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Identification and antibiotic sensitivity of microbial contaminants from callus cultures of garlic *Allium sativum* L. and *Allium longicuspis* Regel

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Abstract

Microbial contaminants were isolated from in vitro callus cultures of garlic *Allium sativum* L. and *Allium longicuspis* Regel and identified as *Bacillus circulans*, *Staphylococcus xylosus* and *Staphylococcus warneri*. The isolation procedure was based on microbial enrichment in different media and on microscopic preparations using Gram- and Methylene Blue stainings. Pure cultures of isolates were grown and then identified using commercially available identification kits (API 50 CHB and API Staph) as well as a series of specific physiological tests. Screening experiments with a broad spectrum of antibiotics have shown that the growth inhibition of contaminants was strain-dependent, thereby displaying different minimum inhibitory (bacteriostatic effect) and critical bactericidal concentrations. Phytotoxicity effects and persistence of the antibiotics were examined with a selection of three substances (erythromycin at 10 mg/l, gentamycin at 10 mg/l, imipenem at 5 mg/l) supplemented to the in vitro media and subsequently used for culturing of the calli. Neither phytotoxic effects nor visible bacterial growth (colony formation) were observed with the cultures, but rod-shaped bacteria identified as *B. circulans* could still be isolated from microbial enrichment samples of the plant material. Although the antibiotics exhibited pronounced bacteriostatic effects, endospores from bacilli may survive such a treatment.

Keywords: Garlic; Callus cultures; Microbial contaminants; Antibiotic sensitivity

1. Introduction

Garlic (*Allium sativum* L.) is a crop traditionally used as an essential ingredient of various foods. It is well recognized because of its antibiotic [1,2] as well as medicinal (e.g. Ref. [3]) properties. Garlic is a sterile plant which does not form seeds, except in very few cases [4]. It is, therefore, propagated almost exclusively asexually by means of underground cloves or vegetative topsets in the inflorescence. The problem of garlic sterility was reviewed recently by Pooler and Simon [5]. These authors have put forward the hypothesis that mycoplasma- or rickettsia-like organisms may cause a disease of the garlic tapetum interfering with sexual

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reproducibility [6,7]. Furthermore, vegetative reproduction of garlic may lead to chronic contamination with viruses [8,9] and other microorganisms. The absence of sexual reproduction of garlic also lessens its genetic variability. For these reasons, regeneration of plants from in vitro cultures and particularly garlic protoplasts may be a useful method to overcome the inability of sexual reproduction. Garlic is known to contain some antibacterial substances [10]. However, cultures often exhibit a chronic contamination by microorganisms possibly involved in growth defects of the plant [11]. Although comprehensive papers concerning *Allium* cultures have been published (for review see Ref. [12]), limited knowledge exists regarding the nature and the persistence of microbial pathogens hosted in the plants. As demonstrated in previous studies [13,14], in vitro cultures of sterile species *A. sativum* and *Allium longicuspis* were highly contaminated, whereas with fertile species of *Allium porrum* and *Allium schoenoprasum* only a low contamination level was evident. To avoid such problems with in vitro cultures of the genus *Allium*, Fellner [15] proposed to use antibiotics as growth-inhibiting supplements to the plant medium.

This study was undertaken primarily to examine the nature of microbial contaminants present in callus cultures of two garlic species, i.e. cultivated *A. sativum* and its putative progenitor wild type *A. longicuspis*. Additionally, the suitability of different antibiotics to inhibit the growth of the isolated and identified bacteria was examined.

2. Materials and methods

2.1. Plant material

Cultivated garlic *Allium sativum* L. cv. 'Moravan' and wild type garlic *Allium longicuspis* Regel (collected in the area of Issyk-Kul lake in Uzbekistan) from the germplasm collection of the Department of Botany, University of Palacky, Olomouc, were used. *A. sativum* is a pollen sterile plant, while its progenitor *A. longicuspis* [5,16,17] has fertile pollen but sterile ovules. Virus-free garlic plants, originally obtained from the meristem-tip [8] were used as the source of in vitro cultures. They germinated and grew in the greenhouse under natural light conditions at 23–27°C.

