Phytotests - a method to fast evaluation of the biostimulant potential of different organic industrial waste

Fitotesty - metoda szybkiej oceny potencjału biostymulacyjnego różnych organicznych odpadów przemysłowych

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Abstrakt
W procesie produkcji skóry powstają duże ilości odpadów stałych, zawierających niebezpieczne, ale i wartościowe składniki. Dlatego głównym przedmiotem zainteresowania badawczego jest eliminacja zanieczyszczeń i wykorzystanie w jak najbardziej efektywny sposób stałych odpadów skórzanych. Również odpady drzewne są potencjalnie ważnym źródłem surowców. Tego rodzaju opady powstają jako produkt uboczny podczas pozyskiwania i przetwarzania drewna na całym świecie. Zastosowanie kory ma wyjątkowe znaczenie ze względu na jej unikalne składniki chemiczne i niezwykłą strukturę. Kora drzewa iglastego jest wykorzystywana do produkcji chemikaliów i materiałów, a także bioenergii. W wyniku modyfikacji organicznych odpadów, chemicznych bądź biologicznych, można uzyskać produkty o wartości dodanej – biostymulatory roślin. W pracy zaprezentowano wyniki badań z użyciem testów Phytotoxikit, które mogą służyć nie tylko jako cenne narzędzie w badaniach ekotoksykologicznych, ale również mogą stanowić doskonałą metodę szybkiej oceny działania biostymulatorów organicznych.

Abstract
High amounts of solid waste containing hazardous and high-value components are generated during the leather making process. Therefore, the elimination of the pollution and resource utilization of leather solid waste is the primary research interest. In addition, wood waste is also a potentially important source of raw materials. This type of rainfall is produced as a by-product of timber harvesting and processing around the world. The use of bark is of exceptional importance due to its unique chemical components and unusual structure. Conifer bark is used to produce chemicals and materials as well as bioenergy. As a result of the modification of organic waste, chemical or biological, it is possible to obtain products with added value - plant biostimulants. The paper presents the results of research with the use of Phytotoxikit tests, which can serve not only as a valuable tool in ecotoxicological research but also can be an excellent method for a quick assessment of the effects of organic biostimulants.

Słowa kluczowe: *odpady skórzane, kora, fitotesty, biostymulatory*

Keywords: *leather waste, bark, phytotests, biostimulants*

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1. Introduction

Millions of tons of leather waste from the tanning process in the leather industry are released at onshore locations. These solid wastes might contain large amounts of trivalent chromium Cr (III). Although Cr (III) is not considered hazardous, its oxidation to the highly toxic hexavalent chromium Cr (VI) due to burning poses environmental concerns. Therefore, conventional disposal options, such as burning and landfilling, are not environmentally beneficial approaches to this waste disposal. The effective conversion of these wastes into valued products can be a viable option [1]. For example, recovery and reuse of protein components of the waste can reduce the amount of landfilled waste which will reduce the negative impact on the natural environment. [2]. Numerous research of the treatment and application of leather wastes have been reported [3-5]. Shavandi et al. described the dissolution, extraction, and biomedical application of keratin [5]. The leather wastes can also be used in the production of biodiesel, biogas, biopolymers, adsorbent materials, agriculture applications, and others [4]. Using these waste in agriculture seems to be of particular interest. In the work of Nabavinia et al., the influence of tannery waste on chemical and physicochemical properties of soil and the yield of radish was investigated [6]. The addition of waste in fertilizers increased the content of nitrogen and phosphorus in the soil, causing an increase in fresh and dry matter of vegetables and tannery waste can also be used to produce vermicompost (the end-product of the breakdown of organic matter by earthworms) to increase the amount of fruit or plants [7]. Another interesting solution is the use of collagen hydrolysates from tannery waste in the coating process of leguminous seeds. Numerous studies show that the coating containing the collagen preparation provides the plant with favorable conditions in the early growth phase [8-10]. The rational and effective use of natural resources, including the improvement of existing technologies is currently an important task [11]. Along with other natural materials, stored waste bark can be another example of wasting a valuable raw materials. Most of hundreds million tons of waste bark are incinerated, landfilled or used for thermal energy production [12]. Burning the waste bark is not cost-effective due to its low heating value and its high ash and moisture content. However, tree bark contains important extractives, and large-tonnage waste bark is a huge raw material resource for manufacturing valuable chemical products [11]. As reported in the literature, the wastes from wood processing have been used for air purification (biofilter) and water purification, which allows for the binding of different harmful ions (lead, mercury, cadmium). Bark can be also used as a substrate for anaerobic fermentation. In addition, the bark is used to obtain substances with bactericidal and hydrophobic properties, such as wax, which is widely used in the cosmetic industry. The bark is also successfully used to create effective environmentally friendly insulation materials. What is more, waste bark can be used in agriculture for mulching which reduces evaporation from the soil surface. As a consequence, the soil needs fewer additional artificial fertilizers. All mentioned advantages of bark, easy availability and the problem of its use, prompted the authors to use the bark as an additive to the soil in plant cultivation.

