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EFFECTS OF DROUGHT ON SYMBIOTIC NITROGEN FIXATION, PHOTOSYNTHESIS, AND PRODUCTIVITY OF RED CLOVER (*TRIFOLIUM PRATENSE* L.) UNDER CO-INOCULATION WITH RHIZOBIA AND PLANT GROWTH-PROMOTING RHIZOBACTERIA

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Background. Red clover is an important forage crop that enhances soil fertility. Pre-sowing inoculation of legume seeds with nitrogen-fixing microorganisms is an effective way to increase their productivity. It is known that co-inoculation with rhizobia and plant growth-promoting bacteria helps protect plants from abiotic stresses, particularly drought. The aim of this study was to investigate the effects of drought on nitrogen-fixing activity, net CO₂ assimilation rate, and productivity of clover plants inoculated with microbial complexes based on nodule bacteria and free-living soil microorganisms.

Materials and Methods. The study was conducted on symbiotic systems involving clover plants (*Trifolium pratense* L.) of the Tina variety and nodule bacteria *Rhizobium leguminosarum* bv. *trifolii* 348a strain, as well as in their combinations with *Azotobacter chroococcum* T79, *Pseudomonas fluorescens* 33, and *Neorhizobium galegae* MC-1 strains. Research methods – microbiological, gasometric, gravimetric, statistical analysis.

Results. Drought suppressed both the nitrogen-fixing activity (NFA) of root nodules and the net CO₂ assimilation rate (A_N) in clover leaves, with a stronger effect on NFA than on A_N . A significant increase in leaf photorespiration (R_{light}) was observed in drought-exposed plants, and R_{light}/A_N ratio remained elevated compared to control plants even after drought termination. Drought was also found to weaken the correlation between NFA and A_N , which was strong under optimal water conditions. A stronger correlation was observed between A_N and whole-plant dry weight ($r = 0.84$) than between



NFA and dry weight ($r = 0.71$). The data clearly show that neither NFA nor A_N returned to well-watered control levels within seven days of drought termination. Seed treatment with the combination of *Rhizobium leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1 stimulated NFA earlier in plant development than other treatments, maintaining peak activity from the eight-leaf stage onwards, whereas treatments with *R. leguminosarum* bv. *trifolii* 348a alone and in combination with *A. chroococcum* 79 reached comparable NFA values only at the budding stage.

Conclusions. The results indicate that inoculation of clover plants with different combinations of nitrogen-fixing microorganisms increased their productivity and improved their performance under drought conditions, although to varying extents. The combination *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC1 was the most effective in stimulating clover productivity under both optimal and stress conditions. The *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33 treatment was the worst-performing inoculant combination.

Keywords: *Trifolium pratense* L., *Rhizobium leguminosarum* bv. *trifolii*, *Neorhizobium galegae*, *Azotobacter chroococcum*, *Pseudomonas fluorescens*, drought, nitrogen-fixing activity, photosynthesis, productivity

INTRODUCTION

Legumes play a key role in addressing global food security challenges by providing a stable source of protein and bioactive compounds (Uçar, 2023). The inclusion of legumes in agricultural systems helps to diminish negative impacts on soils by reducing the use of mineral nitrogen fertilizers and decreasing greenhouse gas emissions.

Red clover (*Trifolium pratense* L.) is an important forage crop that improves soil fertility (Bastruk-Hlodan, 2023). Its value lies in its ability to form symbiotic relationships with rhizobia for biological nitrogen fixation. Clover can fix 100–150 kg/ha of atmospheric nitrogen per growing season and has high nutritional value for animals. However, this crop is sensitive to drought (Egan *et al.*, 2021; Heslop *et al.*, 2023; Hendrickson *et al.*, 2025).