2.2. Callus cultures

For isolation and identification of microbial contaminants from garlic callus cultures derived from basal parts of young leaves on culture, MS medium [18] supplemented with 2.5 μM 2,4-D, 10 μM IAA and 10 μM kinetin [14] was used. Derived garlic calli were maintained in the dark at 25°C on BDS medium [19] supplemented with 30 g/l sucrose, 1 μM 2,4-D and 5 μM kinetin (adjusted to pH 5.7 before autoclaving) and solidified with agar (0.8% w/v). Calli were transferred to fresh media monthly. During long-term culture, some of those calli of both species showed visible microbial contamination appearing as yellowish turbidity (formation of microbial colonies) at the point of contact of the calli with the medium. Contaminated calli as well as calli free of visible infection were used as the source material for the experiments presented in this paper. In addition, visibly non-contaminated calli of *A. longicuspis* maintained under light (16-h period, 3200–3600 lx) were also used.

2.3. Isolation and identification of microbial contaminants

2.3.1. Isolation procedure

One-gram portions of the subcultured calli were harvested and transferred into 100-ml Pyrex bottles containing 50 ml sterile physiological sodium chloride solution. The bottles were closed and shaken vigorously for approximately 2 min and left for another 10 min, before pipetting aliquots of 0.5 ml into a second series of bottles containing 50 ml Caso broth (Merck) (enrichment of bacteria) and Worth broth (Merck) (enrichment of yeasts). The samples were incubated in duplicates for 2 weeks at 25°C and 30°C, respectively. During this period, the enrichment flasks were inspected daily for their turbidity indicating microbial growth, under defined light conditions. In cases of highly-contaminated plant material already exhibiting visible colonies on the surface of the callus culture medium, swabs were drawn and directly transfer-

red into flasks with Caso broth, followed by incubation under analogous conditions. From those enrichment broths showing turbidity, 100-μl amounts were taken and used for preparing Gram and Methylene Blue stainings for microscopic evaluation (total magnification 1000×). In addition to the macroscopic examination of colonies of isolates produced on the agar medium and the morphological criteria checked microscopically (i.e. shape of cells and colonies, Gram reaction), different tests were carried out to identify the micro-organisms. For this purpose, pure cultures containing single bacterial strains were produced by using fractionated loop-streakings on a solid substrate. For Gram-positive rods, the medium described by Mundt and Hinkle [20] (1 l medium contained the following ingredients: peptone from meat, 5 g; meat extract, 5 g; yeast extract, 3 g; glucose, 5 g; cycloheximide 2 g, agar-agar, 17 g) and, for Gram-positive cocci, Caso agar (Merck) was used.

2.3.2. Identification of Gram-positive rods

Identification of Gram-positive rods was carried out based on the API 50 CHB identification kit (BioMérieux, Marcy-l'Etoile, France), in combination with the APILAB software. In addition to this basic differentiation, further morphological and physiological tests as outlined in Bergey's Manual of Systematic Bacteriology [21] were necessary to identify the isolates. Pure cultures were checked microscopically for their motility, based on hanging-drop slides prepared from 24-h-old cultures grown in Caso broth. To detect and to localize endospores, endospore stainings were prepared from surface cultures of the same age grown on Caso agar, using aqueous solutions of Malachite Green 5% (w/v), Eosin G 2.5% (w/v) and Safranin 0.5% (w/v). Catalase activity was detected on a slide, based on the observation of oxygen bubbles during mixing of bacterial cells (taken from the agar surface) with a drop of 3% (v/v) H_2O_2 with a loop. Tubes containing 5 ml standard I broth (Merck) were used for examining the growth behaviour of the bacteria at different temperatures. For this purpose, tubes were inspected for their turbidity caused by bacterial growth, after incubation for 48 h at 10, 30, 40 and 50°C, respectively, using thermostated waterbaths. The same basal medium was taken for testing the compatibility of the bacteria to different sodium chloride concentrations. Five-millilitre portions of broth supplemented with 2%, 5%, 7% and 10% (w/v) NaCl were inoculated with a loop of a 24-h-old bacterial culture, then incubated for 48 h at 30°C and then checked for bacterial growth. Amylolytic activity of bacteria was detected based on loop-streakings on Standard Plate Count agar (Merck) enriched with 2% (w/v) soluble starch (Merck). After incubation for 48 h at 30°C, starch hydrolysis was examined based on the formation of a clear halo around the bacterial colonies when layering 2 ml of Lugol's solution on the surface. Calcium caseinate agar according to Frazier and Rupp (Merck) and enriched with 0.5% (w/v) skim-milk powder was used for the assessment of the proteolytic activity of isolates. After loop-streaking the isolates on the agar and incubated for 48 h at 30°C, proteolytic bacteria produced a clear hydrolysis zone around the colonies.