Chemical methods which are used to assess the impact of the soil environment on plant growth, are not always quick and cheap. Therefore, the practice of environmental monitoring more often uses biological methods such as biotests, which in most cases meet such conditions. As a consequence, biotests are becoming a supplement to routine laboratory practice [13]. Plants, mainly seed plants, are commonly used to determine the degree of soil contamination with heavy metals, pesticides, or PAHs. Contamination assessment includes seed germination, seedling growth, and root inhibition. The growth of seedlings is based on the analysis of the phytotoxic effects of pollutants on plants at the stage of seed germination and seedling development [14,15]. These tests can also be used to investigate the effect of soil constituents that are intended to support the growth of seeds. For this reason, this paper presents the results of toxicological research on the impact of the admixtures of waste from the leather and wood industry in soil on the growth of sugar peas using popular, cheap and effective phytotests. It is worth noting that the study also examined the influence of both types of waste after microbiological modifications with the use of a selected yeast isolated from the natural environment.

2. Materials and Methods

2.1. Waste materials

The bark used in research is a product widely available on the Polish market. The purchased coniferous bark combines various fractions of pine bark, dry matter content min. 60%, the pH is 4. Before starting the tests, the bark was ground into fine dust in an electric grinder with a power of 2400 W for 1 min. The material prepared in this way was introduced directly into the soil, as well as it was added to the liquid culture for microbiological treatment. The chrome-tanned leather shavings were also ground on an electric grinder. This material, which was only added to the soil, had the typical characteristics of semi-finished leather: moisture 51%, total ash 8.6%, total nitrogen 16.5%, chromium oxide 4.4%, and pH of aqueous extract 4.2. The picture below shows the appearance of the bark of conifers.

Fig. 1. Bark of conifers.

2.2. Biological material

2.2.1. Microorganisms

Yeast were isolated from the soil sample contaminated by petroleum hydrocarbons and high content of heavy metals. The isolation was carried out in the Łukasiewicz Research Network - Institute of Leather Industry. The standard method of isolation from soil i.e. diluting the population of organisms in a physiological solution of sodium chloride was used. Then, the diluted suspensions were inoculated on Petri dishes with an appropriately selected solid medium that stimulated the growth of the organisms sought. Reduction inoculations were performed on the appropriate media for fungi (YPG medium) and bacteria (TSA medium), obtaining single colonies. A preliminary study was carried out by biochemical methods identification of microorganisms by determining their properties. So far, it has been shown that yeast is much more suitable for industrial biotechnological processes due to its rapid growth and greater resistance to environmental stress than bacteria. For this reason, an isolated and biochemically identified yeast strain of the *Dipodascaceae* family was selected for further research.

2.2.2. Biological modified methods of waste

Yeasts from the *Dipodascaceae* family were stored on slants in at 4°C on a modified YPG medium composed of 1.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 2% (w/v) agar. The slant over with 10 ml of saline and left for 10 minutes was poured. Then, after washing the microorganism cells from the slant, the optical density (OD 600) was measured on a spectrophotometer at a wavelength of 600 nm. The resulting suspension diluted with saline until the culture density was OD600 = 1,000. The 0.4 ml stock suspension prepared in this way was transferred to 100 ml flasks containing 40 ml sterile YPG medium and the waste bark in the amount of 1% (w/v). The biological modified was carried out for 48 h at 30°C on an orbital shaker (190 rpm). The pH of the medium before sterilization (121°C, 45 min) was 6.0.

