SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2015, V. 50, № 1, pp. 3-15 ISSN 2313-4836 (Online)

Reviews. Recent advances. Challenges

UDC 633/635:581.4:579.64

doi: 10.15389/agrobiology.2015.1.3rus
doi: 10.15389/agrobiology.2015.1.3eng

BACTERIAL MICROORGANISMS ASSOCIATED WITH THE PLANT TISSUE CULTURE: IDENTIFICATION AND POSSIBLE ROLE
(review)

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Received October 8, 2013

Abstract

Effective sterilization of plant explants and antiseptics rules compliance do not exclude the presence of so-called covert (endophytic) bacteria in in vitro cultures. But the role of these bacteria in tissues cultures has been not enough studied whereas it was related to the explants regeneration capacity and the possibility of animal and human cells transformation under in vitro cultivation. Bacterial strains pathogenic to humans can be stably maintained in cultivated tissues and ex vitro plants. The broadening of bacterial environments creates ecological and genetic risks leading to necessity of careful monitoring of endophytic communities in plants used as raw food and at use of in vitro technologies in practical plant growing and food production. Identification of bacterial microorganisms colonizing in vitro plant cultures allows studying the bacteria effect on the host, realizing special chemotherapy and developing the microorganisms' databases. Two methods of identification are the most widespread: more available traditional one that does not allow detecting non-cultured forms (its base is the use of cultural and morphological characteristics as well as chemical and biochemical reactions) and molecular-genetic one. At the second approach different 16S rRNA sequences are studied using metagenomic DNA and appropriate specific primers; these sequences have conserved sites identical for all prokaryotes and variable ones suitable for species specific regions identification. Internal transcribed spacers (ITS) are being mainly used to distinguish the microorganisms at the species level and even at strains one. Taxonomy of in vitro cultures' bacterial endophytes indicates to their diversity and absence of specific composition as for cultures of plants belonging to different taxa as for different plant organs explants. Among identified endophytic bacteria potentially useful for intact plants *Streptomycete*, *Pantoea agglomerans* and others were found as well as those pathogenic for humans, e.g. *Ralstonia mannitolytica*, *Staphylococcus epidermidis*, *Corynebacterium amycolatum*, *Bacillus neonatiensis, Salmonella* and *Nocaridia* spp. At in vitro plant cultivation durable symptomless bacterial presence is caused on the one hand by bacterial growth repression with factors accompanying plant explants cultivation (pH, temperature below bacterial optimum, activation of the defense mechanisms), and on the other hand by simultaneous bacteria support due to exudates secreted by plant explants. The rapid bacterial cells proliferation can begin even at small changes in initial conditions, at increase in plant exudates concentrations and per se in consequence of in vitro cultivation as a stress at the absence of whole organism regulatory role. As the number of subcultivations increases a portion of plant cultures with latent bacterial contamination increases too; no-cultured endophytes have been reported to acquire the status of cultured ones. Covert bacterial contamination could depress regeneration, micropropagation, cause death of in vitro cultivated objects, restrict the protocols repeatability and concern induction of epigenetic somaclonal variability. For instance *Acinetobacter* and *Lactobacillus plantarum* filtrates extracted from degrading calluses strongly reduced shoot regeneration at inoculation in explants or addition into a medium; bacteria *Mycobacterium obuense* and *M. aichiense* repressed seeds development in in vitro cultures. The article accents the problem of gnotobiological plant cultures (specifically in in vitro collections of plants genetic banks) development caused by difficulties in identification and elimination of bacterial microorganisms.

Keywords: plant tissue culture, bacterial microorganisms, antibacterial therapy.

When working with in vitro plant tissue culture, the presence of bacterial contamination is largely determined by the quality of sterility [1, 2]. However, an effective sterilization of plant explants and compliance with the antiseptics

rules do not exclude the presence of covert bacteria in in vitro cultures (without visual growth and specific symptoms) [3-5]. Bacterial organisms, the native habitat of which is air, soil, plants and human, are detected and identified using microbiological, molecular and genetic and biochemical methods both in the long-term passaged plant cultures and plant cultures initiated in vitro [6-14]. Latent bacterial infections, defined by many researchers as internal or endophytic, are detected in calli and microplants cultivated in vitro, as well as in various explants such as shoot apices, buds, and meristems [15-22]. Bacterial endophytes performing a number of functions that are important for plants have always been and continue to be the subject of numerous studies [23]. At the same time, the role of endophytic bacteria in tissue cultures is less well studied, but it is of utmost interest both in fundamental and applied aspects. Specifically, bacterial endophytes are considered as a key factor that defines the regenerative capacity of explants along with the genotype and cultivation conditions [18]. They are studied as a possible promising source of new components for the use in the microbiology and medicine practices [24]. Moreover, attention is drawn to bacterial endophytes due to the accumulation of data indicating the conventionality of historical division of microorganisms into phytopathogenic, pathogenic for animals (human) and non-pathogenic [25]. It was shown that human pathogenic bacterial strains can be steadily preserved in passaged cultures and ex vitro plants [14], and bacteria *Agrobacterium tumefaciens* can transform in vitro cultured human cells [26] and sea urchin embryos [27]. The enhanced bacteria habitat creates environmental and genetic risks that necessitate careful monitoring of endophytic communities, especially in plants used as raw food [14, 28]. This problem is relevant for the plant tissue culture as well, since in vitro techniques are widely used in plant growing practices and food production.

