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EXPERIMENTAL ARTICLES

The Formation of Artificial Nitrogen-Fixing Symbioses with Rape (*Brassica napus* var. *napus*) Plants in Nonsterile Soil

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Abstract—The treatment of rape plants grown in nonsterile soil with 2,4-dichlorophenoxyacetic acid (auxin-like growth-promoting substance) or their inoculation with the bacterial association *Micrococcus* sp. + *Rhodococcus* sp. and/or with the mixed nitrogen-fixing culture *Azotobacter nigricans* + *Bacillus* sp. led to the formation of paranodules on the rape roots. The introduced bacteria were detected both in the intercellular space and inside the cells of the paranodules and the rape roots. The nitrogen-fixing activity of the paranodulated plants was two times higher than that of the inoculated plants lacking paranodules and five times higher than that of the control (i.e., not inoculated) plants. The paranodulation led to a 40% increase in the crop yield of rape plants and provided for a statistically significant increase in the total nitrogen as well as protein nitrogen contents of the plants.

Key words: rape, paranodules, 2,4-dichlorophenoxyacetic acid, bacterial association, nonleguminous plants, symbiosis, nitrogen fixation.

One of the important problems of modern microbiology is the improved supply of nonleguminous plants with available nitrogen using soil nitrogen-fixing microorganisms [1]. This can be done by different methods: (1) the direct transfer of *nif* genes from bacterial into plant cells [2]; (2) the inoculation of plants with genetically engineered *nif*-expressing bacterial strains [3, 4] or bacterial strains producing auxin-like substances [5]; (3) the association of cultivated plants with endophytic nitrogen-fixing microorganisms isolated from natural symbiotic associations [6]; (4) the creation of plants colonized by diazotrophs through the stage of a mixed callus (callus cells with diazotrophs) using the totipotential property of plant cells [7]; and (5) the formation of artificial symbiotic structures (paranodules) on the nonlegume roots [8, 9].

The most efficient biosystems for the fixation of molecular nitrogen are the symbiotic associations of leguminous plants with bacteria of the genus *Rhizobium*, nonlegumes (such as alder, sea buckthorn, and raspberry) with actinomycetes of the genus *Frankia*, and fern *Azolla* with cyanobacteria *Anabaena azolla*. In these associations, nitrogen-fixing bacteria grow in specific structures providing beneficial conditions for diazotrophs [10]. The creation of artificial nitrogen-fixing symbiotic associations with plants that are unable to form them in a natural way is one of the most promising approaches to enhance the efficiency of the biological fixation of nitrogen in agriculture. The feasibility of such an approach is based on the conception that all plants form associations with rhizosphere microorganisms, which propagate from the rhizosphere to the rhizoplane and then to the root tissues, including the epidermis, cortex, endodermis, and vascular system [11].

Paranodulation, i.e., the artificial formation of nodules on the roots of nonleguminous plants (such nodules are known as paranodules) can be induced by auxin-like substances (such as 2,4-dichlorophenoxyacetic acid, chloramben, and chlorsulforn) [8, 12–14] and enzymes (as cellulase or pectinase) [15]. Using such an approach, paranodulation was induced in wheat, rice, maize, and rape plants [14, 16]. However, to be able to fix molecular nitrogen, paranodules must be colonized by soil diazotrophic bacteria, which is not always the case.

As a biogenic nodulating agent, we used the bacterial association *Micrococcus* sp. + *Rhodococcus* sp. inoculated together with a mixed nitrogen-fixing culture. Earlier laboratory studies showed that the induced paranodules of axenic plants provide beneficial conditions for the accumulation and growth of diazotrophic microorganisms and for the nitrogen metabolism of rape plants [17].

