Endogenous phthalates in plants and their alleged participation in defense response against phytopathogens

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Abstract. Endogenous phthalates (esters of o-phthalic acid) have been revealed in plant in situ and in vitro. Phthalates reduced biofilm formation and growth of Clavibacter michiganensis ssp. sepedonicus and changed morphology of colonies. So phthalates were considered to be a part of plant defense against bacterial phytopathogens. Meanwhile, phthalates have been found in the cells of phytopathogenic bacteria. It was suggested that the physiological and biochemical role of phthalates can be much more complex and not be limited to the participation of plant organisms in the protective process.

1. Introduction and Background

Phthalates (esters of o-phthalic acid) are best known as products of the chemical industry. For a long time, they were considered purely xenobiotics and pollutants [1,2]. For a human and animal organisms phthalates (PEs) are characterized as a very dangerous substances. PEs are known as a mutagenic, teratogenic, carcinogenic agents and as an endocrine disruptors[3,4]. Phthalates have also a phytotoxic effect: at presence of PEs normal physiological reactions in barley seedlings were disturbed [5].

PEs are widely spread in sewage sludges and soils, including agricultural areas, due to their ability to be complexed with humic substances and to became soluble [6]. In addition to above mentioned, another point of view take place. To date a lot of information has appeared on the presence of biogenic phthalates in organisms of various phyla. PEs have been detected in microorganism [7,8], algae [9-12], fungi [13] and higher plants [14-16]. Direct evidence of phthalate biosynthesis from labelled precursors has been obtained [10, 12]. The aim of current work to detect endogenous PEs in various plant objects and bacteria and to propose their physiological role.

2. Materials and Methods

Plant material in situ was collected in different regions of Russia (Crimea and Siberia) in locations distant from central industrial areas. Plants in vitro and cell cultures and also Rhizobium rhizogenes (R-Ri) (strain RiC58C1), Rhizobium radiobacter (R-Ti) (strain TiC58) were obtained from the collection of the Bioresource Center for Collective Use of the Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch, Russian Academy of Sciences (Irkutsk). Cultures of the Clavibacter michiganensis ssp. sepedonicus (Cms) (strain Ac-1405) and Pectobacterium carotovorum ssp. carotovorum (Pcc) (strain V1247) were obtained from the All-Russian Collection of Microorganisms (Pushchino).
Plant and bacterial samples were analyzed for dibutylphthalate (DBP) and di-2-(ethylhexyl) phthalate (DEHP) content by gas-liquid chromatography with a mass spectrometric detector using 7000QQQ/7890A chromatography mass spectrometry (Agilent Technologies, United States).

Culturing of Cms was carried out in the dark while shaking at 26°C using the C medium. After 72 h of culturing, 10 µl of bacterial suspension (OD\text{650} = 0.7) was dropped into center of Petri dish on solid medium C (agar 0.7%) with addition DBP. Concentration of DBP was 10, 20, 30 and 60 µM. Plates were placed in thermostat at 37 °C for seven days. Daily dishes were photographed and areas of CMS colonies were measured. Plates with linear gradient of DBP agar medium were prepared as was described in [17]. Experiments were carry out in triplicate.

3. Experimental Section

PEs (DBP and DEHP) were detected in a number of plant (table 1). It should be noted that plants in situ were of different phyla, different ecological specializations and grew in different regions of Russia.

| Specie                                | DBP  | DEHP |
|---------------------------------------|------|------|
| Pleurozium schreberi Willd. Ex Brid   | 98±3 | 19±1 |
| Equisetum sylvaticum L. (aboveground shoots) | 14±2 | 19±1 |
| Lycopodium clavatum L.                | 8±1  | 37±1 |
| Athyrium filix-femia Roth et Mert     | 19±1 | 9±1  |
| Pinus sylvestris L.                   | 16±2 | 23±2 |
| Pinus brutia var. stankeviczii (Sukaczew) Frankis* | 32±2 | 39±1 |
| Betula platyphylla Sukaczew           | 8±1  | 14±2 |
| Fagus sylvatica L. *                  | 23±2 | 32±3 |
| Magnolia grandiflora L. *             | 118±5| 14±2 |
| Populus tremula L.                    | 21±3 | 23±2 |
| Quercus pubescens Willd*              | 74±3 | 23±3 |
| Elodea canadensis Michx               | 22±1 | 120±2|
| Allium victoriais L.                  | 13±3 | 5±1  |
| Triticum aestivum L. (etiolated seedlings) | 42±2 | 36±3 |

*plant collected in South Crimea; \(M \pm m; n=3\)

PEs were detected not only in plants taken from natural habitats but in closed experimental systems with controlled growth conditions – in plants and cell cultures grown in vitro (table 2).

| Specie                                | DBP  | DEHP |
|---------------------------------------|------|------|
| Plant in vitro                        |      |      |
| Nicotiana tabacum L.                  | 47±7 | 100±11|
| Oxytropis triphylla (Pallas) Pers.    | 9±6  | 18±1 |
| Solanum tuberosum L. (cv.Lugovskoy)   | 50±1 | 63±2 |
| Cell cultures                         |      |      |
| Aconitum baikalense Turcz. ex Rapaics | 24±2 | 332±20|
| Aconitum barbatum Patr. ex Pers.      | 10±2 | 246±3|
| Nicotiana tabacum L.                  | 15±2 | 76±4 |
| Saussurea controversa DS              | 9±1  | 124±11|
| Scorzonera hispanica L.               | 85±3 | 82±2 |
| Solanum tuberosum L. (cv.Lugovskoy)   | 13±2 | 43±2 |

\(M \pm m; n=3\)
PEs were also detected in bacteria (table 3).

