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EXPERIMENTAL ARTICLES

The Effect of the Plant Growth Stimulant Bactozole on *Rhizobium leguminosarum* bv. *viciae* 250a and Its Nitrogen-Tolerant Mutant M-71 under Different Nitrogen Supply Conditions

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Abstract—The effect of the plant growth stimulant bactozole on the growth of *Rhizobium leguminosarum* bv. *viciae* 250a and its nitrogen-tolerant mutant M-71 and the synthesis of extracellular carbohydrates was studied. At a low content of nitrate (6 mM) in the medium, all three bactozole concentrations tested (0.001, 0.01, and 0.1%) exerted similar stimulating effects on the growth of the parent strain 250a (about 1.5-fold) and the synthesis of extracellular carbohydrates (about 2-fold). At a high content of nitrate (20 mM) in the medium, when the growth of the parent strain and the synthesis of extracellular carbohydrates were inhibited, bactozole at all three concentrations exerted only a growth-stimulating effect. At the same time, mutant M-71 showed better growth at higher concentrations of bactozole, whereas the ability of the mutant to synthesize extracellular carbohydrates decreased with increasing bactozole concentration. The cell biomass of the mutant accumulated at 20 mM nitrate was 1.8–2.5 times greater than it was at 6 mM nitrate. Bactozole enhanced the symbiosis of legume plants with both parent and mutant strains, raising the mass of plants and enhancing nodulation and the nitrogen-fixing activity of root nodules. The symbiotic parameters of mutant M-71 were better (irrespective of whether bactozole was present or not) when its inoculum was grown at a high nitrogen content (20 mM nitrate), whereas the respective parameters of the parent strain were better when it was grown at 6 mM nitrate. The inference is made that the better physiological characteristics of the mutant in the high-nitrate medium are due to its higher nitrate reductase activity (as compared with the parent strain) in both the free-living state and in legume nodules.

Key words: *Rhizobium*, nitrogen fixation, legume–rhizobial symbiosis, plant growth stimulant.

Recently, various synthetic (such as N-oxides of pyridine, phosphorylated azoles, and chemical analogues of phytohormones) and naturally occurring (such as auxins, gibberellins, cytokinins, brassinolipids, and fusicococcine) plant growth stimulants have come into use to increase the productivity of agricultural plants [1–4]. The mechanism of action of these substances on rhizosphere microorganisms is far from being well understood, although the knowledge of it is very important for the invention of new plant growth stimulants and for the proper ecological appraisal of the known plant growth stimulants.

One of the promising approaches to increasing plant productivity is to enhance nitrogen fixation in agrobiocenoses by introducing into them symbiotic microorganisms and microbial associations in combination with biologically active substances, such as rhizobial exopolysaccharides (EPSs) [5–7]. The latter may affect the virulence of homologous strains, stimulate nitrogenase in legume nodules, enhance the efficiency of symbiosis [5, 6], and influence the rhizogenesis of pea plants by exerting phytohormone-like action [7]. This prompted us to study bactozole, a plant growth stimulant with the EPS of *Beijerinckia* sp. IBX-84 as the principle [8].

Taking into account the fact that the functional activity of rhizobia strongly depends on the level of bound nitrogen in the medium, this work was aimed at studying the effect of bactozole on the growth of *Rhizobium leguminosarum* bv. *viciae* 250a and its nitrogen-tolerant mutant M-71 in the free-living state and on the efficiency of their symbioses with pea plants under different conditions of mineral nitrogen supply.

