Study of the quality and safety of microbiological bioformulations for plant protection and growth stimulation

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Abstract. Bioformulations containing microorganisms that have various useful properties for plants are widely used as an alternative to chemical pesticides. Methods for evaluating the quality of such biological products have not yet been standardized. Composition of bioformulations used for protecting plants and stimulating plant growth was studied with molecular methods. Toxicity of bioformulations was evaluated on laboratory populations of bumblebees (Bombus terrestris L.). The number of bacteria in the two bioformulations was significantly less than the manufacturer's stated. The declared composition of microorganisms in 33% of bioformulations did not fully or partially correspond to the identified composition. Klebsiella sp. and Citrobacter sp. bacteria were found among non-target bacteria. It is known that some species of these bacteria genera are opportunistic for humans. Not one of the studied bioformulations did not cause the death of bumblebees. However, it was found that 5 bioformulations out of 18 reduced the flight activity of bumblebees, which indicates their toxic effect. The data obtained by us shows that it is necessary to develop standardized methods for assessing the quality and safety of microbiological bioformulations that are available for public sale.

1. Introduction

The growth of the human population on the planet requires increasing efforts in food production, which requires the introduction of intensive agrobiotechnologies and the use of pesticides [1]. It is difficult to produce organic food in such conditions. Currently, bioformulations containing certain microorganisms, both prokaryotic and eukaryotic, are becoming more widely used as an alternative to pesticides [2, 3, 4]. It is believed that bioformulations are safer for humans and animals than chemical plant protection products [5]. Microbiological bioformulations can be used as an alternative to fungicides [6, 7] and insecticides [8]. Bacteria such as Bacillus subtilis [9], Bacillus amyloliquefaciens [10, 11], and some species of Pseudomonas genera are considered as bacterial agents against pathogenic microflora of soils and plants [12, 13]. Species of Trichoderma genus are most often considered as fungal bioformulations [14, 15]. Bioformulations may include various combinations of prokaryotic and eukaryotic microorganisms.
Bioformulations that contain bacteria that can fix nitrogen, such as *Bradyrhizobium* sp, are becoming increasingly common. [16, 17], which allows them being considered as a biofertilizer for plants, especially soybeans. Biofertilizers may also include other non-nitrogen-fixing bacteria, such as *B. megaterium* [18] and *Anabaena azollae* [19]. Bioformulations that have an insecticidal effect may contain *B. thuringiensis* [20, 21] and *Beauveria bassiana* [22].

Standardized methods for evaluating the quality of bioformulations have not been developed. The problem is both the estimation of the number of bacteria and eukaryotic microorganisms in such bioformulations, especially complex ones, and the taxonomic identification of microorganisms in the bioformulations. Molecular identification of bacteria based on sequencing of the 16S rRNA gene and identification of fungi based on sequencing of a DNA segment that includes the 18S rRNA, 28S rRNA, 5.7 S rRNA genes, and intergenic regions ITS1 and ITS2 as marker sites for identifying microorganisms [23, 24] can be an effective tool for identifying microorganisms in biological products. The purpose of this work was to study the composition of bioformulations used for protecting and stimulating plant growth by molecular methods, as well as to assess their toxicity on laboratory populations of bumblebees (*Bombus terrestris* L.).

2. Materials and methods

2.1 Samples

The object of the study was commercially available bioformulations (18 samples) for plant protection (biofungicides and bioinsecticide) and microbiological bioformulations for stimulating plant growth (biofertilizers).

2.2 Microbiological seeding

Seeding of bioformulations on Petri dishes was carried out on two solid media: GRM-agar for counting bacterial colonies and Saburo medium for counting fungal colonies. The composition of the GRM-agar included: pancreatic hydrolysate of fish meal – 24 g/l, sodium chloride – 4 g/l, microbiological agar – 10.0±2.0 g/l. The Saburo medium included: pancreatic hydrolysate of fish meal – 10 g/l; pancreatic hydrolysate of casein – 10 g/l; yeast extract – 2 g/l; NaH$_2$PO$_4$ – 2 g/l; D-glucose – 40 g/l; microbiological agar – 10 g/l, chloramphenicol – 0.05 g/l. Seeding of bioformulations was carried out in 5 dilutions: 1:100, 1:1000, 1:10000, 1:100000 and 1:1000000.

