Modern microbiological methods of phytosanitary monitoring

M V Nikolenko¹, V V Trigub² and O V Enotkaeva³

¹Tyumen State Medical University, Tyumen, Russia
²Industrial University of Tyumen, Tyumen, Russia

nikolenko-marina@mail.ru, trigubvv@tyuiu.ru, pechkanova@mail.ru

Abstract. In this work, an express method is proposed, based on the registration of changes in the impedance of the nutrient medium, which occurs under the influence of the processes of growth and vital activity of microorganisms in the sample under study. The research was carried out on the analyzer "BioTrack 4250". This device is highly productive, as it examined 21 samples simultaneously for several hours. For testing the indirect impedance technique, fodder, medicinal and weed plants were selected as objects of study. The sanitary condition of the samples was assessed by the total mesophyll aerobic and optional-anaerobic microorganisms (QMA&OAMO), sanitary-indicative bacteria of the E. coli group, and the number of yeast fungi. It was proved that plants collected in the central part of the city of Tyumen with a high population density had high QMA&OAMO indices and high indicators of contamination with Candida sp. On the contrary, fodder plants growing in the Tyumen region were characterized by high indices of sanitary-indicative microorganisms, which indicated an increased risk of feed contamination. Thus, in contrast to the classical methods, the proposed method allows obtaining objective results, shortening the research time, reducing labor costs, and significantly reducing the cost of analysis.

1. Introduction

Currently, much attention is paid to assessing the phytosanitary situation in the regions, which is important for obtaining high and stable yields and intensifying agricultural production [1, 2].

The most significant and dangerous causes of sanitary destabilization of plants are: changes in the structure of populations of bacterial-fungal associations in Russia, increased pathogenicity of microorganisms, changes in distribution areas, and the possibility of increasing the development of soil infections (fusarium, late blight, candidiasis) [3, 4]. The use of high doses of mineral fertilizers contributes to an increase in the development of bacterial diseases, pests, weeds.

An overdose of pesticides has become a dangerous phytosanitary problem with the incorrect use of intensive technologies, leading to an increase in the resistance of pathogens to protective agents, as well as to plant toxicosis. An important reason for phytosanitary problems is the imperfection of domestic agricultural technologies [5, 6].

Classical methods for determining the sanitary and bacteriological state of plants, plant materials, and soils require a lot of labor and time and produce results within a few days. Traditional methods of analysis reduce the efficiency of obtaining information.

Revolutionary technologies for the detection of pathogens in plant-based food are based on the latest advances in molecular cloning, methods of studying recombinant DНС, and PCR analysis. [7].

The advent of gene probe methods has made it possible to develop bacterial identification tests based on gene amplification. PCR analysis is an undeniably reliable and sensitive method. However, this is an expensive, high-tech process that requires compliance with the strictest rules for equipping
the laboratory and can give a false positive result due to the presence of DNC fragments of various living organisms in the air [8]. Considering the importance of the microbiological state of the biotope, it is essential to use universal methods of rapid analysis for the presence or absence of pathogenic microorganisms that are neither laborious nor costly [9]. This paper proposes an innovative methodological approach using indirect impedance for phytosanitary monitoring.

The aim of the study is to test modern microbiological methods for phytosanitary monitoring.

2. Materials and methods
To test the method, 5 samples were selected as objects of research.

Sample No. 1—inflorescences with pollen from awnless brome grass (*Bromopsis inermis Holub.*) of the grass family (*Poaceae sp.*). The plant is widespread in western and eastern Siberia [10]. This valuable fodder crop cultivated for hay and green fodder is widely used on long-term pastures [11].

Sample No. 2—grass of meadow timothy (*Phleum pratense*) of the grass family (*Poaceae sp.*). It is an important fodder crop for cattle, horses, rabbits, chinchillas, and degus [12, 13].

Sample No. 3—leaves and inflorescences with pollen from the cocksfoot or orchard grass (*Dáctylis glomeráta*) of the grass family (*Poaceae sp.*). It is a widely-cultured hay and pasture fodder plant. Samples 1-3 were collected in the meadow area of the Tyumen region allocated for hay making 15 km off Tyumen along the Moskovsky tract (highway) in June 2019.