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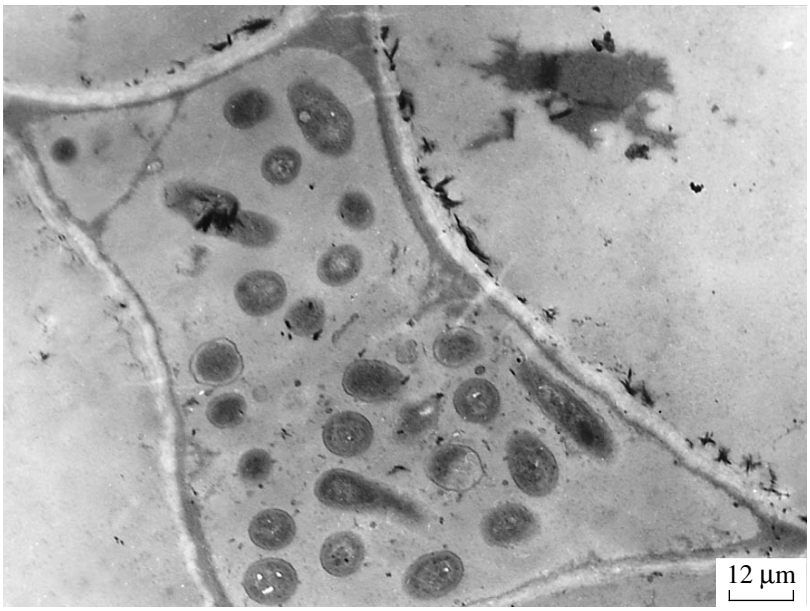

Fig. 1. A fragment of the intercellular space in a paranodule formed under the combined action of an abiogenic nodulating agent (DCPA) and the nitrogen-fixing association *Azotobacter nigricans* + *Bacillus* sp.

The aim of the present work was to induce artificial symbiosis in rape plants grown in nonsterile soil, i.e., under conditions close to natural ones.

MATERIALS AND METHODS

Experiments were performed with the winter rape *Brassica napus* var. *napus*, a valuable oil-yielding and fodder plant from the family *Cruciferaceae*. Some biological features of this plant makes it invaluable in experiments of such type [14].

As an abiogenic nodulating agent (ANA), we used 2,4-dichlorophenoxyacetic acid (DCPA), which possesses herbicidal activity at high concentrations (20–30 kg/hectare) and plant growth–promoting activity at low concentrations (about 20 g/hectare).

As a biogenic nodulating agent (BNA), the natural association *Micrococcus* sp. + *Rhodococcus* sp. isolated from the rhizosphere of the cycad *Cycas revoluta* was used. Since the nitrogen-fixing activity of this association is low, rape plants were additionally inoculated with the mixed nitrogen-fixing bacterial culture *Azotobacter nigricans* + *Bacillus* sp., which has a high nitrogenase activity.

Induction of paranodules. Rape plants were grown in nonsterile limed soddy podzolic moderately loamy soil (pH 6.0) taken from the arable soil horizon at the Chashnikovo agricultural experiment station in the Moscow region.

The experimental variants were as follows: (1) control rape plants without any treatment; (2) rape plants treated with the ANA; (3) rape plants treated with the BNA; (4) rape plants inoculated with the mixed nitrogen-fixing culture; (5) rape plants treated with the BNA and inoculated with the mixed nitrogen-fixing culture; and (6) rape plants treated with the ANA and inoculated with the mixed nitrogen-fixing culture.

In accordance with these six variants of the experiment, rape seeds were soaked for 1 h in (1) a nutrient medium + Ashby medium; (2) an ANA solution (0.1 μg/ml) in the nutrient medium; (3) a BNA suspension containing 10^{11} cells/ml; (4) a mixed nitrogen-fixing culture containing 10^{11} cells/ml; (5) the BNA suspension (10^{11} cells/ml) + the mixed nitrogen-fixing culture (10^{11} cells/ml); and (6) the ANA solution in the nutrient medium + the mixed nitrogen-fixing culture (10^{11} cells/ml). During sowing, the soil contained in seedling pans was supplemented with, correspondingly, (1) 93 ml of the sterile nutrient medium + 67 ml of Ashby medium; (2) 93 ml of the ANA solution; (3) 93 ml of the BNA suspension; (4) 67 ml of the mixed nitrogen-fixing culture; (5) 93 ml of the BNA suspension + 67 ml of the mixed nitrogen-fixing culture; and (6) 93 ml of the ANA solution + 67 ml of the mixed nitrogen-fixing culture. The BNA suspension and the mixed nitrogen-fixing culture were prepared using 4-day-old bacterial cultures grown in liquid media. Rape plants were cultivated in seedling pans (each of the six variants of the experiment was carried out using five pans) containing 2 kg of one of the aforementioned types of soil. The pans were incubated in an open-air lattice shed.