2.3.3. Identification of Gram-positive cocci

The API Staph identification kit (BioMérieux) was used for basic identification of the isolated cocci. Catalase activity of pure cultures was examined as described for the rod-shaped bacteria. Filter disks impregnated with dimethyl-*p*-phenylenediamine (BioMérieux) were used for detecting oxidase activity. Columbia mutton blood agar (BioMérieux) served as a medium indicating haemolytic activity of isolates, Kanamycin-aesculin-azide medium (Oxoid) was used for visualizing the aesculinase activity. Lysostaphin resistance of bacterial strains was checked by means of agar diffusion testing. For this purpose, 0.1 ml of a 24-h-old bacterial culture were suspended in liquefied Caso agar at 50°C in a Petri dish. After solidification of the medium, sterile filter disks impregnated with an aqueous lysostaphin (Sigma Chemicals, St. Louis, USA) solution at 0.5 mg/ml were transferred onto the agar surface using sterile forceps. Plates were incubated at 37°C for 48 h and then checked for a halo appearing around the disks. A clear inhibition zone was taken as an indicator for a bactericidal effect of lysostaphin (i.e. non-lysostaphin resistance) against the isolate

examined. The Slidex Staph agglutination kit (BioMérieux) was applied to distinguish within the genus *Staphylococcus* by assessing coagulase and clumping activities. Growth behaviour of cocci at 15°C and 45°C and at NaCl concentrations of 10%, 12% and 15% was examined under conditions analogous to those applied for the examination of the rods.

2.4. Maintenance of bacterial isolates and preparation of endospore suspensions from bacilli cultures

Cultures of bacterial isolates were maintained in Caso broth throughout and propagated either 24 h before being used in different test series or at least weekly, by inoculating a freshly prepared medium with the preceding culture. In the latter mode, cultures were stored intermittently at 5°C until reactivation. Endospore suspensions of bacilli were produced by pasteurizing a suspension of bacilli for 10 min at 80°C in a thermostated water-bath. The suspension of bacilli was prepared from surface cultures on Caso agar which were transferred with a loop into a sterile 0.85% (w/v) sodium chloride solution.

2.5. Viable count determination

For the examination of bacterial counts as well as endospore counts, appropriate decimal dilutions of bacterial and endospore suspensions in sterile 0.85% (w/v) sodium chloride solution were prepared and transferred into liquefied Caso agar. Solidified plates were then incubated for 72 h at 30°C.

2.6. Antibiotic sensitivity of bacterial isolates

Growth inhibition of bacteria by antibiotics was screened using different techniques. In a series of agar diffusion tests, bacterial cultures pre-grown in Caso broth for 24 h at 30°C were streaked radially on the agar surface, beginning at the centre of the plate. Filter disks impregnated with an inhibitory substance were placed on these sectors. They contained cefotaxime, cephalothine ciprofloxacine, cloxacillin, chloramphenicol (200, 100, 50, 20 μg/ml, respectively), imipenem (20, 10, 5, 2 μg/ml, respectively), kathon (30, 15, 7.5, 1.5 μg/ml, respectively), neomycine, novobiocin, polymyxine B (200, 100, 50, 20 μg/ml, respectively), plumbagin (10, 5, 2, 0.5 μg/ml, respectively) and streptomycin (200, 100, 50, 20 μg/ml, respectively). Antibiotics were of analytical grade and purchased from Sigma, except imipenem which was provided by Merck-Sharp and Dohme (Rahway, USA) and kathon by Rohm and Haas (Vienna, Austria). Plates were incubated for 48 h at 30°C and then checked visually for inhibition zones around the filter disks. From these tests, the inhibitory effect as well as the effective concentration of the antibiotics were estimated. In a second test series, the commercially available ATB G+ antibiogramme kit was used. For these tests, the advice given by the manufacturer was followed throughout.