MATERIALS AND METHODS

The parent strain *Rhizobium leguminosarum* bv. *viciae* 250a was obtained from the collection of rhizo-

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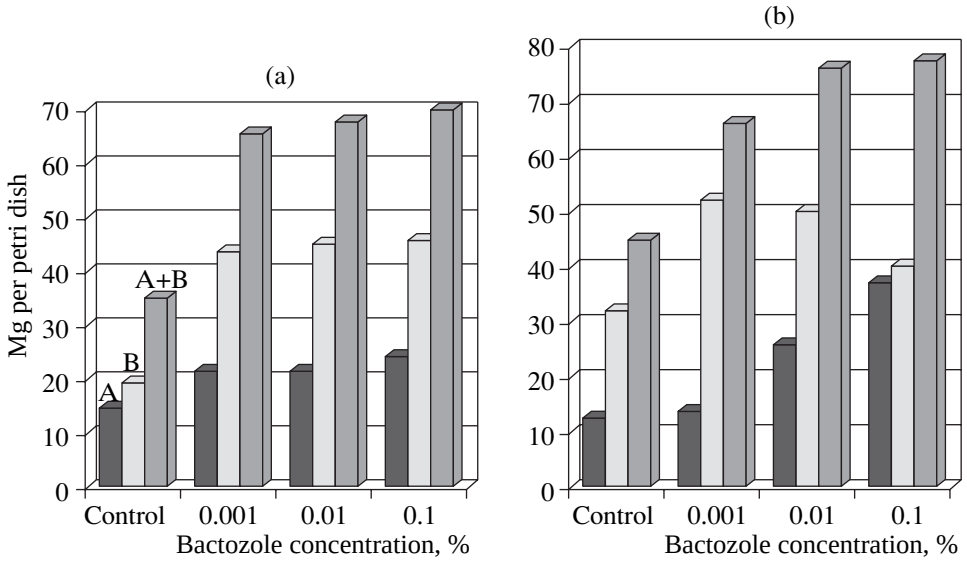

Fig. 1. The effect of bactozole on the growth of (a) *R. leguminosarum* bv. *viciae* 250a and (b) its nitrogen-tolerant mutant M-71 and synthesis of extracellular carbohydrates in medium 1 (contains 6 mM nitrate): (A) biomass, (B) extracellular carbohydrates, and (A + B) biomass + extracellular carbohydrates. The results are expressed in mg per petri dish (15 ml of agar medium).

bia at the All-Russia Research Institute of Agricultural Microbiology, St. Petersburg. The mutant strain M-71 was obtained by chemical mutagenesis using ethyl methanesulfonate [9].

In the free-living state, the strains were grown at 26–28°C for 3 days in petri dishes containing 15 ml of a mannitol agar medium [10] supplemented with either 6 mM (0.6 g/l) KNO_3 (medium 1) or 20 mM (2.02 g/l) KNO_3 (medium 2).

Bactozole, a plant growth stimulant with the EPS of *Beijerinckia* sp. IBX-84 as the principle, was obtained from V.M. Bagnyuk, Institute of Botany, National Academy of Sciences of Ukraine. Bactozole is prepared by precipitating EPS with ethanol from the *Beijerinckia* sp. IBX-84 culture liquid [8].

Bactozole was added to the cell suspension (10^9 cells/ml) used for inoculation at concentrations of 0 (control), 0.001, 0.01, and 0.1%. Each petri dish was inoculated with 1 ml of the inoculation suspension (accordingly, one petri dish contained either 0, 0.01, 0.1, or 1 mg bactozole). Each bactozole concentration was tested in triplicate plates. After 3 days of incubation, bacterial cells were thoroughly washed off from the plates with physiological saline solution. The resultant cell suspension was centrifuged at 15000 *g* for 40 min. The cell pellet was desiccated at 103–105°C and weighed. The content of extracellular polysaccharides in the supernatant was determined by reaction with phenol and sulfuric acid [11]. The results were expressed as the amount of polysaccharides per petri dish (or 15 ml of agar medium). The data presented in the paper are the means of quadruplicate measurements.

Vegetative experiments were performed using sand as the substrate. The sand was supplemented with a

Table 1. The effect of bactozole on the ability of *R. leguminosarum* bv. *viciae* 250a and its nitrogen-tolerant mutant M-71 to synthesize extracellular carbohydrates in media with different nitrate concentrations

Bactozole, %	Extracellular carbohydrates, % of the mass of dry cells			
	Strain 250a		Mutant strain M-71	
	Medium 1 (6 mM nitrate)	Medium 2 (20 mM nitrate)	Medium 1 (6 mM nitrate)	Medium 2 (20 mM nitrate)
0 (Control)	133	77	246	69
0.001	210	55	371	58
0.01	205	79	192	52
0.1	192	80	108	18
LSD_{05}	18	13	21	10

Note: LSD stands for “least significant difference.”