The cytological investigation of paranodules was carried out by light, electron transmission, and electron scanning microscopy using an Opton light microscope, two (a Jeol-100B and an HU-11C) transmission electron microscopes, and an S-405A scanning electron

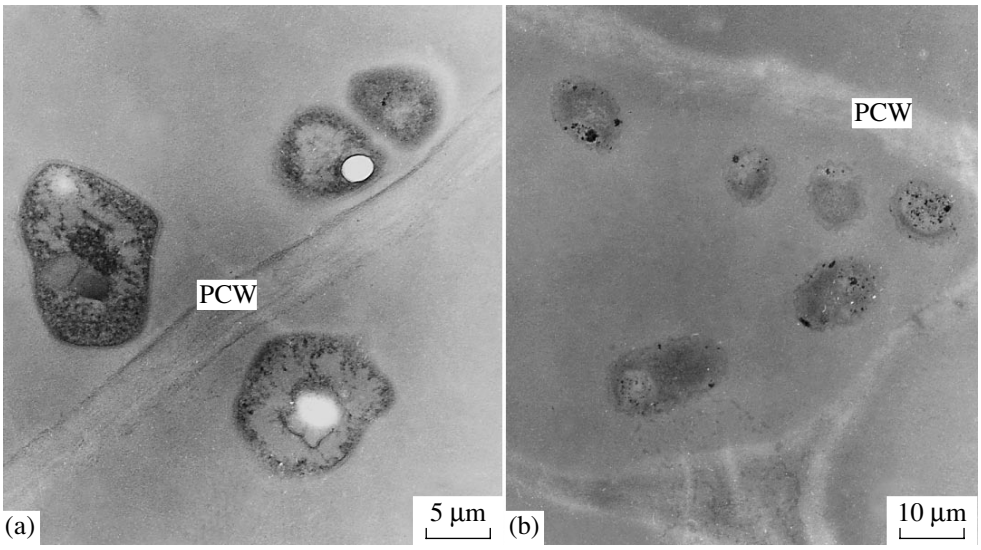

Fig. 2. Cell fragments and the cell wall in a paranodule formed under the action of a nodulating agent (*Micrococcus* sp. + *Rhodococcus* sp.) PCW is plant cell wall.

microscope. The paranodule specimens for transmission electron microscopy were prepared by two methods. In the first method, paranodules were fixed with a 2.5% solution of glytaraldehyde and a 0.8% solution of formaldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) containing 15 mg/ml sucrose, postfixed with a 1% solution of OsO_4 in the same buffer, dehydrated in a series of alcohol solutions of increasing concentration and then in pure acetone, and embedded in Epon-812 epoxy resin as described by Gornung *et al.* [18]. In the second method, paranodules were fixed with a 2% solution of glytaraldehyde in a Millonig or cacodylate buffer, postfixed with a 2% solution of OsO_4 in the corresponding buffer for 4.5 h, dehydrated in a series of alcohol solutions of increasing concentration, and embedded in araldite epoxy resin [19]. Thin sections were then contrasted with lead citrate [20]. The material for scanning electron microscopy was fixed for 30 min in a 2% solution of glytaraldehyde in phosphate buffer, dehydrated in a series of alcohol solutions of increasing concentration and then in pure acetone, and dried in an HCP-2 Critical Point Dryer in an atmosphere of CO_2 at 35°C at a pressure of 100 atm. The material was then coated with a platinum–palladium alloy in an argon atmosphere using an IB-3 Ion Coater. The coating thickness was 150 Å.