Based on the above described screening tests, the minimum inhibitory concentration (MIC) indicating the critical bacteriostatic concentration of an antibiotic was determined as described by Drews [22]. For this purpose, serial dilutions of tubes containing 6 ml nutrient broth (1 l of medium contains: tryptone 5.0 g; yeast extract 1.5 g; meat extract 1.5 g; glucose 1.0 g; NaCl 3.5 g; KH_2PO_4 3.5 g; KH_2PO_4 3.7 g) supplemented with different antibiotics were inoculated with 24-h-old bacterial cultures at a strength of 0.01% (v/v). Tubes were checked visually for bacterial growth after incubation for 48 h at 30°C. Besides the MIC, the critical bactericidal concentration (CBC) of the inhibitory substances was also examined by performing plate counts on Caso agar of aliquots taken from the incubated antibiotic-containing broths as used for MIC testing. A viable count reduction of at least 5 log-units compared with the non-antibiotic-containing blind sample was accepted as an indicator for a distinct bactericidal effect.

Tubes containing 10 ml Caso broth enriched with antibiotics selected (erythromycin, gentamycin and imipenem) were used for additional inhibitory tests under long-term storage conditions. Bacterial growth was checked based on turbidity of the medium as well as on the formation of surface colonies when plating sub-cultures on Caso agar (incubation conditions: 72 h at 30°C).

2.7. Phytotoxicity and bacterial inhibitory effects of antibiotics supplemented to the in vitro medium

Callus cultures were grown on MS as well as on BDS medium containing selected antibiotics at defined concentrations, based on above described inhibitory tests. Cultures were inspected for growth defects of the plant material as well as for the appearance of bacterial colonies on the medium during an incubation period of 12 weeks. Microbiological samples were drawn from the colonies by using the swab method (see Section 2.3) and from rinsing-solutions obtained when washing 5-g portions of the calli with sterile aqueous sodium chloride solution 0.85% (w/v), after 6 and 12 weeks of incubation. Samples were enriched bacteriologically under analogous conditions and then examined microscopically.

3. Results and discussion

3.1. Identification and characterization of contaminating micro-organisms

Turbidity of microbial enrichment broths was taken as a primary indicator for microbial growth and only observed with those callus cultures that showed microbial colonies on the in vitro culture medium, regardless of the incubation temperature used. Plant cultures exhibiting no visible contamination were found to be microbiologically sterile. Purified single cultures from the turbid enrichment broths were differentiated bacteriologically as listed in Table 1. No yeasts were found in the material examined. Callus cultures of *Allium sativum* were contaminated with a single strain of rod-shaped bacteria producing endospores, those of *A. longicuspis* contained two different strains of cocci. Based on further biochemical testing (Table 2), the rods were identified as *Bacillus circulans* and the cocci as *Staphylococcus xylosus* and *Staphylococcus warneri*. As reported by other authors [23–26], bacteria of these species are associated frequently with in vitro plant cultures.

Table 1
Basic identification results of bacterial isolates A, B and C

Test criteria	Isolates[a]		
	A	B	C
Gram reaction	+	+	+
Morphology of cells	Short rods	Cocci	Cocci
Endospores	+	n.d.	n.d.
Position	Central		
Shape	Ellipsoidic		
Sporangium	Swollen		
Motility	+	–	–
Starch hydrolysis	+	n.d.	n.d.
Casein hydrolysis	+	n.d.	n.d.
Catalase activity	+	+	+
Oxidase activity	n.d.	–	–
Aesculinase activity	n.d.	–	–
Haemolytic activity	–	–	–
NaCl tolerance			
2%	+	+	+
5%	+	+	+
7%	(+)	+	+
10%	–	+	+
12%	–	+	+
15%	–	(+)	(+)
Temperature tolerance			
10°C	–	–	+
15°C	+	+	+
30°C	+	+	+
40°C	+	+	+
45°C	–	(+)	+
50°C	–	–	(+)
Lysostaphin resistance	n.d.	–	–
Nitrofurantoin resistance	n.d.	–	–
Result	*Bacillus circulans*	*Staphylococcus xylosus*	*Staphylococcus warneri*

[a]One rod-shaped bacterium designated A was isolated from cultures of *Allium sativum*, two strains of cocci designated B and C were isolated from cultures of *Allium longicuspis*.