The purpose of this survey was to collect and organize data related to detection, identification, structure, dynamics, possible role, and elimination of latent bacterial contaminations in the plant tissue culture.

In literature, bacterial microorganisms, the presence of which in the in vitro cultivated plant objects is not accompanied by visual displays and specific symptoms, are referred to as latent, covert, endogenous, internal, and endophytic, and often these terms are used as synonyms. Most often, these bacterial microorganisms in the plant tissue culture are called latent. One of the papers [29] emphasizes that the term «latent» is borrowed from the plant pathology, where it is used to describe asymptomatic pathogens, while bacterial microorganisms in the plant tissue culture are not necessarily pathogens (they can exert either negative, positive or no impact). The author of the cited paper [29], along with other researchers [30, 31], believes that it is more appropriate to use the term «covert» for these bacterial microorganisms. Many researchers call covert bacterial microorganisms «endophytes» due to their presence in the culture of plant objects that underwent surface sterilization. We will use the term «endophytic bacteria» as it is used by the authors of the cited papers.

According to the widely used definition, endophytes are microorganisms that live inside the plant during the whole or part of the life-cycle and do not cause symptoms of diseases [32]. In nature, they enter the plant through the stomata, wounds, and root system. A significant role in the formation of endophytic microflora is played by transfer of microorganisms through seeds, as well as their introduction by vector organisms, the invertebrates and fungi [28, 32]. Introduced microorganisms may be included in the plant microflora at the point of entry and/or distributed throughout the plant [32], and obligation is not a prerequisite [33].

Endophytic bacteria have been found in cell cytoplasm, intercellular

space [34] and vascular system [35] of plants. In numerous papers the presence of endophytic microorganisms in in vitro cultivated plant explants was documented by light and electronic microscopy, and using in situ hybridization [15, 16, 21, 36-38].

Sources of bacterial microflora. Endophytic bacteria are derived from epiphytic associations of plant rhyzosphere and phytosphere. The initial explants mostly are the causal factors of endophitic infection during in vitro cultivation. Aseptic explants are hardly prepared from rosette, woody and perennial plants [12, 38], in case of wet habitats or sampling when the weather was wet and warm, and also from the diseased plants [21, 39]. Infection can occur when specific explants are used, in particular, the underground organs (root, rhizome, corm) [40, 41], the buds which are tightly covered with multilayer scales, the fragments of epidermis, especially hairy one [42, 43]. Some bacterial epiphytes can remain inaccessible to disinfecting agents, particularly in the closed stomata, in folds on the surface of the root cuttings, or in the epidermal intercellular space [5, 9].

Systemic infection of in vitro plant culture can also be due to bacterial contamination of the operator's position or the operator himself, glassware and instruments used [2, 44]. Spores of some bacterial species remain viable after autoclaving [36] and in ethanol [37].

Approach to detection and identification. There are different ways to reveal a latent bacterial contamination. In particular, selective media, physiological tests, bacteriophages, specific fatty acid and protein assay are commonly used. Besides, recently improved MALDI TOF (Matrix assisted laser desorption/ion-ization time of flight) mass spectrometry and molecular markers (i.e. RAPD-PCR — random amplified polymorphic DNA polymerase chain reaction, REP-PCR — repetitive extragenic palindromic polymerase chain reaction, AFLP — amplified fragment length polymorphism, ARDRA — amplified ribosomal DNA restriction analysis, 16S rRNA) are successful in bacterial typing. All they are specific at different taxonomic levels, being mostly suitable for the estimation at family, genus and species levels. For subspecies, biovars and strain attributing, current biochemical and molecular genetic techniques are preferable [45).