2.2.3. Plants material

The research was conducted with one legumes cultivars (Iłuviecki sugar pea in Latin *Pisum sativum*) grown in laboratory conditions at the Łukasiewicz Research Network – Łódź Institute of Technology. These grains were purchased from W. Legutko Breeding and Seeding Company Sp. z o.o. (Poland). The purchased sugar peas are characterized by the formation of fine and fiberless pods. Plants grow up to 50 cm. The picture below shows the appearance of the grains.

Fig. 2. Sugar pea seeds.

2.3. Biological assay

Phytotoxkit – set for the germination and early plant growth testing. Assay plates were filled with 100 g (65 ml) of tested soil and moistened with distilled water to 100% water capacity and covered with filter paper. Then, to soil different organic biostimulators were added (Tab. 1). The next step procedures the test plant seeds (Iłuviecki sugar pea in Latin *Pisum sativum*) were applied at 10 pieces per plate.

Tab. 1. Description of tested variants.

Description	Code
100 g of soil and 0.35 g bark	A 1
100 g of soil and 0.2 g biological modified bark	A 3
100 g of soil and 0.35 g (biological modified bark + biomass yeast)	A 5
100 g of soil and 15 ml bark supernatant after centrifugation	A 7
100 g of soil and 15 ml (biological modified bark/yeast supernatant after centrifugation)	A 9
100 g of soil and yeast supernatant after centrifugation	A 11
100 g of soil	Control 1
100 g of soil and 0.2 g chrome-tanned leather shavings + 0,2 g bark	B 1
100 g of soil and 0.3 g biological modified bark	B 3
100 g of soil and 0.2 g (biological modified bark + biomass yeast)	B 5
100 g of soil and 5 ml bark supernatant after centrifugation	B 7
100 g of soil and 2.5 ml (biological modified bark/yeast supernatant after centrifugation)	B 9
100 g of soil and 2.5 ml yeast supernatant after centrifugation	B 11
100 g of soil	Control 2

Assay plates were incubated vertically at 25 for 3-5 days. The tests were carried out in triplicate for each variant. The reaction of plants to the presence of organic wastes and stimulant substances intermediates of their metabolism was determined based on the degree of the inhibition or stimulation of seed germination and root growth. All measurements were done using an "Image J" analysis program. The idea of Phytotoxikit tests is shown in Figure 3.

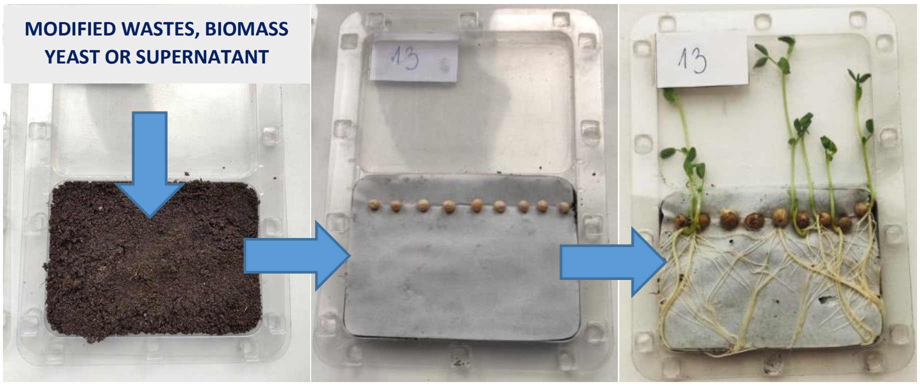

Fig. 3. The process of conducting the Phytotoxikit assay.