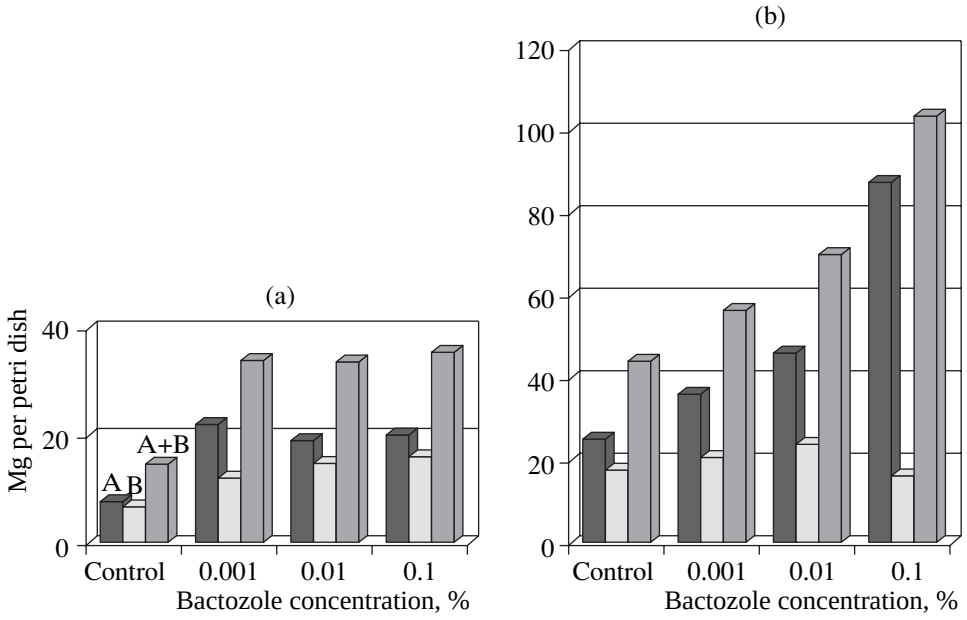

Fig. 2. The effect of bactozole on the growth of (a) *R. leguminosarum* bv. *viciae* 250a and (b) its nitrogen-tolerant mutant M-71 and the synthesis of extracellular carbohydrates in medium 2 (contains 20 mM nitrate). Designations as in Fig. 1.

nutrient medium containing mineral nitrogen in amounts corresponding to 0.2 and 0.8 of the standard nitrogen requirement (16 and 64 mg N/kg sand, respectively). Nitrogen was added to the medium in the form of $Ca(NO_3)_2 \cdot 4H_2O$.

Pea seeds of the Nord cultivar were surface-sterilized with concentrated sulfuric acid and sowed in petri dishes with legume agar. After 3 days of cultivation, calibrated pea seedlings were bacterized with a rhizobial suspension containing 10^9 cells/ml and 0.1% bactozole, after which they were planted in pans 0.5 kg in capacity. Each experiment was performed in six replicates and lasted 30 days. The estimated parameters were the wet mass of overground plant parts and plant roots, the number of root nodules, and their nitrogenase activity [12].

The nitrate reductase (NR) activity of root nodules was determined in vitro by the method of Kennedy *et al.* [13] modified by L'vov and Safaraliev [14]. The nodules were fractionated as described by Morell and Copeland [15]. The nitrate reductase activity of the nodule cytosol was determined with NADH, whereas that of bacteroides, with methyl viologen. The NR activity of rhizobia was determined with methyl viologen using cells washed with physiological saline solution. Nitrites were assayed with *N*-(1-naphthyl)ethylenediamine dihydrochloride using $NaNO_2$ as the standard.

Data were statistically processed as described in the handbook by Dospekhov [16].

