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Inoculation of soybean seeds by rhizobia with nanometal carboxylates reduces the negative effect of drought on N_2 and CO_2 assimilation

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Abstract: The effect of individual nanometals (Co, Fe, Cu, Ge) carboxylates (NMC) as components of the suspension for seeds inoculation with rhizobia on the nitrogen fixation rate and the parameters of CO_2 and H_2O gas exchange in soybean plants grown under different water conditions was investigated. The scheme of trials included the following variants: 1 – seeds + strain B1-20; 2 – seeds + (strain B1-20 + nano-cobalt carboxylate); 3 – seeds + (strain B1-20 + nano-ferrum carboxylate); 4 – seeds + (strain B1-20 + nano-cuprum carboxylate); 5 – seeds + (strain B1-20 + nano-germanium carboxylate). The results showed that during the flowering period, drought (30% field capacity) significantly reduced the rates of nitrogen fixation (N_{fx}), CO_2 net assimilation (A_n), and transpiration (T_r) in soybean plants. Inoculation of seeds by rhizobia with NMC before sowing reduced the negative effect of drought on these physiological processes. Close correlations were found between the rates of N_{fx} and A_n and the stomatal conductance for CO_2 and A_n rates. It was concluded that pre-sowing treatment of seeds by rhizobia with NMC mitigates the negative effect of drought on the main components of soybean-rhizobia symbiosis productivity formation – nitrogen fixation and CO_2 assimilation, and also contributes to their recovery after the removal of the stressor. The most effective for this was the use of Ge and Fe nanoparticle carboxylates.

Keywords: *Glycine max* (L.) Merr.; *Bradyrhizobium japonicum*; nanotechnology; water deficit; photosynthesis

Water deficit is one of the most common abiotic environmental factors, which significantly limits the genetic potential of crop productivity in many parts of the world (Lesk et al. 2016). Global climate change, accompanied by rising temperatures and aggravated uneven distribution of precipitation both by region and during the growing season, enhance these risks (Senapati et al. 2019). Therefore, the issue of the development of new technologies for growing crops under drought conditions is becoming increasingly important.

During the evolution of legume-rhizobium symbiosis, a well-coordinated system of metabolism and energy has been developed between macro- and micro-partners (Adams et al. 2016). With active nitrogen fixation, about 30% of the carbohydrates synthesised by plants during photosynthesis are spent by nodules on binding atmospheric nitrogen. Therefore, all methods that improve the photosynthetic activity of legumes also lead to an increase in the amount of fixed molecular nitrogen by root nodules and a rise in the productivity of these crops.

Of particular importance is the study of photosynthetic apparatus functioning under drought conditions and the search for ways to mitigate the negative impact of this stressor on soybean. For this purpose, it is promising to use nanotechnologies (Wang et al. 2016) and seed priming (Liu et al. 2022) that can give significant results at the lowest material cost. Seed nano-priming is an effective process that alters seed metabolism and signalling pathways, affecting not only germination and seedling rooting but the

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entire plant life cycle and increasing stress tolerance (Pereira et al. 2021).

The inoculation of leguminous plant seeds with effective strains of nodule bacteria is an indispensable component of modern technologies for their cultivation. In this regard, it should be noted that the effectiveness of the combined use of technologies for seed inoculation with rhizobia and priming with nano preparation of various metals to increase the resistance of legumes to drought almost has not been studied. Kots et al. (2021) have investigated and discussed possible mechanisms of various nanometal carboxylates (NMC) effects on rhizobia and soybean seeds treated with them at the formation of symbiotic systems parameters (nodules number and mass, their nitrogen-fixing activity) under conditions of optimal and insufficient water supply. It was shown that carboxylates of nano-Ge, nano-Co and nano-Fe exhibited the most pronounced protective effect, which enhanced the rhizobia nodulation activity, fixation of molecular nitrogen by soybean symbiotic systems under insufficient water supply, and stimulated their recovery after stressor cessation.

In the presented work, atmospheric nitrogen fixation is of interest primarily as one of the two main components of soybean plant productivity. The second component is photosynthetic CO_2 assimilation.

Thus this study aimed to determine the impact of nanometal carboxylates as components of the suspension for seeds inoculation with rhizobia on the nitrogen fixation rate and the parameters of CO_2 and H_2O gas exchange in soybean plants grown under different water conditions.