A conventional approach to bacteria detection and identification is based on their cultural and morphological properties, as well as the biochemical tests [46] carried out with no expensive equipment. However, the methods of classical microbiology are more available but thrivelles in case of non-cultivated forms unable to metabolize the nutrient substrate. Molecular identification of the genotypes is based on the analysis of conservative rRNA genes which present in all bacterial cells and are genus-specific in most microorganisms [23]. For identification, the genes of 23S rRNA of ~3000 bp, 16S rRNA of ~1500 bp and Internal Transcribed Spacers (ITS) should be sequenced [47]. In the 16S rRNA genes there are both conservative regions characteristic for all prokaryotes and species-specific sites suitable for identification [48, 49]. The sequences of 16S—23S rRNA ribosomal spacers are even more informative due to their high variability in size and structure compared to the genes themselves. Thus, the ITS are preferably used to attribute the microbial species and strains [50]. The ITS and 16S rRNA gene fragments are amplified in PCR with metagenomic DNA and specific primers [13, 51, 52]. After sequencing PCR products their homology to DNA sequences deposited in GenBank database should be estimated [53] for taxonomic identification. According to A.V. Pinevich [54], genome sequencing has been reported for 60 bacterial species while their total number is 5007.

Identification of bacteria in in vitro culture. Identifi-

cation of bacterial colonization of in vitro plant tissue culture allows us to study effects of microorganism on the host cells, to apply specific chemotherapy, and to create databases with regard to microorganisms associated with plant tissue cultures. In early papers there were data mostly obtained by classical methods including study of growth on different media, Gram staining, morphology and color of the colonies [4, 6, 9, 10, 46]. Due to advances in studying taxonomic diversity among bacteria associated with plant tissue cultures by means of molecular methods, the database of these microorganism progressively increases. In the Table there is a taxonomic composition of bacterial endophytes from in vitro plant culture for a relatively limited range of the samples tested which indicates a diversity of bacterial form able to colonize plant tissue cultures as a very specific niche quite different from the natural one. It also should be noted the absence of specific bacterial composition in case the plants were from different systematic groups and the explants derived from different organs. The data on bacterial identification reported earlier for plant tissue cultures allow us to make the same conclusion [4, 6, 9, 10].

Among identified endophytes there are those potentially useful for intact plants, namely *Streptomycete*, *Pantoea agglomerans*, etc., as well as pathogenic for humans, in particular, *Ralstonia mannitolytica*, *Staphylococcus epidermidis*, *Corynebacterium amycolatum*, *Bacillus neonatiensis*, *Salmonella* and *Nocaridia* spp. [13].

Dynamics of bacterial expression. Long symptomless presence of endophytic bacteria in in vitro plant cultures is due to two opposite processes, the growth limitation and the support of viability. In the course of cultivation the bacteria growth and reproduction are suppressed by some factors, such as acidification, suboptimal temperature (25 °C), and probable activation of defense mechanisms against microorganism in the tissue culture [55]. At the same time, the exudates secreted by explants support the viability of bacteria, since most bacteria, despite the heterotrophy, are not sustainable in the absence of plant [12, 55]. As a result, the number of bacteria associated with the plant tissue culture is at a medium level leading to symptomless and long persistence of infectious agents which are difficult to remove.

Rapid proliferation of bacterial cell can occur under slight modification of the conditions, such as an increased temperature, changes in acidity or nutrient contents (in particular, due to additional N from destroyed explant tissues) [55, 56] or at high cytokinin levels in the media for subcultivation of old cultures [8, 57]. Proliferation is induced by activated secretion of exudates by explants which, in turn, can be stimulated by temperature, growth of the culture, or transfer to the rooting medium [55]. Intensified bacterial growth can lead to visible symptoms in in vitro culture and/or observed growth on the media used for explant cultivation [3]. When bacteria migrate from the cultivated tissues into the medium, they usually form a turbid halo observed by many researchers [7, 10, 11, 58, 59]. In vitro cultivation could be a stress factor stimulating growth of endophytes which under these conditions are not controlled in the same way as in an intact plant [16].

In numerous papers it is noted that the more is passage number, the higher rate of latent contamination can observed, while the rate of pronounced infection decreases. Besides, the composition of microbial community can also change, i.e. the number of Gram-positive microorganisms increases as well as the rate of those capable to growth on a nutrient media [4, 13, 21]. Thus, in micropropagated banana plants the endophytes were nonculturable for three passages and detected only by sequencing 16S rRNA gene (viable but nonculturable bacteria — VBNC). Nevertheless, from 4^{th} to 18^{th} passage the culturable bacteria were

found in the same microplant [13, 21]. The authors of the cited papers suggested that in the course of plant cultivation the VBNC endophytes can change their status.