RESULTS AND DISCUSSION

Bearing in mind that the stimulation of plant growth is accompanied by the activation of nitrogen metabolism and hence requires a sufficiently high level of nitrogen in the medium and that a high level of nitrogen in the medium suppresses the symbiotic activity of rhizobia, we chose for investigations two rhizobial strains—the parent strain 250a with a normal tolerance to nitrogen and its nitrogen-tolerant mutant M-71. The growth characteristics of these strains and their ability to synthesize extracellular carbohydrates were studied

Table 2. The nitrate reductase activity of the free-living and symbiotic (in pea root nodules) cells of *R. leguminosarum* bv. *viciae* 250a and its nitrogen-tolerant mutant M-71

Strain	Free-living cells	Pea root nodules	
	Methyl viologen–dependent NR	NADH-dependent NR	Methyl viologen–dependent NR
250a	2.35 ± 0.13	2.84 ± 0.15	6.13 ± 0.17
M-71	6.44 ± 0.38	5.24 ± 0.21	8.92 ± 0.24

Note: NR activity is expressed in μg NO_2^--formed in 30 min per g of wet bacterial biomass or pea root nodules. The data are the means of triplicate experiments.

Table 3. The effect of bactozole on the symbiosis of *R. leguminosarum* bv. *viciae* 250a and its nitrogen-tolerant mutant M-71 with pea plants grown on a substrate with 1.2 mM nitrate

Inoculation variants			Root nodules		Mass of wet overground plant parts, g per plant	Mass of wet roots, g per plant
no.	strain	nitrate (mM) in the growth medium for inoculum	number per plant	nitrogen-fixing activity, nmol C_2H_4/h per plant		
Without bactozole						
1	Control (nonbacterized) plants	0	0	0	1.4	0.7
2	250a	6	25	602	2.7	1.7
3	250a	20	15	432	2.6	1.1
4	M-71	6	11	185	2.6	1.4
5	M-71	20	17	232	3.1	1.6
Bactozole, 0.1%						
6	Control (nonbacterized) plants	–	–	–	2.2	1.2
7	250a	6	41	2004	5.3	1.6
8	250a	20	17	558	3.4	1.8
9	M-71	6	16	248	2.9	1.3
10	M-71	20	21	296	2.9	1.3
LSD_{05}			5	43	0.6	0.3

at different concentrations of mineral nitrogen in the medium.

When grown in medium 1, with a nitrogen content of 6 mM, both strains accumulated equal amounts of biomass, although the ability of the mutant to synthesize extracellular carbohydrates was higher than that of the parent strain (246 and 133% of the mass of dry cells, respectively) (Table 1, Fig. 1).

At all three concentrations tested, bactozole increased the biomass of strain 250a by 1.4–1.6 times and the amount of extracellular carbohydrates by more than 2 times (to 192–210% of the mass of dry cells).

At the same time, the effect of bactozole on the growth and the synthesis of extracellular carbohydrates in the mutant strain M-71 depended on the concentration of bactozole. At the concentration 0.001%, bactozole enhanced the synthesis of extracellular carbohydrates from 246 to 371% of the mass of dry cells and did not influence the growth of the mutant. At the concentration 0.01%, bactozole increased the growth of the mutant by about 2 times, but diminished the synthesis of extracellular carbohydrates to 192% of the mass of dry cells. At the concentration 0.1%, bactozole promoted further the growth of the mutant (by about 3 times) and diminished further the synthesis of extracellular carbohydrates, to 108% of the mass of dry cells (Fig. 1).

Of interest is the fact that, in the presence of 6 mM nitrate, the effects of all three bactozole concentrations on the total biosynthesizing activity (the biomass + extracellular carbohydrates) of the parent and mutant strains were almost the same.

The high concentration of nitrate (20 mM) in medium 2 suppressed both the growth of strain 250a and the synthesis of extracellular carbohydrates by this strain (Fig. 2), which is in agreement with our earlier observations [17].