The activity of nitrogen fixation by the rape roots was assayed on the 19th, 25th, 28th, and 33rd days of plant growth by the standard procedure. The measurements were performed in triplicate.

The total nitrogen and protein nitrogen contents of the overground parts of the plants was determined in triplicate by the Kjeldahl method.

The results were **statistically processed** using Microsoft's Excel 2000 program.

RESULTS AND DISCUSSION

The treatment of rape seedlings with the ANA (experimental variant 2) and BNA (experimental variant 3) caused the formation of paranodules on the rape roots, which resembled short thickened lateral roots grown in the root hair zone.

In experimental variant 2, the formation of paranodules was noticeable on the 10th day of plant growth. The 18-day-old plants contained from 9 to 15 paranodules about 1 mm in diameter, whose number did not change in the course of the further cultivation of the plants. At the same time, in experimental variant 3, the formation of paranodules was noticeable only on the 18th day of plant growth. The number of the paranodules gradually increased in the course of plant cultivation, so that the 33-day-old plants contained from 15 to 20 paranodules about 1 mm in diameter.

In experimental variants 5 and 6 (the combined treatment of rape seedlings with the ANA or BNA and the mixed nitrogen-fixing culture), the time of the appearance of paranodules also depended on the type of nodulating agent used but did not depend on the presence of the mixed nitrogen-fixing culture. Nor did this culture considerably influence the number and the diameter of the paranodules. For instance, in experimental variant 6 (treatment with the ANA and the mixed culture), the formation of paranodules was noticeable on the 10th day of plant growth. The 18-day-old plants contained from 9 to 15 paranodules 1–1.5 mm in diameter, whose number did not change in the course of the further cultivation of the plants. In experimental variant 5 (treatment with the BNA and the mixed culture), the formation of paranodules was noticeable on the 18th day of plant growth. The number of the paran-