Pre-sowing inoculation of legume seeds with nitrogen-fixing microorganisms is an effective way to increase their productivity (Monalisha *et al.*, 2025). It should be noted that different legume species form symbiotic relationships with specific bacterial species (Santos *et al.*, 2019). For example, common bean (*Phaseolus vulgaris* L.) can form symbiosis with *Rhizobium leguminosarum* bv. *phaseoli*, red clover – with *R. leguminosarum* bv. *trifolii*, goat's rue – with *Neorhizobium galegae*. The latter bacterium deserves special attention, as there is evidence of its resistance to various environmental stress factors, attributed to the production of large amounts of polysaccharides and specific growth-promoting metabolites. This has been demonstrated in studies involving the pre-sowing inoculation of lupine seeds (Omelchuk *et al.*, 2024) and the influence of mono- and complex inoculants on the morphometric parameters of seedlings and the sowing qualities of red clover seeds (Kominarets *et al.*, 2025).

However, the plant rhizosphere is typically inhabited not only by microorganisms that directly form symbiotic relationships with roots but also by many other microbial species. The root system can be colonized by plant growth-promoting rhizobacteria (PGPR), which have a positive effect on the functioning of the legume–rhizobia symbiosis (Alemneh *et al.*, 2020). These bacteria enhance nitrogen-fixing activity, promote nodule formation on legume roots, and mitigate the harmful effects of stress factors (Koza *et al.*, 2022).

Among the most well-known representatives of PGPR are bacteria from genera such as *Azotobacter* and *Pseudomonas*, among others (Ibrahim & El-Sawah, 2022). For example, *A. chroococcum* produces auxins, gibberellins, and phenolic compounds, thereby increasing plant height, biomass, and nitrogen content. *A. vinelandii* is capable of fixing atmospheric nitrogen and making it available to plants in the form of ammonium compounds. *Pseudomonas putida* is an effective phosphate-mobilizing bacterium that is resistant to high temperatures and hydrolyzes phosphate into forms available to plants, thereby promoting their growth and development (Costa-Gutierrez *et al.*, 2022).

Previously, rhizobia were most often used as inoculants. As an exception, two compatible strains of the same species were occasionally used. However, the use of inoculants containing microorganisms of different species has recently become significantly more widespread (Alemneh *et al.*, 2020). The application of mixed inoculants is referred to as co-inoculation. The effectiveness of co-inoculation depends on the selection of compatible strains, cell concentration, and the method of microbial application (Santos *et al.*, 2019).

Drought is the most common and harmful abiotic stress factor worldwide. It negatively affects physiological processes in plants, including a reduction in nitrogen-fixing activity in legumes (Vleugels *et al.*, 2024). As a result, nitrogen availability to plants decreases, which can also affect the efficiency of the photosynthetic apparatus, in addition to the direct effects of drought on it, and the productivity of legume plants decreases. It has been shown that soil microorganisms can induce trait changes in red clover, suggesting that they play an important role in plant responses to drought, in maintaining plant productivity, and in prolonging plant vitality (Tello-García *et al.*, 2023). Literature data indicate that co-inoculation of legume plants with PGPR bacteria and rhizobia is an effective strategy for mitigating abiotic stresses, particularly drought (Fahde *et al.*, 2023; Goswami & Suresh, 2020; Chandra *et al.*, 2021).

The aim of this study was to investigate the effect of drought on nitrogen-fixing activity, net CO₂ assimilation rate, and the productivity of clover plants inoculated with microbial complexes composed of nodule bacteria and free-living soil microorganisms.

MATERIALS AND METHODS

The study was conducted on red clover plants (*T. pratense*) of the Tina variety (originator – the Institute of Feed Research and Agriculture of Podillia, NAAS of Ukraine). Seed inoculation was performed using one- or two-component microbial preparations containing nitrogen-fixing and associative bacteria from the museum collection of nitrogen-fixing microorganisms at the Institute of Plant Physiology and Genetics of the NAS of Ukraine.