+, positive reaction; (+), weakly positive reaction; –, negative reaction.

n.d., not determined.

3.2. Bacterial growth inhibition and phytotoxicity by antibiotics

To suppress microbial contamination it is common to use antimicrobials as supplements to the plant culture medium [27,28]. Supplementation with antibiotics may, however, lead to phytotoxicity problems and to the resistance of micro-organisms against the inhibitory substance. It

Table 2
Additional identification results of bacterial isolates A, B and C as obtained using specific test kits API 50 CHB and API Staph

Test criteria	Isolates[a]		
	A	B	C
Glycerol	+	n.d.	n.d.
Erythrol	–	n.d.	n.d.
D-Arabinose	–	n.d.	n.d.
L-Arabinose	+	n.d.	n.d.
Ribose	+	n.d.	n.d.
D-Xylose	–	n.d.	n.d.
Adonitol	–	n.d.	n.d.
β-Methyl-xyloside	+	n.d.	n.d.
Galactose	+	n.d.	n.d.
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	–	n.d.	n.d.
Rhamnose	–	n.d.	n.d.
Dulcitol	–	n.d.	n.d.
Inositol	–	n.d.	n.d.
Mannitol	+	+	+
Sorbitol	–	n.d.	n.d.
α-Methyl-D-mannoside	–	n.d.	n.d
α-Methyl-D-glycoside	–	–	–
N-Acetyl-glucosamine	+	+	–
Amygdaline	+	n.d.	n.d.
Arbutine	+	n.d.	n.d.
Esculine	+	n.d.	n.d.
Salicine	+	n.d.	n.d.
Cellobiose	+	n.d.	n.d.
Maltose	+	+	+
Lactose	+	+	+
Melobiose	+	+	–
Sucrose	+	+	+
Trehalose	+	+	+
Inuline	–	n.d.	n.d.
Melizitose	–	n.d.	n.d.
D-Raffinose	+	–	–
Amidon	+	n.d.	n.d.
Glycogen	+	n.d.	n.d.
Xylitol	–	+	–
β-Gentibiose	+	n.d.	n.d.
D-Turanose	+	n.d.	n.d.
D-Lyxose	–	n.d.	n.d.
D-Tagatose	–	n.d.	n.d.
D-Fucose	–	n.d.	n.d.
D-Arabitol	–	n.d.	n.d.
L-Arabitol	–	n.d.	n.d.
Gluconate	–	n.d.	n.d.
2-Ketogluconate	–	n.d.	n.d.
5-Ketogluconate	–	n.d.	n.d.
Nitrate reduction	n.d.	–	–
Phosphatase alkaline	n.d.	–	–
Acetylmethylcarbinol	n.d.	+	+
Arginine dihydrolase	n.d.	+	+
Urease	n.d.	+	+
Result	*Bacillus circulans*	*Staphylococcus xylosus*	*Staphylococcus warneri*

[a] One rod-shaped bacterium designated A was isolated from cultures of *Allium sativum*, two strains of cocci designated B and C were isolated from cultures of *Allium longicuspis*; results shown in Table 1.

+, Positive reaction; –, negative reaction.

n.d., not determined, depending on the necessity to be included for bacterial identification.

should, therefore, be applied to a reduced extent [29]. Nevertheless, alternatives to this technique are obviously limited so far.