2.4. Data analysis

The percentage inhibition or stimulations (1) of seed germination (GI) and root growth inhibition or stimulations (RI) for the plants was calculated according to the formula:

$$(1)\ \text{GI or RI} = \frac{A-B}{A}\ x\ 100\%$$

where:
A is the mean seed germination level or root length in the control soil;
B is the mean seed germination level or root length in the test soil.
The effect of each variant on GI and RI was analyzed using the Statistica 10.0 program. Significance was set at p = 0.0.

3. Results and Discussion

The main aim of this study was to investigate the possibility of using phytotests to assess the performance of biostimulators produced by biotechnological methods. It was focused on the analysis of the root length as an indicator of the effect of modified wastes added to the soil. Particular attention was paid to the growth of roots which are more sensitive to biostimulators while seed germination is much less sensitive. Preliminary experiments revealed that in both the reference soil and soil with biostimulators the number of seeds that did not germinate was comparable. Furthermore, it was noted that the germination of seeds alone did not guarantee further growth of plant roots. Literature data prove that germination capacity is only slightly affected by petroleum substances, heavy metals, or other pollutants added to soil [16-18]. Literature reports that biostimulants have a positive effect on germination capacity when seeds were soaked. Most biostimulant effects of modified organic wastes refer to the amelioration of root nutrition, via different mechanisms [19-21]. For this reason, our studies focused on the control of the percentage inhibition or stimulation of root growth as a key factor affected by the presence of biological modified wastes in soil.

The biological material used in the study was sugar pea (*Pisum sativum* L.). The value of sugar pea (*Pisum sativum* L.) as a crop can be assessed in two ways. Firstly, as the seeds contain 20–24% protein, they are a valuable food and feed source. Secondly, the crop residues that remain in the field after cultivation favorably affect the physical, chemical and biochemical properties of the soil. Sugar pea is considered one of the most important vegetable crops belonging to the legume family [22-24]. The effects of the biostimulators (modified or not biological modified organic wastes) on the pea plants were variable between variants and depended on dose of substances in soil conditions (Fig. 4, Fig. 5 and Fig. 6).

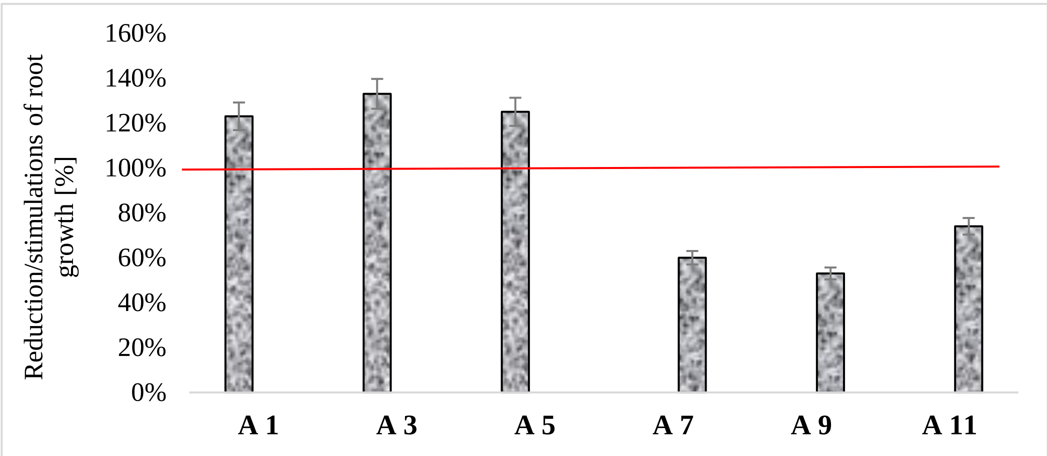

Fig. 4. Results of % reduction or stimulations growth of sugar peas in soil with additives (A1-A11) over control after three days of the test.