MATERIAL AND METHODS

Materials. Symbiotic systems formed with soybean plants cv. Almaz and nodule bacteria with the addition of chelated nanometals (Co, Fe, Cu, and Ge) to their cultivation medium were studied. Co, Fe, and Cu are parts of the various enzyme cofactors, while Ge exhibits antioxidant properties in biological systems. Nodule bacteria *Bradyrhizobium japonicum* B1-20 strain from the collection of symbiotic and associative nitrogen-fixing microorganisms of the Institute of Plant Physiology and Genetics NAS of Ukraine were used in this work.

The chelated nanometals were provided by LLC Scientific and Production Company Avatar (Kyiv, Ukraine). They were obtained in two stages: 1 – obtaining an aqueous colloidal solution of nanoparticles by dispersing granules of the corresponding highly purified metals with electric current pulses in deionised water; 2 – obtaining NMC by the reaction of direct interaction of the obtained nanoparticles with citric acid.

Experimental conditions. Before sowing, soybean seeds were sterilised with a solution of ethanol (70%) and washed with running water, after which they were inoculated by moistening them for 1 h with a suspension of rhizobia contained nanometals. Rhizobia were cultivated in test tubes on yeast agar for 6 days and then washed off with saline and inoculated into Erlenmeyer flasks (at a concentration of 2% of the volume) with liquid mannitol-yeast medium, which in the respective variants contained chelated metals in a ratio of 1:1 000. Cultivation of a freshly prepared suspension of rhizobia contained nanometals was carried out for 6 days on a shaker. The bacterial titer of the suspension for seeds inoculation was 10^8 cells/mL; in the respective variants, it contained chelated metals in a ratio of 1:1 000.

Soybeans were grown in 4 kg pots filled with washed river sand under optimal (60% field capacity (FC), control) and insufficient (30% FC, drought) water supply, natural light and temperature. The source of mineral nutrition was the Hellriegel (Hellriegel and Wilfarth 1888) nutrient mixture with 0.25 nitrogen norm. The pots were placed on shelving with a transparent polyethylene film roof. In half of the pots from each variant with treatment by NMC, drought was created for two weeks (from the stage of three true leaves to the flowering of plants); after that, watering was restored to 60% FC. The parameters of plant nitrogen fixation and leaf gas exchange were measured in two stages: flowering (the second week of drought) and pod formation (one week after the optimal watering resumption). A more detailed description of the experimental variants is given in Table 1.

The number of repetitions. For each NMC treatment, 16 pots with 7 plants were set up. Thus, the number of biological repetitions for each variant of combinations of NMS treatment, the water conditions, and the sampling period was 4-fold. The repeatability of gas exchange and nitrogen-fixing activity determination was 4-fold.

Measurement methods. The nitrogen-fixing activity (N_{fx}) of symbiotic systems was determined by the acetylene method (Hardy et al. 1968) on gas chromatograph Agilent GC system 6850 (Santa Clara, USA). The CO_2 net assimilation (A_n) and transpiration (T_r) rates were recorded under controlled

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Table 1. The nitrogen-fixing activity of symbiotic systems, gas exchange parameters of soybean leaves at flowering (part of plants under drought 30% field capacity (FC)), and pod formation (all plants at optimal humidity 60% FC) after inoculation of seeds by rhizobia with nanometal carboxylates (NMC) (in parentheses is the ratio (%) of the parameter measured in drought-treated plants to the corresponding index in plants grown at optimal substrate moisture all the time) (x ± standard error, n = 4)