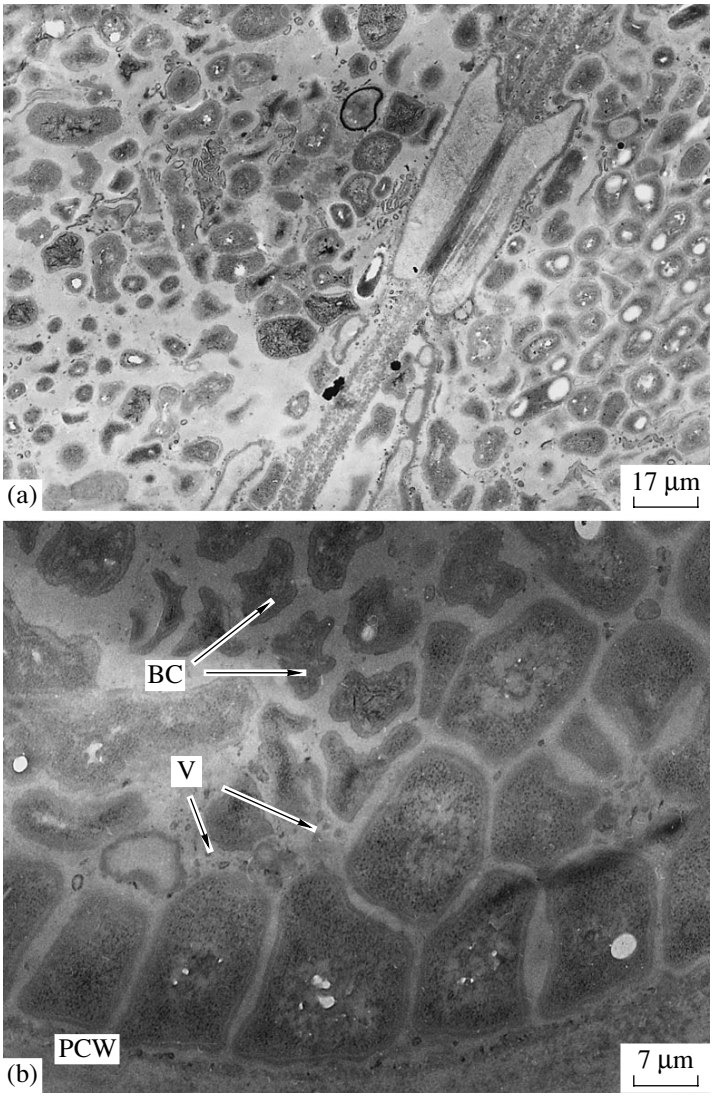

Fig. 3. Fragments of tracheids in the rape roots: (a) general view and (b) immobilization of bacterial cells on the inner surface of the plant cell wall (BC, bacterial cells of an irregular shape; V, vesicles; and PCW, plant cell wall).

odules increased in the course of plant cultivation. The 33-day-old plants contained from 15 to 20 paranodules.

Some differences were observed in the morphology of paranodules. In experimental variant 6, the paranodules resembled short rudimentary lateral roots. In the root elongation zone at the base of the paranodules, a root callus composed of large parenchymatous loosely bound cells with increased intercellular spaces colonized by bacterial cells was observed (Fig. 1). It is known that it is the root elongation zone that primarily responds to the action of auxins by changing the properties of the cells of epidermis, cortical parenchyma, endodermis, pericycle, and the central vascular cylinder [14]. This may lead to the penetration of bacteria to conducting plant tissues and eventually to their colonization by the bacteria.

In experimental variant 5, the intercellular space of paranodules was not increased, as occurred in experimental variant 6. As a rule, bacterial cells were observed inside the paranodule cells, close to their walls (Fig. 2). In other words, the type of nodulating agent influenced not only the formation time of paranodules and their morphology but also the distribution of microorganisms in the plant tissues.

The investigation of the distribution of microorganisms in the root tissues of nodulated plants showed that, in experimental variant 6, bacterial cells colonized not only the intercellular space but also the tracheids of the primary xylem (Fig. 3a), which provide for the transport of water and dissolved minerals from the roots to the leaves. Bacterial cells inside the tracheids were immobilized on a newly formed secondary envelope (Fig. 3b). In the center of the plant cells, heteromorphic bacterial cells, which differed from normal bacterial