Clover rhizobia – *Rhizobium leguminosarum* bv. *trifolii* 348a (production strain), goat's rue-specific nodule bacteria *Neorhizobium galegae* MC-1, as well as associative microorganisms – *Azotobacter chroococcum* T79, *Pseudomonas fluorescens* 33 were used. The bacteria were grown at a temperature of +28 °C for 5 days on the following media: *R. leguminosarum* bv. *trifolii* 348a and *N. galegae* MC-1 – on the Yeast Extract Mannitol (YEM) medium (g/L): K₂HPO₄ (0.5), MgSO₄·7H₂O (0.4), NaCl (0.1), mannitol (10.0), yeast extract (0.5), agar (15.0–17.0), distilled water, pH 6.8–7.2, sterilization at 1 bar for 30 min. *A. chroococcum* 79 was cultured on Ashby medium (g/L): KH₂PO₄ (0.2), MgSO₄·7H₂O (0.2), K₂SO₄ (0.1), NaCl (0.2), CaCO₃ (5.0), sucrose (20.0), agar (20.0), pH 7.3–7.6, sterilization at 0.75 bar 10 min. *P. fluorescens* 33 – on LPJ medium (g/L):

0.5 % bacpeptone (5), 0.3 % yeast extract (3), 0.5 % glucose (5), agar (16), pH 7.0, sterilization at 0.5 bar, 20 min.

The bacterial biomass was washed from the surface of the agar medium, suspended, transferred into flasks containing liquid medium, and cultivated for 4 days at +28 °C under constant aeration. The optical density of the inoculum suspensions was adjusted to a uniform level of 0.5 relative units by adding sterilized water and monitored using a Shimadzu UV-1900 spectrophotometer (Japan) at a wavelength of 600 nm.

Seeds were inoculated for 1 hour. The inoculated seeds were then sown in Wagner pots, previously sterilized with a 20% hydrogen peroxide solution, and filled with 8.5 kg of non-sterile river sand. Hellriegel's nutrient solution was used as the mineral nutrition source at one-quarter of the standard nitrogen rate (full rate: 0.708 g $\text{Ca}(\text{NO}_3)_2$ per kg substrate), with microelements added accordingly.

The pots were arranged on outdoor racks at the Institute grounds, covered with a transparent polyethylene film canopy to exclude rainfall. Plants were grown under natural light and temperature conditions, with fifteen plants per pot.

The experiment scheme included the following treatments: 1 – without inoculation; 2 – inoculation with *R. leguminosarum* bv. *trifolii* 348a; 3 – inoculation with *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79; 4 – inoculation with *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33; 5 – inoculation with *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1. At the stage of 8 true leaves, drought was induced in half of the pots of each inoculation treatment by stopping irrigation. When substrate moisture decreased to 40 % field capacity (FC), it was maintained at this level for one week through controlled watering, after which moisture was restored to the optimal level (70 % FC). Control plants were maintained at optimal substrate moisture throughout the experiment. Thus, the experimental design included 5 inoculation treatments $\times$ 2 watering regimes $\times$ 7 replicates = 70 pots. Soil moisture during drought was determined gravimetrically by weighing the pots every morning and evening.

The nitrogen-fixing activity of root nodules (NFA), leaf net CO_2 assimilation, and photorespiration rate were determined before the onset of drought at the 5 true-leaf stage, at the end of the drought at the 8 true-leaf stage, and seven days after the end of the drought at the budding stage (the restoration of watering). Since each pot contained 15 plants, nitrogen-fixing activity was measured by grouping plants into sets of three, with four replicates (12 plants in total), and the acetylene reduction activity of the nodules was determined. Intact leaves from 4 different plants per pot were used to measure gas exchange parameters.

The nitrogen-fixing activity of root nodules (NFA) was determined by the acetylene method (Hardy *et al.*, 1968) using an Agilent Technologies 6850 chromatograph (USA) with a flame ionization detector.