Sample No. 4—leaves of the greater plantain (*Plantágo májor*) of the plantain family (*Plantaginaceae sp.*). *P. májor* leaves contain polysaccharides, iridoid glycoside, aucubin, bitter substances, carotenoids, ascorbic acid, choline and are used as a wound healing, anti-inflammatory, hemostatic, hypnotic, analgesic, bactericidal and anti-allergic agent [14, 15].

Sample No. 5—inflorescence with pollen of the creeping soft grass (*Hólcus móllis*) of the grass family (*Poaceae sp.*). It is an invasive, weed plant [16, 17]. *P. májor* leaves and *H. móllis* inflorescences with pollen were collected in the local area of the Central District of Tyumen in June 2019.

The samples obtained were studied on a "BioTrack 4250" device manufactured by SY-LAB Gerate GmbH (Austria) according to the MUK 4.2.2578-10 method “Sanitary-bacteriological studies by the separated impedance method”. The "BioTrack 4250" device is an automated express system for rapid quantitative and qualitative assessment of the degree of microbial contamination of food raw materials, food products, cosmetic and pharmaceutical products, drinking water, soil and other environmental objects.

This device is highly efficient, due to the fact that it allows for simultaneous examination of 21 samples of the samples taken. The express analyzer "BioTrack 4250" recorded changes in the electrical resistance of the nutrient medium under the influence of the growth and vital activity of microorganisms in the test sample. The microbiological analyzer has a unique ability to measure two parameters: electrode impedance (E-parameter) and medium impedance (M-parameter), which made it possible to carry out a wide range of qualitative and quantitative studies.

The M-parameter measurement was the relative decrease in the impedance of the medium, expressed as a percentage of the initial measurement. The M-parameter mainly reflected the part of the impedance associated with the active conductance. It was directly influenced by the ionic composition of the nutrient medium, as well as the sample used for analysis. For these reasons, with a high electrical conductivity of the nutrient medium, this parameter lost its significance. The composition of the nutrient medium did not affect the value of the E-parameter, which is very important in cases when media with a high salt content are used, therefore, the E-indicator was chosen for the study. When the specified threshold values for the E-parameter were crossed, the sample was evaluated using a traffic light system—red, yellow, green (if time intervals were set) and/or an automatic counting of microorganisms in the test sample (if a calibration file was used).

The sanitary state of the samples was assessed by the total mesophyll aerobic and optional-anaerobic microorganisms (QMA&OAMO) in 1 cm³ (ml). The total number of microorganisms was
determined on a BiMedia 001B nutrient medium (manufactured by HiMedia) at a temperature of 30°C.

Sanitary-indicative bacteria of the *Escherichia coli* group (*Escherichia sp.*, *Shigella sp.*, *Salmonella sp.*, *Citrobacter sp.*) were cultivated on an enrichment medium—buffered peptone water, where contamination parameters were determined, and then they were subcultured onto BiMedia 205A, 401A, XLD (Xylose-Lysine-Desoxycholate Agar), Ploskirev’s, Kligler’s, Simmons’ and Christensen’s nutrient media to identify the genus and species. The saccharolytic activity of bacteria, their ability to break down lactose and proteolytic activity, and the ability to break down protein with the formation of hydrogen sulfide were evaluated on Kligler's medium. Microbes using citrate as their sole carbon source grew on Simmons' medium. Microorganisms that break down urea alkalized Christensen's medium, as a result of which it was colored. All crops were cultured at a temperature of 37°C. To culture molds and yeasts, BiMedia 501B medium with pH: 7±0.2 was used.

The species identification of fungi was carried out according to a set of characteristics: the appearance of the colonies, the chlamydospore formation, the test for the formation of growth tubes, and the assimilation capacity of strains [18].

The "Auxacolor 2" assimilation colorimetric test system by Bio Rad (France) includes 13 sugars: glucose (GLU), maltose (MAL), sucrose (SAC), galactose (GAL), lactose (LAC), raffinose (RAF), inositol (INO), cellobiose (CEL), trehalose (TRE), adanitol (ADO), melibiose (MEL), xylose (XYL), arabinose (ARA). The objects of the study were assessed without restrictions on sanitary and bacteriological indicators in the absence of pathogenic bacteria and spoilage fungi [19]. For control, a nutrient medium (without inoculum) was used.