In the first series (Fig. 4), the best results were obtained for the A3 test. The stimulations of root growth reached 33% as compared to the control sample. The introduction of 0.35 g of biologically modified bark and yeast biomass was effective. These insights are consistent with other works [25, 26]. Yeast extract or yeast biomass is a source of amino acids or cytokines. Amino acids are an easy source of nitrogen and carbon for soil microorganisms [27]. Cytokinins are one of the main groups of plant hormones that stimulate anabolic processes as well as cell growth and division [28]. The modified bark has more nutrients that are readily available to plants. Such bark is free from phenols and tannins, and it also stores water better. Surprisingly, after the addition of culture supernatants, inhibition of the growth of the root length was observed (A7, A9, and A11). Inhibition on average was greater by over 40% compared to the control sample. This could be caused by an overdose of substances rich in phenols and tannins, or proteins [29]. It was decided to repeat the experiment by changing the doses of additives. The results of the second series of experiments are presented in Fig. 5 and Fig. 6.

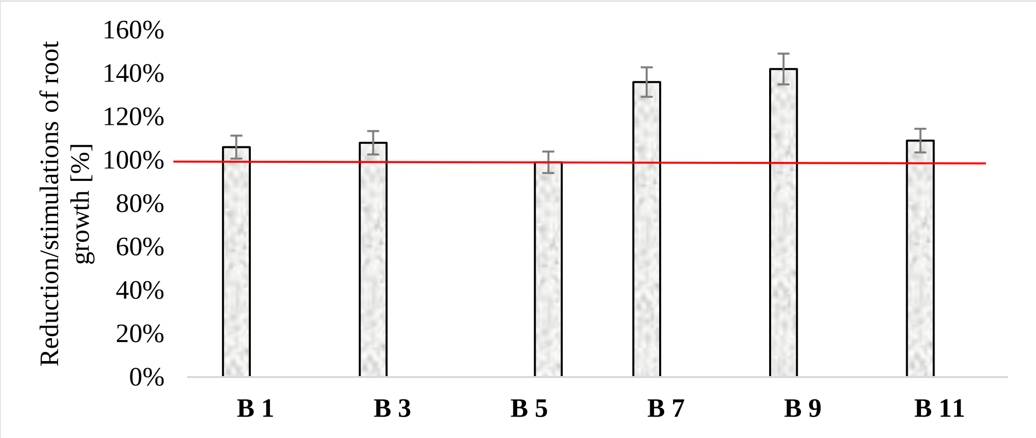

Fig. 5. Results of % reduction or stimulations growth of sugar peas in soil with additives (B1-B11) over control after three days of the test.

The best results were obtained for the B9 test. The percentage of root length growth stimulation was 42% as compared to the control sample (Fig. 5). The addition of a smaller dose of post-cultivation supernatants to the soil resulted in a significant stimulation of root growth and expansion of the root zone, which is shown in Fig. 6.

Fig. 6. Sugar pea growth after 5 days of test (B1 - B11 - test samples, 13 -control sample).

Both the supernatant obtained as an effect of biological modification of the bark and yeast supernatant resulted in better plant growth (Fig. 5). These results are in accordance with those obtained on faba bean for yeast extract [30]. The positive response of sugar pea growth to yeast extract may be due to its high content of cytokines, Vitamin B, organic compounds, and nutrients, which increase the distribution and translocation of metabolites from leaves towards the reproductive organs, thereby increasing sugar pea yield [23]. Treatment of waste bark may contain phenolic compounds, mostly gallic acid derivatives and gallotannins, ellagitannins, and ellagic acid derivatives. Other compounds were low molecular weight phenolic acids and aldehydes [1]. Reports in the literature indicate that the compounds may function as stimulators of growth and plant growth and contribute to the improvement of the yield of crops [3]. On the other hand, the introduced smaller doses of bark, chrome-tanned leather shavings, and bark after modification did not cause statistically significant changes or the stimulation was much lower than in the first series (Fig. 5). In the available literature, there are not many papers on the subject [31].

The nature of biostimulants and their action on plants is diverse. The effectiveness of a biostimulator is strongly dependent on the method of application, species, variety of crop, and concentration. Our study proved showed that the effect of biostimulators on grown of sugar peas was closely related to the dose of organic substances, not their nature (solid or liquid).

4. Conclusions

The obtained results show that Phytotoxikit tests represent an effective tool to employ in the evaluation of the initial performance of biostimulants. Therefore, they can be used for future crop management to stimulate plant growth and productivity. Further experiments will be necessary to investigate in depth the effects of organic biostimulants against other parameters.

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