The net CO_2 assimilation (A_N) and photorespiration (R_{light}) rates were measured under controlled conditions using an EGM-5 gas analyzer (PP Systems, Amesbury, USA). An intact, fully expanded middle-tier leaf was placed in a temperature-controlled chamber (25 °C) and illuminated with a 50 W TA-11 LED lamp with a color temperature of 5200 K. The irradiance at the chamber level was 1500 $\mu\text{mol}/(\text{m}^2 \cdot \text{s})$ of photosynthetically active radiation. Atmospheric air was passed through the chamber at a flow rate of 1 L/min. The CO_2 concentration was not controlled but was continuously recorded by the gas analyzer and ranged from 470 to 490 ppm. The photorespiration rate was determined using the post-illumination burst method, based on measuring CO_2 emission from the leaf within one minute after the light was turned off.

After measuring gas exchange parameters, the leaf blades that had been in the chamber were detached from the petioles, scanned using an Epson 3490 Photo scanner; their area was determined using Adobe Photoshop CS6 software via the pixel-counting method. Gas exchange parameters were calculated using standard equations (Busch *et al.*, 2024).

The data obtained were processed by methods of variation statistics using Microsoft Excel 2010. The significance of differences between samples was evaluated by ANOVA, where differences were considered significant at $P < 0.05$ using Tukey's test. The validation analysis of data approximation by the linear functions (illustrated in **Fig. 3** and **Fig. 5**) was performed using Fisher's F-test. All data are presented as means $\pm$ SE ($n = 4$).

RESULTS AND DISCUSSION

Measurements of NFA in clover plants inoculated with different combinations of nitrogen-fixing microorganisms showed that, at the 5 true leaf stage, this parameter was lowest in non-inoculated plants and highest in plants inoculated with *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1 (**Fig. 1**). Plants subjected to other inoculation treatments exhibited intermediate values between these extremes. The presence of measurable nitrogen-fixing activity in non-inoculated plants can be explained by spontaneous colonization by local rhizobia races, as the substrate was not sterilized.

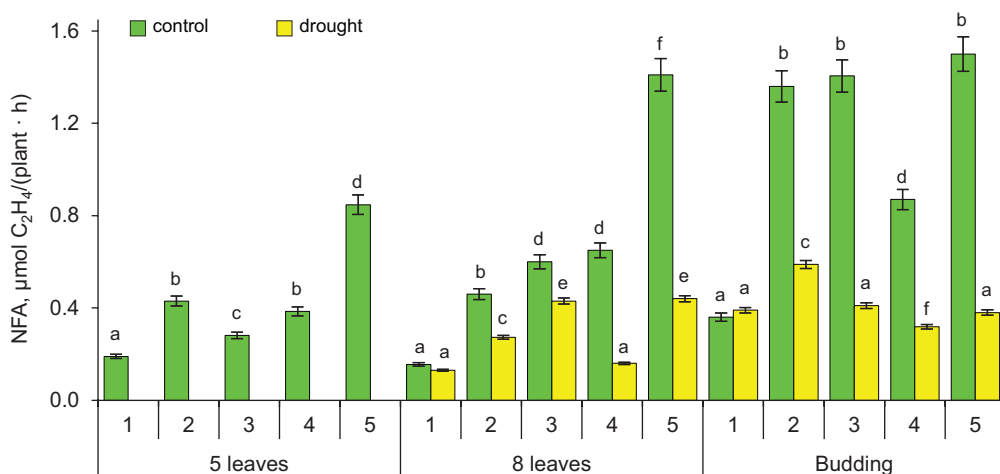


Fig. 1. Nitrogen-fixing activity (NFA) of clover plants inoculated with different combinations of nitrogen-fixing microorganisms under optimal substrate moisture (70 % FC, control) and drought-treated at the stage of 8 true leaves (40 % FC, drought): 1 – without inoculation; 2 – inoculation with *R. leguminosarum* bv. *trifolii* 348a; 3 – *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79; 4 – *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33; 5 – *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC 1 (here and in **Figs. 2, 4, 6**: different letters indicate significant differences among treatments at the same developmental stage, as determined by Tukey's post-hoc test following ANOVA ($P < 0.05$); values are means $\pm$ SE ($n = 4$))