A representative spectrum of antibiotics were screened for growth inhibition of bacterial isolates. As can be seen from Table 3, various substances were, in principle, capable of affecting the growth of the bacteria. To obtain more detailed information about the effect of antibiotics on the bacteria, the bacteriostatic (MIC) as well as bactericidal (CBC) properties of selected antibiotics were examined. Fig. 1 demonstrates that the bacilli were, in general, more resistant against the antibiotics than the staphylococci. As expected, a bactericidal effect of nearly all substances was obtained at distinctly higher concentrations than that sufficient for bacteriostastis. Since survival and germination of endospores of bacilli is a problems during long-term culturing and/or storage of plant cultures, the inhibitory effect of the antibiotics on the endospores was checked separately.

Table 3
Inhibitory effects of various antibiotics on the bacterial isolates

Antibiotics	Concentration (μg/ml)[a]	Isolates		
		A	B	C
		Bacillus circulans	*Staphylococcus xylosus*	*Staphylococcus warneri*
Loop streak (agar diffusion) testing				
Cefotaxim	20	–	+	+
Cephalothin	20	–	+	+
Ciprofloxacin	20	–	+	+
Cloxacillin	20	–	+	+
Chloramphenicol	20	+	+	+
Imipenem	5	+	+	+
Kathon	7.5	+	+	+
Neomycin	20	+	+	+
Novobiocin	20	+	+	+
Polymyxin sulphate	50	+	+	+
Plumbagin	10	+	+	+
Streptomycin	200	–	+	+
ATP antibiogramme testing				
Penicillin G	0.125	–	–	–
Oxacillin	2	–	+	+
Kanamycin	8	+	+	+
Tobramycin	8	+	+	+
Gentamycin	4	+	+	+
Tetracycline	4	+	+	+
Minocyclin	4	+	+	+
Erythromycin	4	–	+	–
Lincomycin	8	+	+	–
Pristinamycin	2	+	–	–
Fosfomycin	32	–	–	–
Nitrofurantoin	100	–	–	–
Quinolone	4	–	–	+
Rifampicin	16	+	+	+
Fusidinic acid	16	+	+	+
Vancomycin	4	+	+	+
Teicoplanine	4	+	+	+
Cotrimoxacole	8	+	+	+

[a]Only the lowest concentrations exhibiting an inhibitory effect on at least one of the isolates are shown.
+, Inhibitory effect observed; –, no inhibitory effect observed.

Freshly prepared endospore suspensions from isolates of *B. circulans* were transferred into Caso broths to give endospore counts of around 10^6 CFU/ml; broths were supplemented with selected antibiotics and then checked for MIC and CBC, after incubation for 48 h at 30°C. It was found that novobiocin exerted a sporicidal effect at a concentration which was five times higher than that necessary for inhibiting the vegetative cells, while no pronounced differences were observed with the other antimicrobials examined (data not shown).

In another test series, tubes containing 10 ml

Table 4
Effect of three selected antibiotics supplemented to the in vitro medium on the plant material and on bacterial growth in/on callus cultures of *Allium* sp. at 25 and 30°C; cultures were inspected during an incubation period of 12 weeks; microbiological tests were performed after 6 and 12 weeks

Antibiotic	Concentration (mg/l)	*Allium sativum*		*Allium longicuspis*	
		Phytotoxicity[a]	Bacteriological examination[b]	Phytotoxicity[a]	Bacteriological examination[b]
Erythromycin	10	–	NP, NT	–	NP, T, G+R
Gentamycin	10	–	NP, NT	–	NP, T, G+R
Imipenem	5	–	NP, NT	–	NP, T, G+R

[a]–, No visible effect of antibiotics on growth of the callus cultures.
[b]NP, no plaque on the culture medium; P, plaques on the surface of the culture medium; NT, no turbidity of bacterial enrichment broth; T, turbidity of bacterial enrichment broth; G+R, Gram-positive rods were identified by microscopical examination of the enrichment broth.