The effect of bactozole on strain 250a grown in medium 2 was the same as in the case of medium 1: bactozole at all three tested concentrations augmented the biomass by 2.3–2.6 times. In this case, the amount of extracellular carbohydrates comprised 55–80% of the mass of dry cells as compared with 192–210% observed in medium 1 (Table 1).

The high concentration of nitrate in medium 2 did not suppress the total biosynthesizing activity of the mutant strain M-71, but shifted it toward protein synthesis (in contrast, medium 1 promoted the synthesis of extracellular carbohydrates). The addition of bactozole at concentrations of 0.001, 0.01, and 0.1% to medium 2 raised the biomass accumulated by mutant M-71 by 1.5, 1.8, and 3.4 times, respectively, and diminished the synthesis of extracellular carbohydrates from 69 to, respectively, 58, 52, and 18% of the mass of dry cells (Table 1). In other words, bactozole shifts metabolic processes in mutant M-71 grown in medium 2 toward the prevalence of nitrogen metabolism.

One of the key enzymes of nitrate assimilation is nitrate reductase, which reduces nitrate to nitrite and thus involves nitrate nitrogen into the next transformation stages (reduction to ammonium ions and incorpo-

Table 4. The effect of bactozole on the symbiosis of *R. leguminosarum* bv. *viciae* 250a and its nitrogen-tolerant mutant M-71 with pea plants grown on a substrate with 4.8 mM nitrate

Inoculation variants			Root nodules		Mass of wet overground plant parts, g per plant	Mass of wet roots, g per plant
no.	strain	nitrate (mM) in the growth medium for inoculum	number per plant	nitrogen-fixing activity, nmol C_2H_4/h per plant		
Without bactozole						
1	Control (nonbacterized) plants	0	0	0	1.4	0.8
2	250a	6	0	0	1.8	0.9
3	250a	20	0	0	1.7	1.8
4	M-71	6	15	384	2.7	1.1
5	M-71	20	23	425	2.8	1.5
Bactozole, 0.1%						
6	Control (nonbacterized) plants	0	0	0	1.9	0.7
7	250a	6	6	69	2.0	0.9
8	250a	20	1	0	1.9	0.8
9	M-71	6	29	457	2.4	1.3
10	M-71	20	39	556	2.5	1.6
LSD_{05}			5	40	0.3	0.3

ration into amino acids and then proteins). Bearing this in mind, we assayed NR in the parent and mutant strains grown in the free-living state on agar media and symbiotically in root nodules. In the root nodules, NR is of two types—NADH-dependent NR of plant origin and methyl viologen–dependent NR of bacterial origin.

When grown on agar media, the mutant strain M-71 showed almost 3-fold higher NR activity than did the parent strain 250a (Table 2). The high level of NR activity allows the mutant to efficiently assimilate nitrate nitrogen. This may explain the high level of cell biomass accumulated by the mutant in medium 2 in the presence of bactozole.

Taking into account the dependence of the physiological activity of the parent and mutant strains on the nitrate content in the medium, the effect of bactozole on the efficiency of the symbiosis of these strains with pea plants was studied at two nitrate concentrations, 1.2 mM (this nitrate concentration favors the symbiosis) and 4.8 mM. The bacterial inocula for these experiments were grown, respectively, in medium 1 and medium 2 (recall that these media contain 6 and 20 mM nitrate).

When pea plants were grown in the presence of 1.2 mM nitrate, both strains were symbiotically active and promoted the formation of root nodules with nitrogen-fixing ability (Table 3, experimental variants 2–5). In this case, the masses of overground plant parts and roots were higher than they were in the case of the control (nonbacterized) plants (variant 1). The symbiotic activity of the parent and mutant strains depended differently on the nitrate content in the medium. When grown in medium 1, strain 250a promoted the formation of a larger number of root nodules with a higher nitrogenase activity (experimental variant 2) than when grown in medium 2 (variant 3). Conversely, the mutant strain M-71 produced more nodules with a higher nitrogenase activity when grown in medium 2 (variant 5) than when grown in medium 1 (variant 4). These data imply that pea plants bacterized with nitrogen-tolerant rhizobial strains are able to assimilate both symbiotrophic and mineral nitrogen.