Impact on colonized in vitro plant cultures. Bacteria associated with plant tissue culture can adversely affect the regeneration of callus, cell suspension and protoplasts [38, 56], depress microclonal propagation, shoot growth and rooting, cause death of samples cultivated in vitro and ex vitro [3, 11, 13, 21, 29, 36, 37, 57, 60]. Latent contamination may negatively affect the reproducibility of protocols [29] and be associated with the emergence of epigenetic somaclonal variants [61]. A probable reason for the negative effect of latent bacterial microorganisms is their increased number. Some researchers consider that fact in a connection with plant tissue culture death after second or third subcultivation [18, 62]. In our investigation, the second and third passages turned out to be critical for in vitro micropropagation of raspberry explants [59]. A depressive effect of bacteria can also be due to changes in medium pH or nutrient level (particularly, because of consumption of saccharose) or synthesis of herbicidal substances [63].

Study of the impact of axenic bacteria on in vitro plant culture is of special interest. It was shown that *Acinetobacter* and *Lactobacillus plantarum* filtrates from degrading callus decreased sharply the shoot regeneration when adding to medium or inoculating explants [56, 63]. *Mycobacterium obuense* and *M. aichiense* depressed seed development in vitro [38].

As far as the helpful effect of endophytes on in vitro plant cultures the researchers began to study later, the data obtained are less numerous. In some papers a positive influence of *Methylobacterium* on induction of organogenesis and embryogenesis was reported [15, 17, 64-68]. Presumably the *Mycobacterium* sp., *Methylobacterium* spp., *Pseudomonas* spp., *Rhodotorula minuta* endophytes detected in pine tissue culture by in situ hybridization can affect positively the in vitro morphogenesis [17] similar to that observed in animal cell culture [69]. The stimulation of somatic morphogenesis by *Bacillus circulans* was reported in *Pelargonium* × *hortorum* Bailey [70].

Thus, bacterial microorganisms associated with plant tissues in in vitro cultures, on one side, are the factors depressing explant growth, development and viability, and on the other side, they can effect them positively.

In gene banks the plants are not preliminarily checked for endophyte contamination before cryoconservation. Gnotobiotic state of certified plant material allows us to avoid transfer of infection under micropropagation and is a criterion of safe storage of the genotypes in the controlled conditions. In the course of certification the main viruses, mycoplasmas and bacterial microflora are analyzed and eliminated from the plant material [39]. Diversity, significant number and changes in bacteria of in vitro plant cultures necessitate its regular checking for contamination by various methods that complicates the procedure in big collections of plant gene banks.

Endophytes of in vitro plant cultures

Plant genus, species (variety)	Culture type (time of in vitro cultivation)	Genus, species of bacteria (frequency, %)	Reference
Chrysanthemum (Arka Swarna)	Microplants (1-7 passages)	Morphtypes of *Curtobacterium citreum*	[8]
Pinus sylvestris	Callus culture	*Hormonema dematioides* (isolates L, M), *Methylobacterium extorquens* (isolate F), *Pseudomonas synxantha* (isolates G, H, J), *Pseudomonas* sp. (isolates K, N), *Rhodotorula minuta* (isolate T)	[16]
Pinus sylvestris	Callus and suspension cultures	*Methylobacterium extorquens*	[18]
Prunus cerasus (Montmorency)	Microplants	*Pseudomonas aeruginosa*	[19]

Table (continued)