2.3 DNA isolation and PCR

DNA was isolated from microbial colonies by Proba-GS kit (DNA technology, Russia). The polymerase chain reaction was performed on a Mastercycler personal device (Eppendorf, Germany). The following components were mixed in a 0.2 ml tube: 5X qPCRmix-HS reaction mixture (Evrogen, Russia) – 5 µl; 5 µM direct primer – 1 µl; 5 µM reverse primer – 1 µl; DNA – 2 µl; deionized water – up to 25 µl. The following temperature cycle was used: 94 °C 4 min, 35 cycles: 94 °C 30 sec 54 °C 30 sec, 72 °C 45 sec, final elongation 72 °C 10 min. The primers used were: direct ITS1 TCCGTAGGTGAACCTGCGG, reverse ITS4 TCCTCAGGTTATGATATGC for identification of fungi and 337F GACTCCTACGGGAGGCAG, 1100R GGGTTGCGCTCCTGTG for identification of bacteria. Visualization of PCR products was performed using electrophoresis in 2% agarose gel.

2.4 Sequencing

Extraction from agarose gel and purification of the amplicon was performed using a commercially available Cleanup Standard kit (Evrogen, Russia). Sequencing of purified PCR products was performed on the Applied Biosystems 3500 genetic analyzer using BigDye Terminator v3.1 Cycle Sequencing Kit. The obtained nucleotide sequences were compared with those already available in the international NCBI GenBank system using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).
2.5 Effect of bioformulations on bumblebee mortality
The test substances were dissolved in water at the concentration specified in the instructions for the bioformulations. A solution of distilled water was used as a control solution. Bumblebees were carefully placed in a test tube with a solution using tweezers for 1 second. After that, they were kept for 2 hours in a specialized garden with filter paper at the bottom of the cage in order to dry out. Next, the bumblebees were placed in cylindrical cages (diameter – 14 cm, height – 7 cm) with a mesh bottom and a lid, 10 bumblebees in each cage. Inverted sugar syrup (60%) was used as feed. Bumblebees were kept at a temperature of 27-28.5 °C and at a humidity of 55-68%. The number of dead bumblebees was registered after 3 days.

2.6 Study of bumblebee flight activity
Bumblebees (3 insects) were placed in a transparent chamber: length – 25 cm, width – 15 cm, height – 20 cm. For lighting, a 600mm 18W D26 G13 daylight neutral white PHILIPS lamp was used. Every 5 seconds, the number of bumblebees that are in the flight process was recorded. The measurement was performed for 20 minutes. After that, the average number of bumblebees that were in flight during this time was calculated.

3. Results
We have studied commercially available bioformulations for plant protection and plant growth stimulation on the Russian market (table 1).

| Bioformulation | Purpose               | Form   | Hazard class for humans | Hazard class for bees |
|----------------|-----------------------|--------|-------------------------|-----------------------|
| 1              | Plant protection      | Liquid | 3B                      | 3                     |
| 2              | Biofertilizer         | Liquid | 4                       | 3                     |
| 3              | Plant protection      | Liquid | 4                       | 3                     |
| 4              | Plant protection,     | Liquid | 4                       | Not specified         |
|                | Biofertilizer         |        |                         |                       |
| 5              | Plant protection      | Powder | 4                       | 3                     |
| 6              | Plant protection      | Powder | 4                       | 3                     |
| 7              | Plant protection      | Powder | 4                       | 3                     |
| 8              | Plant protection,     | Liquid | 4                       | Not specified         |
|                | Biofertilizer         |        |                         |                       |
| 9              | Plant protection,     | Liquid | 4                       | Not specified         |
|                | Biofertilizer         |        |                         |                       |
| 10             | Plant protection      | Liquid | 4                       | 2                     |
| 11             | Plant protection      | Liquid | 3B                      | 3                     |
| 12             | Biofertilizer         | Powder | 4                       | Not specified         |
| 13             | Plant protection      | Powder | 4                       | Not specified         |
| 14             | Plant protection      | Liquid | 4                       | 3                     |
| 15             | Biofertilizer         | Liquid | 4                       | 3                     |
| 16             | Plant protection      | Powder | 4                       | 4                     |
| 17             | Biofertilizer         | Liquid | 4                       | Not specified         |
| 18             | Biofertilizer         | Peat   | 4                       | Not specified         |

Initially, several dilutions (see Materials and methods) of bioformulations were prepared in autoclaved water, after which the prepared samples were seed on universal nutrient media: GRM-agar (for bacterial growth) and Saburo medium (for fungi growth). Next, the grown colonies of microorganisms were counted. Table 2 shows the number of microorganisms that were declared by the manufacturer of the bioformulations and the actual number of colonies.
Table 2. The number of microorganisms grown on the universal nutrient medium

