appl. Environ. Microbiol. 69: 6864–6874

Rius N, Fuste MC, Guasp C, Lalucat J & Loren JG (2001) Clonal population structure of *Pseudomonas stutzeri*, a species with exceptional genetic diversity. J. Bacteriol. 183: 736–744

Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406–425

Salih S, Waterworth H & Thompson DA (2001) Role of plant tissue culture in international exchange and quarantine of germplasm in the United States and Canada. HortScience 36: 1015–1021

Tang WH (1994) Yield-increasing bacteria, (YIB) and biocontrol of sheath blast of rice. In: Ryder MH, Stephens PM & Bowen GD (eds) Improving Plant Productivity with Rhizobacteria (pp. 267–278). Commonwealth Scientific and Industrial Research Organization, Adelaide, Australia

Thomas P (2004) *In vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long term micropropagated triploid watermelon cultures. Plant Cell Tiss. Org. Cult. 77: 173–179

Van den Houwe I & Swennen R (2000) Characterization and control of bacterial contaminants in *in vitro* cultures of banana (Musa spp.). Acta Hortic. 530: 69–79

Vaneechoutte M, Kampfer P, De Baere T, Falsen E & Verschraeren G (2004) *Wautersia* gen. nov., a new genus accomdating the phylogenetic lineage including *Ralstonia eutropha* and related species, and proposal of *Ralstonia* [*Pseudomonas*] *syzygii* (Roberts et al., 1990) comb. Nov. Int. J. Syst. Evol. Microbiol. 54: 317–327

You CB, Song HX, Wang JP, Lin M & Hai WL (1991) Association of *Alcaligenes faecalis* with wetland rice. Plant Soil 137: 81–85

Zelena E, Kutacek M & Cermak V (1988) Fate of root applied indoleacetic acid and its influence on growth of plants. In: Kutacek M, Bandurski RS & Krekule J (eds) Physiology and Biochemistry of Auxins in Plants (pp. 371–376). SPB Academic Publishing, The Hague