**BACTERIAL MICROORGANISMS ASSOCIATED WITH THE PLANT TISSUE CULTURE: IDENTIFICATION AND POSSIBLE ROLE**

A b s t r a c t

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 ne- cessity 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 micro- organisms 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 bio- chemical reactions) and molecular-genetic one. At the second approach different 16S rRNA se- quences 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 microorgan- isms 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 dura- ble 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 subcultiva- tions 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 con- tamination could depress regeneration, micropropagation, cause death of in vitro cultivated objects, restrict the protocols repeatability and concern induction of epigenetic somaclonal variability. For in- stance *Acinetobacter* and *Lactobacillus plantarum* filtrates extracted from degrading calluses strongly reduced shoot regeneration at inoculation in explants or addition into a medium; bacteria *Mycobac- terium 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.

Кeywords: 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 habi- tat 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]. La- tent bacterial infections, defined by many researchers as internal or endophytic, are detected in calli and microplants cultivated in vitro, as well as in various ex- plants 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 ut- most 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 mi- crobiology and medicine practices [24]. Moreover, attention is drawn to bacte- rial endophytes due to the accumulation of data indicating the conventionality of historical division of microorganisms into phytopathogenic, pathogenic for ani- mals (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 moni- toring 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 endo- phytic, 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 microor- ganisms 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 «endo- phytic bacteria» as it is used by the authors of the cited papers.

According to the widely used definition, endophytes are microorgan- isms 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 obliga- tion 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 docu- mented by light and electronic microscopy, and using in situ hybridization [15, 16, 21, 36-38].

S o u r c e s o f b a c t e r i a l m i c r o f l o r a. Endophytic bacteria are de- rived from epiphytic associations of plant rhyzosphere and phytosphere. The initial explants mostly are the causal factors of endophitic infection during in vi- tro 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 oc- cur 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 inter- cellular 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].

A p p r o a c h to d e te c t i o n a n d i d e n t i f i c a t i o n. There are differ- ent ways to reveal a latent bacterial contamination. In particular, selective me- dia, 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 re- action, REP-PCR — repetitive extragenic palindromic polymerase chain reac- tion, AFLP — amplified fragment length polymorphism, ARDRA — amplified ribosomal DNA restriction analysis, 16S rRNA) are successful in bacterial typ- ing. 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 geno- types is based on the analysis of conservative rRNA genes which present in all bacterial cells and are genus-specific in most microorganisms [23]. For identifi- cation, 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 pref- erably used to attribute the microbial species and strains [50]. The ITS and 16S rRNA gene fragments are amplified in PCR with metagenomic DNA and spe- cific 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.

I d e n t i f i c a t i o n o f b a c t e r i a i n i n v i t r o c u l t u r e. 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 mo- lecular 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].

D yn a mic s o f ba c ter i a l ex pre s s i o n. 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 С), 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 cul- ture is at a medium level leading to symptomless and long persistence of infec- tious 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 nutri- ent 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 in- fection 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 micro- propagated banana plants the endophytes were nonculturable for three passages and detected only by sequencing 16S rRNA gene (viable but nonculturable bacte- ria — VBNC). Nevertheless, from 4th to 18th 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. I m p a c t o n c o l o n i z e d in vitro p l a n t c u l t u r e s. Bacteria associ-

ated 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 epige- netic 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 nu- trient 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 spe- cial interest. It was shown that *Acinetobacter* and *Lactobacillus plantarum* fil- trates from degrading callus decreased sharply the shoot regeneration when add- ing 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 *Pe- largonium* ½ *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 ma- terial 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.

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