At the stage of 8 true leaves, under optimal substrate moisture, a general increase in NFA was observed. Under drought conditions, a significant decrease in NFA was recorded in all treatments except spontaneous inoculation. This may indicate that

local rhizobial strains were more resistant to this stress factor; however, their activity remained significantly lower than that of the selected microbial strains. Among the latter, the lowest NFA was in the drought-treated plants inoculated with *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33, where it practically equaled that in plants without inoculation. This parameter was somewhat higher in plants inoculated with *R. leguminosarum* bv. *trifolii* 348a, while the highest values under drought conditions (although still significantly lower than under optimal substrate moisture) were observed in the treatments *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79 and *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1.

At the budding stage (one week after the end of drought), after the restoration of optimal substrate moisture in previously drought-exposed plants, a slight increase in NFA was observed, although this parameter did not return to the control level in plants with artificial inoculation. In plants with spontaneous inoculation, NFA did not differ significantly between the control and previously drought-treated plants. In other cases, the highest NFA in the drought-treated plants was observed under inoculation with *R. leguminosarum* bv. *trifolii* 348a, while the lowest values were recorded under inoculation with *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33.

It should be noted that during this period, a significant increase in NFA was observed in the control plants under inoculation with *R. leguminosarum* bv. *trifolii* 348a (treatment 2) and *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79 (treatment 3). As a result, these treatments reached values that were practically comparable to those of plants inoculated with *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1 (treatment 5). In plants inoculated with *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33, an increase in NFA was also observed in the control plants compared with the previous developmental stages; however, it remained significantly lower than in the other artificially inoculated treatments.

In annual legumes, the highest NFA is usually observed during the budding–flowering period, when the photosynthetic apparatus reaches its maximum performance and provides the plant with a sufficient amount of assimilates for the formation of new nodules and the intensification of functioning of existing ones (Schwember *et al.*, 2019). This may explain the sharp increase in NFA in the control plants in treatments 2 and 3 compared with the previous measurement dates. In treatment 5, the highest NFA was observed throughout the studied period; it reached a near-maximum level at the stage of eight true leaves and remained virtually unchanged until the budding stage. Such early stimulation of NFA is undoubtedly a positive factor for enhancing the productivity of the legume–rhizobial symbiosis.

The nitrogen supply of cultivated plants plays a decisive role in realizing their genetic yield potential. In legumes, this role is further shaped by the interaction between the symbiotic and photosynthetic apparatuses within the source–sink system of the plant organism (Kiriziy *et al.*, 2022; Uçar, 2023). Thus, on the one hand, root nodules supply the plant with nitrogen in the form of organic compounds, which is essential for the development and proper functioning of the photosynthetic apparatus. On the other hand, the photosynthetic apparatus supplies the nodules with products of carbon assimilation (mainly sugars), which provide energy and organic substrates for the fixation of atmospheric molecular nitrogen (Gupta *et al.*, 2026). At the same time, most assimilates are directed toward the growth and development of the plant itself. It is well established that approximately 90% of plant dry weight is derived from photosynthesis,

while the efficiency of the photosynthetic apparatus is strongly determined by the plant's nitrogen status (Evans & Clarke, 2019).

Our measurements of the net CO₂ assimilation rate in clover leaves showed that, although non-inoculated plants generally exhibited lower values than inoculated plants, the difference was less pronounced than that observed for NFA (Fig. 2). This may be explained by the presence of an initial dose of mineral nitrogen in the substrate.