Bactris gasipaes	Microplants	*Brevibacillus* sp., *Moraxella* sp.	[20]
Musa sp.	Microplants (long microcloning)	*Alcaligenes*, *Bacillus* spp., *Brachybacterium*, *Brevibacterium*, *Brevundimonas*, *Corynebacterium*, *Enterobacter, Klebsiella*, *Kocuri*, *Methylobacterium*, *Microbacterium*, *Oceanobacillus*, *Ochrobactrum, Pantoea*, *Pseudomonas*, *Ralstonia*, *Staphylococcus*, *Tetrasphaer* spp.	[21]
Musa sapientum (Chini champa)	Shoot tips (1-2 week cultivation)	Gram-positive: *Bacillus megaterium*, *Cellulomona uda*, *C. flavigena*, *Corynebacterium paurometabolum* Gram-negative: *Erwinia cypripedii*, *Klebsiell* sp., *Pseudomonas* sp.	[22]
Larix, Picea	Suspension culture (6-8 week cultivation)	*Acinetobacter*	[56]
Chrysanthemum (Arka Ravi)	Microplants	*Enterobacter*, *Methylobacterium* spp., *Ralstonia*	[57]
Rubus idaeus, *Fragaria ananassa*, *Cerasus vulgaris*, *Ribes nigrum*	Microplants from in vitro collection	*Arthrobacter* (23.5 %), *Bacillus* (51.5 %) More rare are *Agrobacterium*, *Bacterium*, *Brevibacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Pseudomonas*	[58]
Jatropha curcas	Leaf explants	*Enterobacter ludwigii*	[62]
Fragaria ananassa (Camarosa, Sweet Charlie, Oso-Grande)	Meristem	17 bacterial strains of *Bacillus*, *Sphingopyxis*, *Virgibacillus*	[64]
Musa sp.	Shoot tips	*Bacillus*, *Brevibacillus*, *Paenibacillus*, *Staphylococcus* spp., *Virgibacillus*; *Actinobacteria* (*Cellulomonas*, *Micrococcus*, *Corynebacterium*, *Kocuria* spp.); α-*proteobacteria* (*Paracoccus* sp.); Y-*proteobacteria* (*Acinetobacter* spp., *Pseudomonas*)	[51]
Ilex dumosa	Segments of shoot nodes	*Achromobacter*, *Stenotrophomonas maltophilia*	[71]
Echinacea	Microplants	*Acinetobacter*, *Bacillus*, *Pseudomonas*, *Stenotrophomonas*, *Wautersia* (*Ralstonia*)	[72]
Carica papaya	Shoot tips	*Agrobacterium* (*A. tumefaciens*), *Bacillus* (*B. benzoevorans*), *Brevundimonas* (*B. aurantiaca*), *Enterobacter* (*E. cloacae*), *Methylobacterium* (*M. rhodesianum*), *Microbacterium* (*M. esteraromaticum*), *Pantoea* (*P. ananatis*) (70 %), *Sphingomonas*, *Wautersia* (*Ralstonia*)	[73]
Potato	Microplants	*Bacillus pumilus*	[74]
Carica papaya	Shoot tips (1 month cultivation)	*Lysinibacillus fusiformis*, *Paenibacillus* sp., *Pantoea* sp., *Ralstonia mannitolilytica*, *Sphingomonas* sp.	[75]
Limonium simuatum	Microplants	*Alcaligenes* sp., *Pasteurella multocida*, *Stenotrophomonas maltophilia*	[76]
Ananas comosus	Microplants (5 year cultivation)	*Actinobacteria*, *Alphaproteobacteria*, *Betaproteobacteria*	[77]
Piper nigrum, *Piper colubrinum*, *Taxus baccata* subsp. *wallichiana*, *Withania somnifera*	Callus culture (primary explants)	*Aminobacter*, *Flavobacterium*, *Morococcus*, *Paracoccus Pseudomonas*, *Psychrobacter*, *Rhizobacter*	[78]

Antibacterial treatment. Antibiotics are used to eliminate bacterial microorgamisms [79]. Some antibiotics used for plant chemotherapy are described in a review of G. Seckinger et al. [80]. In order to eliminate bacterial contamination from plant culture the antibiotics should possess bactericide effect, being also inexpensive, non-toxic to humans, soluble in the medium with no influence on pH [9, 61]. The choice of most active antibiotics of wide spectrum (or effective in specific combinations) is more successful if the target bacteria are identified. In case of combination, particularly for synergistic antibiotics, the risk of resistant bacteria emergence decreases, nevertheless, some antibiotics

are the incompatible chemicals neutralizing each other [71, 72]. Most bacteria identified in plant culture are Gram-negative, and they are most hard to eliminate because of, in fact, two layer cell membrane preventing antibiotics input. After the chemotherapy the plant material should be checked for the presence of bacterial contamination for 2-3 passages [71].

Usage of antibiotics is complicated by different reasons. The specific concentration should be optimized, and its effect on plant tissue culture should be taken into account. Antibiotic-resistant strains inevitably occur. Some antibiotics destroy chloroplasts and mitochondria resulting in chlorosis and morphological changes in explants [6, 9]. The advances in antibacterial therapy with regard to plant tissue culture will be largely determined by progress in investigations and development of new generation of antibacterial preparations.

So, diversity of latent bacterial microflora, the endophytes, of in vitro plant cultures is significant and includes forms which can influence on the colonized plant culture both negatively and positively. As the number of plant culture passages increases, the bacteria titer may increase, too, and composition of bacterial association as well as bacterial culturability can change. Complications in obtaining gnotobiotic cultures, particularly in vitro collections of plant gene banks, are caused by difficulties in detection and elimination of bacterial microflora.

Acknowledgement

We thank T.A. Gavrilenko (N.I. Vavilov All-Russian Institute of Plant Industry) and V.I. Safronova (All-Russia Research Institute for Agricultural Microbiology) for valuable comments and discussion of the article.

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