**Table 3.** The content of PEs in bacteria, µg/g dry weight.

| PEs   | Cms   | Pcc    | R-Ri  | R-Ti  |
|-------|-------|--------|-------|-------|
| DBP   | 70-121| 80-121 | 60-90 | 50-80 |
| DEHP  | 7-9   | 35-45  | 11-20 | 8-13  |

When growing *Cms* in Petri dishes on C medium with DBP concentration 20, 30 and 60 µM, growth of bacterial colony was for a time suppressed (figure 1). Remarkably, when growth was resumed, the morphology of colony was changed (figure 2). When growing *Cms* in Petri dishes in medium with a DBP gradient of 0-60 µg/l, growth of bacteria was observed towards the gradient concentration increase (figure 3).

![Figure 1](image1.png)

**Figure 1.** A growth of *Clavibacter michiganensis* ssp. *sepedonicus* colony’s areas on a medium with dibutylphthalate (10, 20, 30 and 60 µM). Legend indicates corresponding concentrations. Line 10 was artificially increased on 5 units to separate it from line 0. M ±SE; n=3.

![Figure 2](image2.png)

**Figure 2.** Morphology of *Clavibacter michiganensis* ssp. *sepedonicus* colonies on a medium with dibutylphthalate (10, 20, 30 and 60 µM).

![Figure 3](image3.png)

**Figure 3.** A growth of *Clavibacter michiganensis* ssp. *sepedonicus* colonies on medium with a dibutylphthalate gradient of 0-60 µg/l. White arrow shows the direction of DBP content increasing. A – medium without organic components; B – complete medium C.
4. Results and Discussion

The experiments were carried out under the conditions that excluded the presence of phthalate impurities in the reagents, materials, and ware, which was carefully controlled. The nutrient media and other materials were tested for the presence of exogenous phthalates. Their content in nutrient media did not exceed 1 µg per 100 mL. All experiments were carried out in glassware. It should be noted also that plant in vitro, cell cultures and bacteria studied for the presence of endogenous phthalates were obtained from collection cultures. Thus, phthalates detected during our experiments were undoubtedly of natural origin. What is a presumable biological function of endogenic PEs in plant? In some studies, the antimicrobial activity of phthalates against gram-positive and gram-negative human pathogens has been found [7]; their cytotoxic properties have been detected [8]. Moreover, the ability of cells to excrete phthalates into the extracellular environment under stress has been disclosed [10]. That may have biological significance in the interaction of various organisms. Earlier we have been revealed that PEs reduced biofilm formation in bacterial phytopathogens [17]. According to this data, adding dibutyl phthalate to the culture medium of bacteria reduced the intensity of biofilm formation both in the biotroph Clavibacter michiganensis ssp. sepedonicus and in the necrotroph Pectobacterium carotovorum ssp. carotovorum. The effect was similar when using di-2-ethylhexyl phthalate. Biofilm formation, the initial stage of plant colonization, is known to determine the display of virulence of pathogens increasing their hardness to plant resistance factors and blocking the xylem flux. In current study we obtained the similar results (figure 1 and 2). These facts suggest that these substances can potentially be used as protective compounds in plants. It should be noted that the effect of phthalates on phytopathogenic bacteria was practically not studied earlier. Meanwhile, phthalates have been found in the cells of phytopathogenic bacteria (table 3). When growing Clavibacter michiganensis ssp. sepedonicus in Petri dishes in minimal (without organic components) medium with a DBP gradient of 0-60 µg/l, growth of bacteria was observed towards the gradient concentration increase (figure 3A). That indicates the physiological reaction of bacteria to the presence of phthalates. This fact can be explained by that bacteria use PEs as a nutrient source. Nevertheless, the same picture was observed when complete medium C was used (figure 3B). Rather ambiguous results suggest that the physiological and biochemical role of phthalates can be much more complex and not be limited to the participation of plant organisms in the protective process.

5. Summary and conclusion

Additional data on presence of PEs in plant and bacteria are obtained. The detection of PEs in closed experimental systems with controlled growth conditions validate the proposition on endogeneity of PEs in plant and bacteria. In experiments phthalates reduced and modified colonies of phytopathogen Clavibacter michiganensis ssp. sepedonicus. On the data obtained, it can be assumed that phthalates are involved in the protection of plants from infections suppressing the process of growth and biofilm formation of bacterial phytopathogens – the initial stage of plant colonization. The biological function of phthalates is thought to be of main interest.

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