Treatment	Nitrogen fixation rate (µmol C_2H_4/plant/h)	Net assimilation rate (µmol $CO_2/m^2/s$)	Transpiration rate (mmol $H_2O/m^2/s$)	Stomatal conductance (mmol $CO_2/m^2/s$)
10. 07. 2020 (flowering)				
Rhizobia without NMC	6.2 ± 0.8	13.17 ± 0.40	2.24 ± 0.07	257 ± 8
Rhizobia + Co (all time under optimal humidity)	6.5 ± 0.6	14.66 ± 0.44*	2.41 ± 0.07	300 ± 9*
Rhizobia + Fe (all time under optimal humidity)	12.8 ± 1.1*	15.93 ± 0.48*	2.76 ± 0.08*	381 ± 11*
Rhizobia + Cu (all time under optimal humidity)	16.9 ± 1.6*	13.63 ± 0.41	2.49 ± 0.07	305 ± 9*
Rhizobia + Ge (all time under optimal humidity)	10.1 ± 0.9*	16.75 ± 0.50*	2.54 ± 0.08*	318 ± 10*
Rhizobia without NMC (drought)	1.7 ± 0.2#	5.35 ± 0.16# (40.6)	0.86 ± 0.03# (38.2)	69 ± 2#
Rhizobia + Co (drought)	2.6 ± 0.2*#	8.05 ± 0.24*# (54.9)	1.33 ± 0.04*# (55.0)	122 ± 4*#
Rhizobia + Fe (drought)	3.6 ± 0.6*#	9.01 ± 0.27*# (56.6)	1.30 ± 0.04*# (47.0)	117 ± 4*#
Rhizobia + Cu (drought)	4.2 ± 0.4*#	6.79 ± 0.20*# (49.8)	1.08 ± 0.03*# (43.4)	91 ± 3*#
Rhizobia + Ge (drought)	4.7 ± 0.5*#	12.83 ± 0.38*# (76.6)	2.14 ± 0.06*# (84.2)	236 ± 7*#
17. 07. 2020 (pods formation)				
Rhizobia without NMC	5.4 ± 0.6	13.78 ± 0.41	2.28 ± 0.07	258 ± 8
Rhizobia + Co (all time under optimal humidity)	9.7 ± 0.7*	13.88 ± 0.42	2.34 ± 0.07	266 ± 8
Rhizobia + Fe (all time under optimal humidity)	14.3 ± 1.2*	14.98 ± 0.45*	2.39 ± 0.07	277 ± 8
Rhizobia + Cu (all time under optimal humidity)	2.5 ± 0.4*	10.45 ± 0.31*	1.81 ± 0.05*	184 ± 6*
Rhizobia + Ge (all time under optimal humidity)	13.2 ± 0.7*	14.82 ± 0.44*	2.28 ± 0.07	239 ± 7
Rhizobia without NMC (optimal humidity after a drought)	2.7 ± 0.3#	10.61 ± 0.32 (77.0)	2.08 ± 0.06 (91.3)	206 ± 6
Rhizobia + Co (optimal humidity after a drought)	6.9 ± 0.6*#	12.48 ± 0.37*# (89.9)	1.76 ± 0.05*# (75.3)	168 ± 5*#
Rhizobia + Fe (optimal humidity after a drought)	5.2 ± 1.1*#	10.92 ± 0.33*# (72.9)	1.93 ± 0.06# (80.9)	190 ± 6#
Rhizobia + Cu (optimal humidity after a drought)	3.7 ± 0.2*#	9.37 ± 0.28# (89.6)	1.68 ± 0.05* (92.5)	155 ± 5*#
Rhizobia + Ge (optimal humidity after a drought)	5.8 ± 0.5*#	12.66 ± 0.38*# (85.4)	1.95 ± 0.06# (85.4)	194 ± 6#

*significant difference compared to the inoculation of seeds by rhizobia without nanometals at P < 0.05; #significant difference compared to plants under continuous optimal water supply at P < 0.05

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conditions (temperature 25 °C, photosynthetically active radiation 1 800 μmol/m²/s, air humidity 10 mbar, CO_2 concentration 400 ppm) with the gas analyser EGM-5 (PP Systems, Amesbury, USA). Gas exchange parameters were calculated according to Laisk and Oja (1998).

Statistical analysis. The obtained data were processed by generally accepted methods of variation statistics using Microsoft Excel (Redmond, USA). The significance of the difference between treatments was evaluated using ANOVA. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

NMC impact on plants' physiological indices under different water regimes. The N_{fx} rate in drought-treated plants was significantly lower than in control plants (which grew all the time at optimal substrate moisture) (Table 1). At the same time, it should be noted that in plants inoculated by rhizobia with NMC and subjected to water stress, this index was usually higher than in those inoculated with a pure culture. The A_n rate sharply decreased under drought and increased after the resumption of optimal substrate moisture. Calculations of the studied parameters values ratio under drought to the corresponding indices in control plants revealed that inoculation by rhizobia with NMC significantly reduced the negative effect of drought on the A_n rate in soybean leaves both during its action (flowering period) and a week after the soil moisture resumption to the optimum (pods formation stage).

The relationships between nitrogen fixation and CO_2 net assimilation rates. During the flowering period, the relationship between the arrays of all data on the N_{fx} and A_n rates was generally described by quadratic function (Figure 1). At the same time, this relationship was formed by two groups of points related to different conditions of the water regime: the first part refers to the parameters of plants subjected to drought and can be approximated by a linear function, the second part – to control plants, and is approximated by a quadratic function. It follows from this that although the water regime was of decisive importance for the functioning of the legume-rhizobium symbiosis, the modification of the nitrogen fixation and CO_2 assimilation processes by the treatment of rhizobia and seeds with NMC had a clearly pronounced positive effect on the formation of a direct relationship between these two processes under drought.