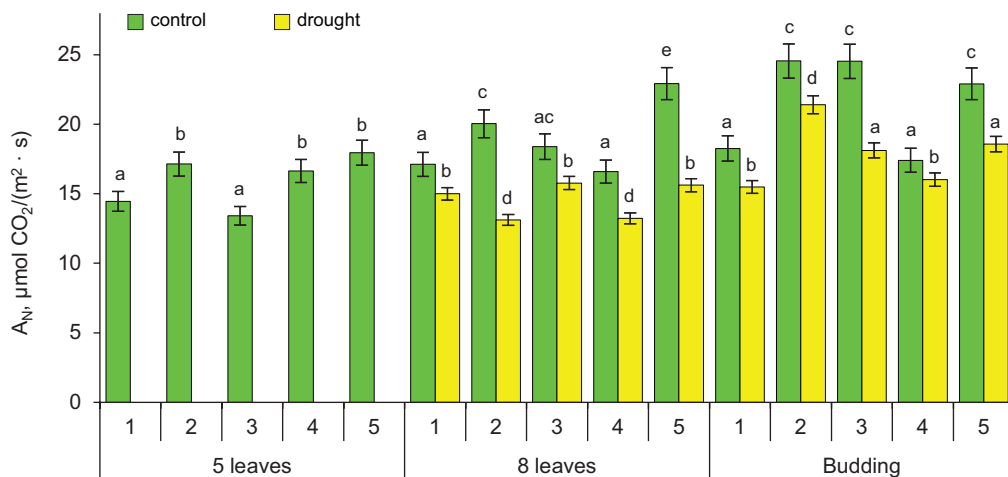


Fig. 2. Net assimilation rate (A_N) in leaves of clover plants inoculated with different combinations of nitrogen-fixing microorganisms under optimal substrate moisture (70 % FC, control) and drought-treated at the stage of 8 true leaves (40 % FC, drought): 1 – without inoculation; 2 – inoculation with *R. leguminosarum* bv. *trifolii* 348a; 3 – *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79; 4 – *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33; 5 – *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC 1 (values are means $\pm$ SE (n = 4))

Under drought conditions, AN was inhibited, although to a lesser extent than NFA. This difference is likely due to distinct mechanisms underlying the response of these processes. Drought reduces leaf photosynthesis by inducing stomatal closure, which limits CO₂ diffusion into the leaf, and by triggering signaling pathways that suppress the biochemical processes of carbon assimilation in chloroplasts (Zahra *et al.*, 2023; Farooq *et al.*, 2024). In addition, these effects are accompanied by increased formation of reactive oxygen species (ROS) and the resulting oxidative stress in nearly all plant tissues (Ehonen *et al.*, 2019).

In nodules under drought conditions, nitrogen fixation is inhibited, firstly due to a reduced supply of assimilates from the leaves, and secondly due to decreased permeability of bacteroid membranes to gases (Kunert *et al.*, 2016; Kibido *et al.*, 2019; Khatun *et al.*, 2021). In addition, drought impairs the respiratory activity of nodule bacteria, which leads to a reduction in their abundance in the rhizosphere/ecotope (Aldasoro *et al.*, 2019; Schwember *et al.*, 2019). Moderate water stress reduces the number of root nodules, while prolonged exposure not only decreases their number but also their size. The negative effect of drought on nodulation and nitrogen-fixing activity also depends on the stage of plant development. Thus, it has been shown that reduced water availability during the vegetative growth of legumes has a more detrimental effect on nodule formation and function than during the reproductive stage (Parvaze *et al.*, 2018).

Thus, in terms of the net CO_2 assimilation rate in the control plants under optimal watering at the eight-leaf stage, inoculation with *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1 showed the highest values compared with all other treatments. At the budding stage, *R. leguminosarum* bv. *trifolii* 348a and *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79 reached similar levels, which was also consistent with the patterns observed for NFA. At the budding stage (one week after the restoration of optimal watering in plants subjected to drought at the eight-leaf stage), A_N did not recover to the control level. This may be attributed not only to direct impairment of the photosynthetic apparatus but also to a pronounced decline in NFA under drought conditions, and consequently to a reduced nitrogen supply to the photosynthetic apparatus.

The relationship between nitrogen fixation and photosynthesis is further supported by the correlations observed between these processes in our experiments (Fig. 3).