| Bioformulation | Declared by the manufacturer | Revealed | Microorganism | Number of colonies |
|----------------|-------------------------------|----------|---------------|-------------------|
| 1              | at least 1×10^7 CFU/cm^3      |          | Bacteria      | 2×10^6 CFU/cm^3   |
| 2              | at least 2×10^7 CFU/cm^3      |          | Bacteria      | 6×10^7 CFU/cm^3   |
| 3              | Not specified                 |          | Bacteria      | 7×10^6 CFU/cm^3   |
| 4              | Not specified                 |          | Fungi         | 2×10^7 CFU/cm^3   |
| 5              | at least 2×10^10 CFU/g        |          | Bacteria      | 8×10^10 CFU/g     |
| 6              | at least 2×10^7 CFU/g         |          | Bacteria      | 7×10^7 CFU/g      |
| 7              | at least 2×10^7 CFU/g         |          | Bacteria      | 8×10^7 CFU/g      |
| 8              | at least 1×10^7 CFU/cm^3      |          | Bacteria      | 9×10^7 CFU/cm^3   |
| 9              | at least 2.5×10^8 CFU/cm^3    |          | Bacteria      | 1×10^11 CFU/cm^3  |
| 10             | at least 2×10^7 CFU/cm^3      |          | Bacteria      | 2×10^7 CFU/cm^3   |
| 11             | at least 1×10^7 CFU/cm^3      |          | Bacteria      | 4×10^10 CFU/cm^3  |
| 12             | at least 1×10^10 CFU/g        |          | Bacteria      | 5×10^10 CFU/g     |
| 13             | at least 1×10^10 CFU/g        |          | Bacteria      | 2×10^10 CFU/g     |
| 14             | at least 2×10^7 CFU/cm^3      |          | Bacteria      | 3×10^10 CFU/cm^3  |
| 15             | at least 2×10^7 CFU/cm^3      |          | Bacteria      | 4×10^10 CFU/cm^3  |
| 16             | at least 8×10^10 CFU/g        |          | Bacteria      | 1×10^11 CFU/g     |
| 17             | at least 1×10^10 CFU/cm^3     |          | Bacteria      | No colonies       |
| 18             | at least 5×10^9 CFU/g         |          | Bacteria      | No colonies       |

In Bioformulation 1, the manufacturer stated the presence of only bacteria, but we detected the presence of eukaryotic microorganisms that grew on the Saburo medium with the antibiotic. Conversely, in Bioformulation 3, the manufacturer stated the presence of only a eukaryotic microorganism, but we detected the presence of bacteria in significant quantities (7×10^4 CFU/cm^3). In Bioformulation 8 and 12, the number of counted bacteria was slightly less than the manufacturer stated. In those bioformulations where the number of microorganisms was not specified, there were significantly fewer bacteria than in those bioformulations where the manufacturer indicated the number of bacteria per 1 cm^3 or 1 g. In general, most often the number of counted colonies of microorganisms exceeded the minimum values stated by the manufacturer of bioformulations. There were no colonies in Bioformulation 17 and 18, probably because GRM-agar is not an optimal medium for the growth of nitrogen-fixing bacteria.

At the next stage, representatives of morphologically homogeneous microbial colonies were isolated from Petri dishes and molecular analysis of the obtained microbial isolates was performed. For bacteria, the 16S rRNA region was sequenced, and for fungi, the region including ITS1 and ITS4 was sequenced. The obtained nucleotide sequences were compared with those already available in the
NCBI GenBank. The results of the comparative analysis of DNA nucleotide sequences are presented in Table 3.

**Table 3. Microorganisms identified by sequencing in microbiological bioformulations.**

| Bioformulation | Declared by the manufacturer | Identified (match in NCBI GenBank) | The similarity of the sequences in the NCBI GenBank, % |
|----------------|-----------------------------|------------------------------------|-----------------------------------------------|
| 1              | *P. fluorescens*             | Lysinibacillus sp.                 | 100.00                                         |
| 2              | *Bradyrhizobium japonicum*   | Galactomyces sp.                   | 96.81                                          |
| 3              | *Trichoderma sp.*            | Citrobacter sp.                    | 99.10                                          |
| 4              | Not specified                | Galactomyces sp.                   | 99.63                                          |
| 5              | *Bacillus subtilis*          | Pseudomonas sp.                    | 99.48                                          |
| 6              | *Bacillus subtilis*          | Bacillus subtilis                  | 99.72                                          |
| 7              | *Bacillus subtilis*          | Bacillus subtilis                  | 99.85                                          |
| 8              | *T. viride, Azomonas agilis, Azotobacter chroococum* | Klebsiella sp. | 98.34                                          |
| 9              | *T. viride, P. koreensis, Bacillus subtilis, Bradyrhizobium japonicum* | Bacillus subtilis | 99.61                                          |
| 10             | *Bacillus thuringiensis*     | Bacillus thuringiensis             | 99.58                                          |
| 11             | *Bacillus amyloliquefaciens* | Bacillus amyloliquefaciens         | 99.49                                          |
| 12             | *Bacillus megaterium*        | Bacillus megaterium                | 99.70                                          |
| 13             | *Bacillus subtilis*          | Bacillus subtilis                  | 99.52                                          |
| 14             | *Bacillus subtilis*          | Bacillus subtilis                  | 99.66                                          |
| 15             | *Bacillus megaterium*        | Bacillus megaterium                | 100.00                                         |
| 16             | *Pseudomonas aureofaciens*   | Pseudomonas aureofaciens           | 100.00                                         |
| 17             | *Bradyrhizobium elkanii*     | Bradyrhizobium sp.                 | 99.87                                          |
| 18             | *Bradyrhizobium japonicum*   | Bradyrhizobium japonicum           | 98.57                                          |