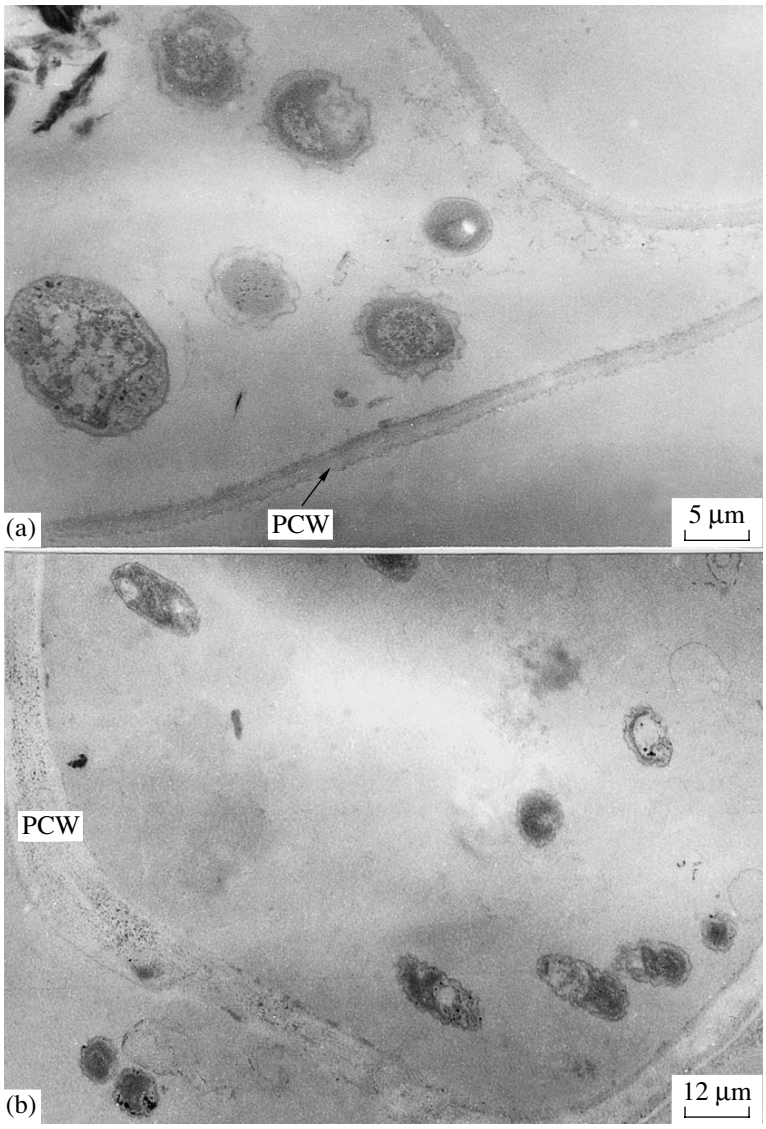

Fig. 4. Fragments of the rape root cells with bacteria (a) in the intercellular space and (b) inside the rape root cells (PCW, plant cell wall).

cells in that they had small sizes and irregular shape were observed. Near the membranes of altered bacterial cells, there was a great number of vesicles, suggesting that these cells occur in an active state. Such vesicles are normally absent in the plant and bacterial cells and, hence, may be considered to be specific to this type of symbiosis, i.e., to paranodules.

In experimental variant 5, bacterial cells were also observed in the intercellular space (Fig. 4a) and inside the rape root cells (Fig. 4b) but, unlike in experimental variant 6, not in the conducting root tissues. In comparison with experimental variant 6, the bacterial colonization of the intercellular space of the rape roots was considerably lower. It is possible that the macrosymbiont (rape plant) controls the population of the microsymbionts, which is an important indication of nonparasitic relations between the symbionts [10].

The assay of the nitrogen-fixing activity of the roots of the rape plants grown in nonsterile soil in experimental variants 5 and 6 showed that their nitrogen-fixing activity was at a maximum on the 28th to 33rd day of plant growth (Table 1), which corresponded to the phase of rape budding and the beginning of the phase of rape flowering. The nitrogen-fixing activity of the nodulated rape plants (experimental variants 5, 6) was about two times higher than the nitrogenase activity of the microorganisms that were attached to the surface of the rape roots (experimental variant 4) and almost five times higher than the nitrogen-fixing activity of the indigenous rhizosphere microflora (experimental variant 1). These data suggest that the paranodulation of the plant roots creates more beneficial conditions for the nitrogen-fixing bacteria than the mere adsorption of these bacteria on the roots.

Table 1. Dynamics of the nitrogen-fixing activity (NFA) of the rape roots in the course of plant growth

Experimental variant	Paranodulating agent	NFA, nmol $C_2H_4/(g_{root}$ h)			
		days of plant growth			
		19	25	28	33
1	Control	2.12 ± 0.06	1.53 ± 0.06	1.25 ± 0.41	2.44 ± 0.07
2	DCPA	2.51 ± 0.06	3.29 ± 0.08	3.36 ± 0.06	3.34 ± 0.08
3	*Micrococcus* sp. + *Rhodococcus* sp.	1.62 ± 0.02	2.24 ± 0.06	3.15 ± 0.03	2.87 ± 0.06
4	*Azotobacter* sp. + *Bacillus* sp.	5.23 ± 0.72	5.18 ± 0.14	5.16 ± 0.08	5.36 ± 0.10
5	*Micrococcus* sp. + *Rhodococcus* sp. + *Azotobacter* sp. + *Bacillus* sp.	8.97 ± 0.90	8.95 ± 1.20	10.34 ± 0.60	10.24 ± 1.46
6	DCPA + *Azotobacter* sp. + *Bacillus* sp.	8.34 ± 0.06	9.81 ± 1.03	9.67 ± 0.60	11.51 ± 1.00

Table 2. The total nitrogen and protein nitrogen contents of the rape biomass upon the use of abiogenic and biogenic nodulating agents