The resumption of normal watering of plants that were under drought during the flowering stage stimulated an increase in the A_n rate of their leaves, which led to a decrease in the slope of the trend line approximating the data for this group, in particular, and for the entire data set for this period (Figure 2). All points related to plants previously exposed to drought were located in the region of the initial part of the curve describing the dependence of A_n on N_{fx} in plants grown all the time at optimal substrate moisture (control group) but below it. Some decrease, compared to the flowering stage, in the A_n rate of plants which all the time grew at the optimum substrate moisture probably occurred due to the starting of nitrogen-containing compounds remobilisation from the leaves to pods (Thilakarathna et al. 2021). As in the flowering period, at the pods' formation stage, the best physiological indices were in plants inoculated by rhizobia with nano-Ge and nano-Fe carboxylates.

The relationships between leaves gas exchange indices. For the entire array of data on treatments and timing of measurement, a close correlation was found between stomatal conductance for CO_2 and the A_n rate of soybean leaves, which was approximated by a logarithmic function (Figure 3). At the same time, in the conductance range from 50 to 200 mmol

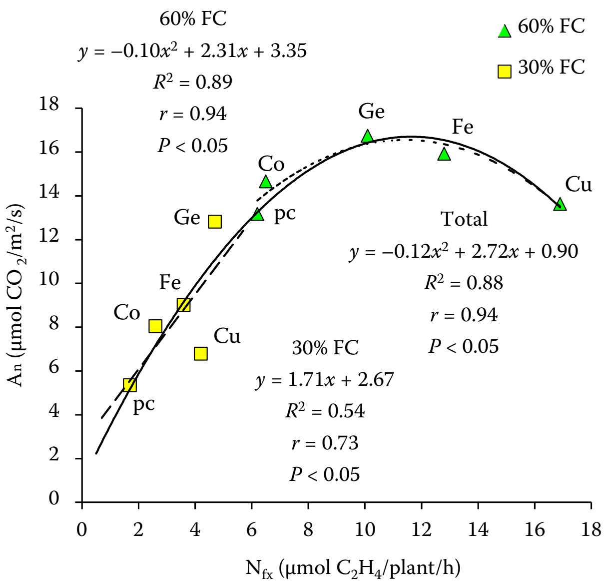

Figure 1. The relationship between the nitrogen fixation (N_{fx}) and CO_2 net assimilation (A_n) rates in soybean plants inoculated by rhizobia with carboxylates of various nanometals under optimal (60% field capacity (FC)) and insufficient (30% FC) water supply at the flowering stage; pc – inoculation with a pure culture of rhizobia

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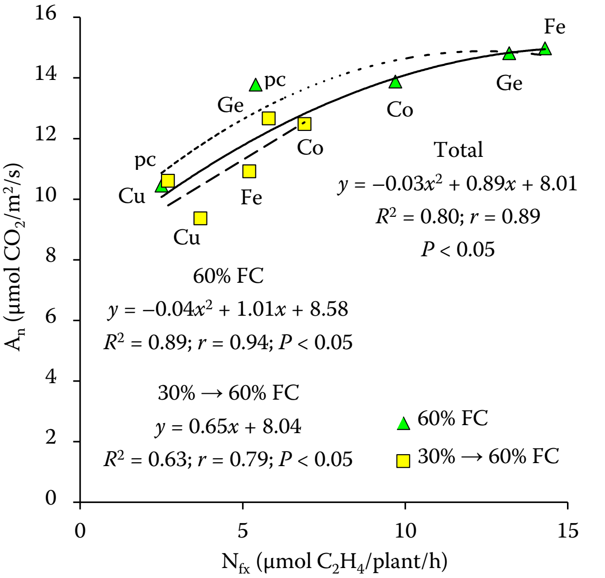

Figure 2. The relationship between nitrogen fixation (N_{fx}) and CO_2 net assimilation (A_n) rates in soybean plants inoculated by rhizobia with carboxylates of various nanometals under optimal (60% field capacity (FC)) water supply during formation; pc – inoculation with pure rhizobia culture; 30% → 60% FC – drought during flowering with the subsequent resumption of optimal water supply at formation stage

$CO_2/m^2/s$, the A_n increased faster than in the range from 200 to 400 mmol $CO_2/m^2/s$. This phenomenon is well known in the literature (Endres et al. 2010) and is explained by an increase in the significance of non-stomatal limitation of photosynthesis against the background of a decrease in the limitation of stomatal conductance (Salmon et al. 2020). Interestingly, the dependence under consideration contingently consists of three groups of points. The first group (Figure 3, (1)) refers to plants that are under drought conditions during flowering, and the second (Figure 3, (2)) – to plants under optimal watering at the pods' formation stage but previously subjected to drought and did not fully restore the functioning of the photosynthetic apparatus, and the third group (Figure 3, (3)) – to plants with the normal water supply at the flowering stage (as well as plants inoculated by rhizobia with Ge, which are under drought during the flowering period) and most of the same plants at the pods' formation stage. The first two groups formed the above-mentioned initial part of the curve (for conductance from 50 to 200 mmol $CO_2/m^2/s$, and the third group formed the second part, where the role of stomatal limitation decreased. Therefore, in plants subjected to drought, the modification of photosynthetic apparatus activity through inoculation by rhizobia with NMC was more effective than in those that grew all the time under conditions of optimal substrate moisture.