A complete match of the declared taxon of the microorganism with the one we identified was found in 11 samples out of 18. In Bioformulation 4, where the manufacturer did not specify the taxon of the microorganism, the bacteria of the *Acetobacter* genus were identified. In six bioformulations, either the declared microorganism did not match the one present in the bioformulation, or the presence of microorganisms not declared by the manufacturer was detected. Thus, the presence of the eukaryotic microorganism *Galactomyces* sp. was detected in Bioformulation 1 and 3. At the same time, Bioformulation 1 contains *Lysinibacillus* sp. instead of *P. fluorescens*. In Bioformulation 3 we found bacteria *Pseudomonas* sp. that was not declared by the manufacturer. In Bioformulation 7, a bacterium of the genus *Paenibacillus* was identified, which was also not specified by the manufacturer. In complex Bioformulation 8, bacteria of the genus *Klebsiella* were identified.

At the final stage, the studied bioformulations were tested on laboratory populations of bumblebees (*Bombus terrestris* L.). The mortality of bumblebees after contact with the bioformulation was estimated (see materials and methods), as well as their flight activity.

In the study of the effect of bioformulations on bumblebee mortality, no difference was found with the control groups of bumblebees. In all groups, the mortality rate was 0%. It was found that
Bioformulation 5, 10, 12 and 16 reduced the flight activity of bumblebees (Fig. 1). Other bioformulations did not have a negative or positive effect on the flight activity of bumblebees.

![Figure 1. Influence of bioformulations on the flight activity of Bombus terrestris L.](image)

The most significant decrease in flight activity was caused by Bioformulation 12 containing *B. megaterium*. The time spent by bumblebees in flight decreased by 5.4 times. Bioformulation 16, containing *P. aureofaciens*, reduced the time spent by bumblebees in flight by 3.8 times. Bioformulation 5 and Bioformulation 10 reduced the time spent by bumblebees in flight by 3.1 and 1.8 times, respectively.

### 4. Discussion

We found that the declared composition of microorganisms in 33% of bioformulations did not correspond fully or partially to the identified microorganisms. The most significant difficulty was to identify the composition of microorganisms in complex bioformulations that contained both *Bacillus* spp. bacteria and nitrogen-fixing bacteria. In this case, using microbiological methods, it is almost impossible to detect the presence of nitrogen-fixing bacteria, since fast-growing bacteria of the *Bacillus* genus, which are usually present in the biological product, occupy all the space on solid nutrient media. The solution of this problem in the future may be the development of specific molecular methods, such as TaqMan qPCR, for the specific identification of nitrogen-fixing bacteria such as *Bradyrhizobium japonicum*.

It is noteworthy that among non-target bacteria, we found bacteria of the *Klebsiella* (Bioformulation 8) and *Citrobacter* (Bioformulation 2) genus. It is known that some species of the *Klebsiella* genus are opportunistic for humans [25]. Similarly, some representatives of the *Citrobacter* genus are also opportunistic microorganisms and can harm human health [26]. Thus, the treatment of plants with such bioformulations will not only be ineffective but can also harm human health. It is interesting to note that we did not find information about useful for crop production signs of the *Acetobacter* sp., which was identified in Bioformulation 4.

We study the bioformulations effect on the mortality and flight activity of bumblebees (*Bombus terrestris L*). Even though not one of the bioformulations did not cause the death of bumblebees, it was found that Bioformulations 5, 10, 12 and 16 reduced the flight activity of bumblebees. This data suggests that these bioformulations should be used with caution since they can harm pollinating
insects and thus reduce the yield of entomophilic crops. It is noteworthy that biofertilizers also hurt the flight activity of bumblebees.

The data obtained by us indicate that it is necessary to develop standardized methods for assessing the quality and safety of microbiological bioformulations that are available for public sale. Standardized methods help to avoid falsification of such products and increase the biological safety of the applied plant protection and growth stimulation products for humans and animals.

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