Experimental variant	Nodulating agent	Total nitrogen content, %	Protein nitrogen content, %	Protein content, %
1	Control	1.45 ± 0.09	1.28 ± 0.01	8.00 ± 0.06
2	DCPA	1.54 ± 0.01	1.43 ± 0.05	8.94 ± 0.31
3	*Micrococcus* sp. + *Rhodococcus* sp.	1.52 ± 0.05	1.41 ± 0.02	8.81 ± 0.13
4	*Azotobacter* sp. + *Bacillus* sp.	1.51 ± 0.05	1.49 ± 0.06	9.31 ± 0.38
5	*Micrococcus* sp. + *Rhodococcus* sp. + *Azotobacter* sp. + *Bacillus* sp.	1.98 ± 0.06	1.91 ± 0.01	11.94 ± 0.06
6	DCPA + *Azotobacter* sp. + *Bacillus* sp.	2.01 ± 0.02	1.97 ± 0.06	12.31 ± 0.38

Investigations of the effect of paranodulation on the yield of the rape biomass and its quality showed (Fig. 5) that the rape biomass yield in experimental variants 5 and 6 (treatment with the ANA and BNA and inoculation with the mixed nitrogen-fixing culture) increased by 20–22% in comparison with experimental variant 4 (mere inoculated with the mixed nitrogen-fixing culture) and by 39–41% in comparison with the control (experimental variant 1). The quality of the rape biomass in experimental variants 5 and 6 also improved (Table 2). Specifically, the total nitrogen and protein nitrogen contents of the biomass in these experimental variants increased by 0.4–0.5% in comparison with experimental variant 4 and by 0.5–0.6% in comparison with the control (experimental variant 1).

The paranodulating effect of the BNA was observed throughout the observation period. Unlike experimental variant 6 with the ANA treatment, when bacterial cells colonized the conducting tissues of the rape roots and thus could cause the death of plants [10], the BNA treatment in experimental variant 5 did not affect the vital functions of the roots, since in this case the introduced bacterial cells did not colonize the root vascular tissue.

In natural symbioses between higher plants and bacteria, the plants can control the population of colonizing bacteria and their physiological activity, provided that this population is not very large [10]. Presumably, such a situation takes place during the BNA-induced paranodulation of the rape roots. The low degree and the specific character of the bacterial colonization of the rape roots, when bacterial cells locate in paranodules and root tissues near the cell walls, as well as the high nitrogen-fixing activity of the introduced bacterial

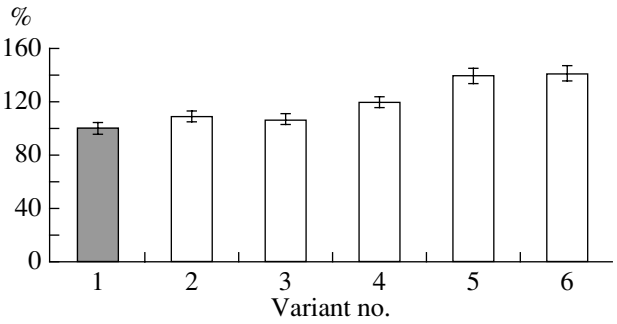

Fig. 5. The effect of the abiogenic (DCPA) and biogenic (the bacterial association *Micrococcus* sp. + *Rhodococcus* sp.) nodulating agents on the rape biomass yield (as a percentage of the control). Experimental variants correspond to those described in the text.

cells, suggest that the relations between the rape plants and the introduced bacteria are symbiotic. The data obtained show that the paranodulating bacterial association *Micrococcus* sp. + *Rhodococcus* sp. enhances the competitiveness of the introduced mixed nitrogen-fixing culture with the indigenous microflora of the nonsterile soil used in these experiments. In experimental variants with the BNA treatment, the introduced bacterial cells were observed only in the intercellular space and inside the dead plant cells, indicating the development of nonparasitic relations between the rape plants and the bacterial cells [10].

The data obtained show that nonleguminous plants can be made capable of nitrogen fixation in situ by means of artificially induced paranodulation.

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