The maximum effect of bactozole on the symbiotic relationship between *R. leguminosarum* bv. *viciae* and pea plants was revealed in experimental variant 7 (the bacterization of pea plants in the presence of bactozole with strain 250a grown in medium 1). As compared with variant 2 (bacterization in the absence of bactozole), the number of root nodules, their nitrogen-fixing activity, and the mass of overground plant parts increased by 1.6, 3, and 2 times, respectively.

In variant 8, in which strain 250a was grown in medium 2 with 20 mM nitrate, the favorable effect of bactozole on symbiosis was considerably lower but statistically significant (cf. variants 3 and 8).

Bactozole also improved the nodulation characteristics (the number and the nitrogen-fixing activity of root nodules) of mutant M-71. However, in total, the symbiotic activity of strain 250a was greater than that of mutant M-71 both in the presence and in the absence of bactozole.

In the next set of experiments (their results are presented in Table 4), pea plants were grown at 4.8 mM nitrate. Under these conditions, strain 250a did not induce the formation of root nodules in the absence of bactozole (variants 2 and 3) and slightly induced nodulation in its presence (variant 7). In the latter case, the nitrogen-fixing activity of nodules was low. Conversely, the nitrogen-tolerant mutant M-71 induced the formation of root nodules with high nitrogen-fixing activity (384–425 nmol C_2H_4/h per plant) and increased the productivity of plants about twofold (variants 4 and 5). In the presence of bactozole (variants 9 and 10), the nodulation activity of mutant M-71 increased by about 1.5 times. In general, the nodulation activity of mutant M-71 both in the presence and in the absence of bactozole was higher when the mutant was grown at 20 mM nitrate (variants 5 and 10) than when it was grown at 6 mM nitrate (variants 4 and 9).

The high nodulation ability of mutant M-71 grown at 20 mM nitrate can be explained by the high level of nitrate reductase activity in the free-living cells of this mutant and in the root nodules formed in the presence of the mutant (Table 2). In the latter case, more active were both NADH-dependent NR of plant origin and methyl viologen–dependent NR of bacterial origin. It should be noted that the activity of bacterial NR exceeded that of plant NR in nodules of both kinds, i.e., those induced by the parent and mutant strains.

The high nitrate reductase activity of mutant M-71 allows it to efficiently assimilate nitrate in the reactions of nitrogen metabolism. Correspondingly, the ability of rhizobacteria to synthesize carbohydrates (most of which are exopolysaccharides [10]) declines (Fig. 1; Table 1). Exopolysaccharides are known to be of crucial importance for the formation of legume–rhizobial symbiosis [18, 19]. This may explain why, under conditions favorable for symbiosis (low-nitrogen medium for strain 250a and high-nitrogen medium for mutant M-71), the symbiosis-promoting effect of bactozole is higher for the parent strain 250a than for the mutant strain M-71. This is due to the fact that the synthesis of extracellular carbohydrates in the mutant grown in the high-nitrate medium is suppressed.

A comparison of the data obtained for two *R. leguminosarum* bv. *viciae* strains, 250a and 263b [20], showed that the effect of bactozole on rhizobia is strain-dependent. Nevertheless, bactozole stimulates the growth of both of these strains at the high nitrate concentration (20 mM). Bactozole enhances the symbiotic activity of strain 250a when the concentrations of nitrate in the cultivation medium of this strain and in the growth substrate of pea plants are low. In contrast, bactozole enhances the symbiotic activity of mutant M-71 when the concentrations of nitrate in the cultivation medium of the mutant and in the growth substrate of pea plants are high.

Thus, the symbiotic potential of nodule bacteria manifests itself when their carbohydrate and nitrogen metabolisms are balanced. The use of plant growth stimulants is efficient if they stimulate the growth of nodule bacteria but do not suppress the synthesis of bacterial exoglycans. The stimulation of the growth of nodule bacteria is of particular interest when high biomasses are required, for instance, for the sake of isolation of valuable products from the cell biomass or culture liquid.

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