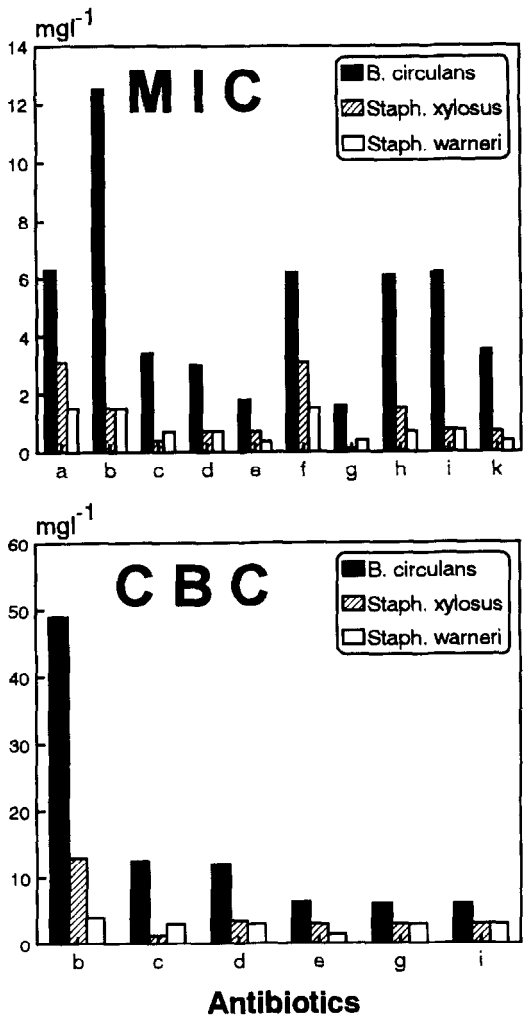

Fig. 1. Minimum inhibitory concentrations (MIC) and critical bactericidal concentrations (CBC) of selected antibiotics exerted against bacterial isolates; a, chloramphenicol; b, ciprofloxacin; c, erythromycin; d, gentamycin; e, imipenem; f, kanamycin; g, kathon; h, neomycin; i, novobiocin; k, penicillin G.

Caso broth supplemented either with 10 mg/l erythromycin, 10 mg/l gentamycin and 5 mg/l imipenem, respectively, were inoculated with the bacterial isolates to give initial viable counts of approximately 10^4 CFU/ml. Vials were incubated for 6 weeks at 30°C and inspected weekly. No turbidity appeared in the media and no surface cultures were observed when plate-cultures were prepared from the broths.

The same antibiotic levels were also applied for assessing phytotoxicity and antimicrobial effects during culturing of contaminated calli of *A. sativum* and *A. longicuspis*, on MS and BDS medium and under different temperature conditions. As can be seen from the results summarized in Table 4, no toxic effects on the calli were evident at the antibiotic concentrations used. No colonies appeared on the surface of the culture media, i.e. these antimicrobials were capable of inhibiting the growth of contaminating cocci and bacilli. Despite this bacteriostasis, the antibiotics applied were, however, not effective enough to exert a bactericidal/sporicidal effect, since turbidity appeared in the enrichment broths containing samples drawn

after 6 and 12 weeks of incubation. As a confirmation of these results, Gram-positive rod-shaped contaminants could be again isolated from this material and identified accordingly. It should, therefore, be taken into consideration that no visual defects of the plant or the substrate is not a guarantee for a real germ-free material since bacterial endospores may survive long-term periods under adverse conditions, even in media containing antibiotics. Moreover, the nature of the medium whether optimized for microbiological or plant culturing obviously affects the viability and resistance of the bacterial contaminants.

References

[1] H.P. Koch, Garlicin — Fact or fiction? The antibiotic substance from garlic (*Allium sativum*). Phytotherapy Res., 7 (1993) 278–280.

[2] S.T. Pai and M.W. Platt, Antifungal effects of *Allium sativum* (garlic) extract against the Aspergillus species involved in otomycosis. Lett. Appl. Microbiol., 20 (1995) 14–18.

[3] M. Fujiwara and T. Natata, Induction of tumor immunity with tumor cells treated with extract garlic (*Allium sativum*). Nature, 216 (1967) 83–87.

[4] T. Etoh, Fertility of garlic clones collected in Soviet Central Asia. Jpn. Soc. Hortic. Sci., 55 (1986) 312–319.

[5] M.R. Pooler and P.W. Simon, True seed production in garlic. Sex Plant Reprod., 7 (1994) 282–286.