Statistical processing of the materials and graphical representation of the results were performed using the following programs: Primer of Biostatics Version 4.03 by Stanton A. Glantz 1998, Microsoft Office Excel 2010.M – the arithmetic mean, δ-the standard deviation, m-the average error of the arithmetic mean were determined, and the data were presented in the form M±m or M±δ. The Student's t-test was used for matching the compared samples to the normal distribution law (in χ²).

The software on the "BioTrak 4250" microbiological analyzer includes: "MicroTrack"—a program that manages data, monitors the progress of measurements and obtains analysis results, and "Micro Assist"—a program for further work with measurement results.

3. Results and discussion

The QMA&OAMO index is an integrated indicator presented by various taxonomic groups of microorganisms. Their total quantity may indicate the sanitary and hygienic state of the object, the degree of its contamination with microbiota. QMA&OAMO is the most common microbial safety test. High values of the indicator can be due to normobiota, while phytopathogens will be absent. Therefore, it is more adequate to assess the total quantity of microorganisms as an indicator of the presence of epiphytic microorganisms. Table 1 provides the results.

**Table 1. The total quantity of mesophyll aerobic and optional-anaerobic microorganisms.**

| Sample No. | Indicators of device, E CFU/ml (QMA&OAMO in 1 ml of object) |
|------------|------------------------------------------------------------|
| 1          | 6.1E+3 CFU* 6.1x10^3 = 6.1x10^4*                          |
| 2          | 5.3E+3 CFU* 5.3x10^3 = 3.3x10^4*                          |
| 3          | 5.0E+3 CFU* 5.0x10^3 = 5.0x10^4*                          |
| 4          | 1.5E+4 CFU* 1.5x10^4 = 1.5x10^5*                          |
| 5          | 1.5E+3 CFU  1.5x10^3 = 1.5x10^4                           |

Note: * - p ≤ 0.05, E-value - electrode impedance, CFU - quantity of colony-forming units

In the course of the work, we found that the samples taken in the meadow area of the Tyumen region had similar indicators of QMA&OAMO. Quantitative values characterize the possible probability of microbial contamination of hay samples. The product of suspicious quality was tested.
After mowing the plants, the permeability of the cells of the integumentary tissues increases, the phytoncidal activity that prevents the penetration of microorganisms into the underlying tissues decreases. All microsymbions of the epiphytic microbiota located on the surface of plants are activated: putrefactive, butyric, lactic acid bacteria, and mold and yeast fungi. With their intensive development, bacterial-fungal associations reduce the quality of the feed and its nutritional value. \textit{Aspergillus sp.} and \textit{Penicillium sp.} change the chemical composition of lipids, and then carbohydrates and proteins; various decay products such as organic fatty acids, ammonia and peptones accumulate in the feed, dramatically changing its smell and taste. These processes are especially active at high humidity and temperature. As a result of the vital activity of microorganisms, decomposition of the constituent parts of the feed occurs, which leads to the loss of nutrients and spoilage of the product. It acquires a putrid odor, the fibers break easily, and their consistency becomes spreadable.

Reliably high QMA&OAMO indicators were found in Sample No.4, which indicates that the object are contaminated, the threshold M was 5% (p <0.05). This fact can be explained by the influence of environmental factors: soil and climatic conditions, frequent sharp fluctuations in meteorological factors, which very often leads to stressful situations for plants. Consequently, the leaves of \textit{P. májor}, according to the requirements of the Pharmacopoeia XI-XIII editions, did not meet the standards and cannot be considered as an herbal dosage form.

Reliably high indicators of bacteria of the \textit{Pseudomonas sp.} genus (p <0.05) were established in all studied samples. The epiphytic microbiota of plants is rather uniform in qualitative composition and is represented mainly by non-spore-forming gram-negative rods \textit{Pseudomonas herbicola aureum} (\textit{P. herbicola}) or \textit{Erwinia herbicola}. \textit{P. fluorensces}. Less common are spore bacteria \textit{Bacillus mesentericus} (\textit{B. mesentericus}), \textit{B. vulgatus}, non-spore lactic acid bacteria \textit{Lactobacterium sp.}, mold and yeast fungi [10]. \textit{Lactobacterium sp} and \textit{Saccharomyces sp.} are the main antagonists of putrefactive microorganisms and fungi.