A close positive correlation was revealed between the T_r and A_n rates, which was approximated by a linear function (Figure 4). In this case, the coefficient at the function argument (5.71) is instantaneous water use efficiency (WUEi) at photosynthesis, while the vapour pressure deficit (VPD) was 22 mbar. At this VPD value, WUEi was 5.71 μmol CO_2/mmol H_2O, i.e. to fix 1 μmol CO_2, 0.175 mmol H_2O must be used (or to fix 1 g CO_2, 72 g H_2O must be used). However, it should be noted that WUEi values are highly variable during the day and growth stage and depend on genotypic features (Lopez et al. 2019, Li et al. 2020). However, in our experiments, measurements were carried out under controlled conditions, and changes in the A_n and T_r rates were obtained due to the direct action and after-effect of water stress at different stages of plant development, as well as seeds inoculation by rhizobia with various NMC. Therefore, a WUEi value of 5.71 μmol CO_2/mmol H_2O can be considered representative of soybean.

In general, the results obtained allow us to state that treatment of seeds by rhizobia with NMC mitigates the negative effect of drought on the main compo-

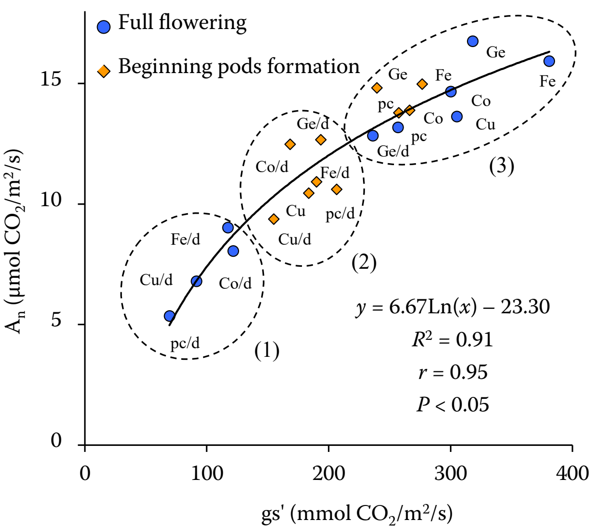

Figure 3. The relationship between stomatal conductance for CO_2 (gs') and CO_2 assimilation rate (A_n) in leaves of soybean plants inoculated by rhizobia with carboxylates of various nanometals, in control and drought-treated (/d) plants; pc – inoculation with pure rhizobia culture

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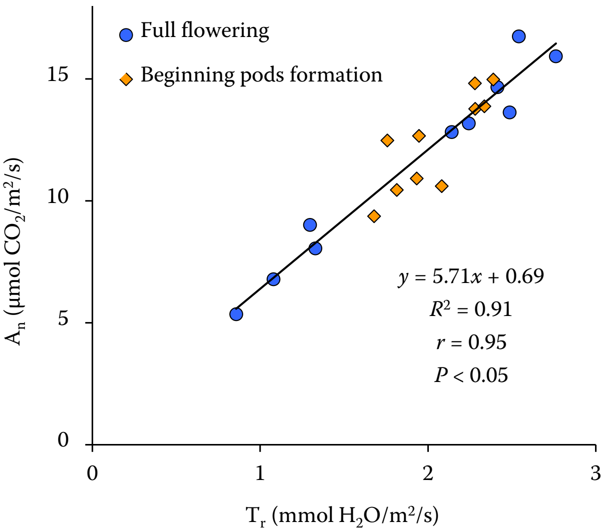

Figure 4. The relationship between the transpiration (T_r) and CO_2 net assimilation (A_n) rates in leaves of soybean plants inoculated by rhizobia with carboxylates of various nanometals in control and drought-treated plants

nents of soybean-rhizobium symbiosis productivity formation – nitrogen fixation and CO_2 assimilation and also contributes to their recovery after the removal of the stressor. These data are useful for the development of technologies for growing one of the leading food crops – soybean.

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