[6] O. Konvicka, Sterility problems of *Allium sativum* L. Biol. Plant. (Praha), 14 (1973) 144–149.

[7] O. Konvicka, F. Nienhaus and G. Fischbeck, Untersuchungen über die Ursachen der Pollensterilität bei *Allium sativum* L. Z. Pflanzenzuecht., 80 (1978) 265–276.

[8] P. Havránek, Virusfree clones of the common garlic obtained by meristem-tip cultures. Ochr. Rostlin, 8 (1972) 291–298.

[9] L. Bos, Viruses and virus diseases of *Allium* species. Acta Hortic., 127 (1983) 11–29.

[10] H. Takagi, Garlic *Allium sativum* L., in: J.L. Brewster and H.D. Rabinowitch (Eds.), Onions and Allied Crops, Vol. III., CRC Press, Inc. 1990, Boca Raton, USA, pp. 109–146.

[11] R. Phillips, S.M. Arnott and S.E. Kaplan, Antibiotic in plant tissue culture: rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of *Helianthus tuberosus*. Plant Sci. Lett., 21 (1981) 235–240.

[12] F.J. Novak, *Allium* tissue culture, in: H.D. Rabinowitch and J.L. Brewster (Eds.), Onions and Allied Crops, Vol. 1, CRC Press, Inc., Boca Raton, USA, 1990, pp. 234–252.

[13] M. Rauber and J. Grunewaldt, In vitro regeneration in *Allium* species. Plant Cell Rep., 7 (1988) 426–429.

[14] M. Fellner and P. Havránek, Culture of protoplasts isolated from leaves and callus cultures of *Allium sativum* and *Allium longicuspis*: a preliminary report. Biol. Zentralbl., 113 (1994) 317–328.

[15] M. Fellner, Influence of the antibiotic ciprofloxacin on culture of *Allium longicuspis* callus derived protoplasts. Ann. Bot. 76 (1995) 219–223.

[16] A.I. Vvedensky, The genus *Allium* in the USSR, (transl.) Herbertia, 11 (1944) 65–218

[17] H.A. Jones and L.K. Mann, Onions and their Allies, Leonard Hill Books, 1963, London, pp. 37.

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

[19] D.I. Dunstan and K.C. Short, Improved growth of tissue cultures of the onion *Allium cepa*. Physiol. Plant., 41 (1977) 70–72.

[20] J.O. Mundt and N.F. Hinkle, Bacteria within ovules and seeds. Appl. Environ. Microbiol., 32 (1976) 694–698.

[21] P.H. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, Bergey's Manual of Systematic Bacteriology, Vol. 2, Williams and Wilkins, Baltimore, London, 1986, pp. 999–1034 and 1104–1138.

[22] G. Drews, Mikrobiologisches Praktikum, Springer Verlag, 3. Aufl., 1976, pp. 148–149.

[23] A. Attafuah and J.F. Bradbury, Pseudomonas antimicrobica, a new species strongly antagonistic to plant pathogens. J. Appl. Bacteriol., 67 (1989) 567–573.

[24] J.J. Finer, W.R. Saxton, B.L. Norris, J.A. Steele and S. Rahnema, Growth and disinfestation of six different bacteria in embryogenic suspension cultures of cotton. Plant Cell Rep., 10 (1991) 380–383.

[25] C. Leifert, W.M. Waites and J.R. Nicholas, Bacterial contaminants in micropropagated plant cultures. J. Appl. Bacteriol., 67 (1989) 353–361.

[26] C. Leifert, W.M. Waites, J.R. Nicholas and J.W.W. Keetley, Yeast contaminants of micropropagated plant cultures. J. Appl. Bacteriol., 69 (1990) 471–476.

[27] W. Kneifel and W. Leonhardt, Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture. Plant Cell Tissue Organ Culture, 29 (1992) 139–144.

[28] Z.A. Wilson and J.B. Power, Elimination of systematic contamination in explant and protoplast cultures of rubber. Plant Cell Rep., 7 (1989) 622–625.

[29] F.R. Falkiner, Strategy for the selection of antibiotics for use against common bacterial pathogens and endophytes of plants. Acta Hortic., 225 (1988) 53–56.