Plant raw materials can be a factor in the transmission of intestinal infections, however, direct detection of pathogenic microbes in a biotope is carried out only when investigating outbreaks of infectious diseases. The data on the fecal contamination of the object are used as indirect indicators. In the course of the study, the intensity of the biological load on objects taken in different regions was established. Bacteria of the \textit{Escherichia coli} group were found in all studied samples (see Table 2).

| Sample No. | Growth on Ploskirev's medium | Growth on XLD medium | citrate | lactose | H$_2$S | urea | Type of microorganism |
|------------|-------------------------------|----------------------|---------|---------|------|------|-----------------------|
| 1          | -                             | -                    | +       | +       | -    | -    | \textit{Citrobacter diversus} |
| Pink colonies | Yellow colonies                |                      |         |         |      |      | \textit{Escherichia coli} |
| 2          | -                             | -                    | +       | +       | +    | -    | \textit{Citrobacter freundii} |
| Pink colonies | Yellow colonies                |                      |         |         |      |      | \textit{Escherichia coli} |
| 3          | -                             | -                    | +       | +       | -    | -    | \textit{Citrobacter diversus} |
| Gray colonies | Yellow colonies                |                      |         |         |      |      | \textit{Citrobacter diversus} |
| 4          | -                             | -                    | +       | +       | -    | -    | \textit{Citrobacter diversus} |
| Pink colonies | Yellow colonies                |                      | +       | +       | +    | -    | \textit{Citrobacter freundii} |

Note: “+” - the feature is present; “-” - the feature is absent.
Thus, the bacteria *Citrobacter diversus* (*C. diversus*), *C. freundii* and *lac+*, *lac - E. coli* were detected at $10^5$ CFU/ml on the *B. inermis* Holub. inflorescence and the *P. pratense* grass. Gram-negative rods with rounded ends were found in smear preparations. The presence of coliform bacteria that do not have oxidase activity and ferment lactose with the formation of acid and gas may indicate fecal contamination of the object (p <0.05). Salmonella was not detected in the test samples by the split impedance method.

It is known that the plant food base and herbal dosage form is a source of sapronotic infections. A characteristic feature of the causative agents of saprozooses is their ability to persist in the environment during the interepidemic period [20]. In this regard, it is important to study the bacterial-fungal microbiota. All samples revealed spore-forming, catalase-negative bacteria *B. cereus*, which cause gastric diseases in animals and humans, as well as septicemia, endocarditis, and lesions of the central nervous system. For these reasons, the control of the named objects for the content of bacteria using reliable accelerated testing methods is becoming more and more important. Yeast fungi of the *Candida* genus were found at 104–106 CFU/ml in all studied objects (see Table 3).

### Table 3. Biochemical properties of *Candida* sp.

| No. | Culture       | GL  | MAL | SAC | GAL | LAC | RAF | CEL | TRE | ADO | ARA |
|-----|---------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1   | *C. albicans* | +   | +   | +   | +   | -   | -   | +/  | +/- | +/- | +/- |
| 2   | *C. tropicalis* | +   | +   | +/- | +   | -   | -   | +/  | +  | +/- | -   |
| 3   | *C. tropicalis* | +   | +   | +/- | +   | -   | -   | +/- | +  | +/- | -   |
| 4   | *C. tropicalis* | +   | +   | +/- | +   | -   | -   | +/  | +  | +/- | -   |
| 5   | *C. albicans* | +   | +   | +   | +   | -   | -   | +   | +/- | +/- | +/- |

Note: "+" - the feature is present; ":-" - the feature is absent.

Plants collected in the central part of the city with a high population density had high QMA&OAMO indices and high indicators of contamination with *Candida* fungi, which indicates the maximum degree of epidemiological danger (p <0.05). Fodder plants growing on the territory of the Tyumen region, on the contrary, were characterized by high indices of sanitary-indicative microorganisms, which, along with sanitary-chemical indicators, signify a problem and an increased risk of feed infection.

### 4. Conclusion

The use of the phytomonitoring method revealed the general patterns of changes in the quantitative and qualitative indicators of microsymbiocenosis. The proposed method based on split impedance makes it possible to obtain fast (within a few hours) and reliable results for a large number of simultaneously studied samples. The undoubted advantage of this device is its portability and small size.

### 5. References

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