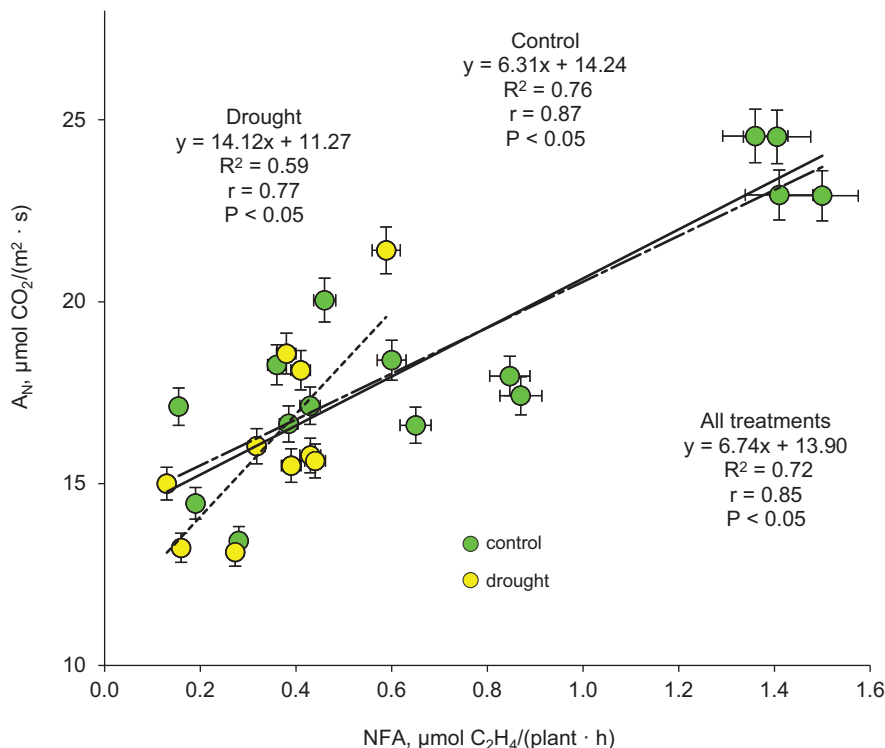


Fig. 3. Correlations between nitrogen fixation activity (NFA) and net assimilation rate (A_N) in clover plants inoculated with different combinations of nitrogen-fixing microorganisms under optimal substrate moisture (70 % FC, control) and subjected to drought (40 % FC, drought) (values are means $\pm$ SE ($n = 4$))

If the entire dataset obtained for these parameters is considered, including both control and treated plants across all developmental stages, a strong correlation is observed between NFA and A_N ($r = 0.85$). When only control plants grown under optimal soil moisture are analyzed, the correlation becomes slightly stronger ($r = 0.87$). However, when NFA and A_N values from the drought-treated plants are considered, both during stress exposure and after the recovery period, the correlation weakens ($r = 0.77$). This indicates that drought disrupts the relationship between nitrogen fixation and photosynthesis. This effect is likely associated both with a slowdown in the exchange

of nitrogen and carbon assimilates between nodules and leaves and with the different stress-induced mechanisms affecting these processes, as discussed above.

In general, based on the results of measurements of the two main components of legume productivity – NFA and CO_2 assimilation – it can be concluded that inoculation with *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1 was the most effective in stimulating these processes, whereas the least effective treatment was *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33. Drought significantly inhibited both processes, and a negative aftereffect was still evident one week after the restoration of optimal watering in previously drought-stressed plants. However, the relative ranking of inoculation treatments remained consistent under these conditions.

Along with photosynthesis, a noticeable rate of photorespiration was recorded in clover leaves (Fig. 4). In C_3 plants, this process results from the oxygenase activity of Rubisco, whereby a portion of ribulose-1,5-bisphosphate is oxidized instead of being carboxylated, leading to the formation of glycolate (Aroca *et al.*, 2023). Glycolate then enters the glycolate cycle, during which CO_2 is released, resulting in a loss of previously assimilated carbon. Photorespiration is an energy-dependent process that consumes ATP and occurs only in the light.

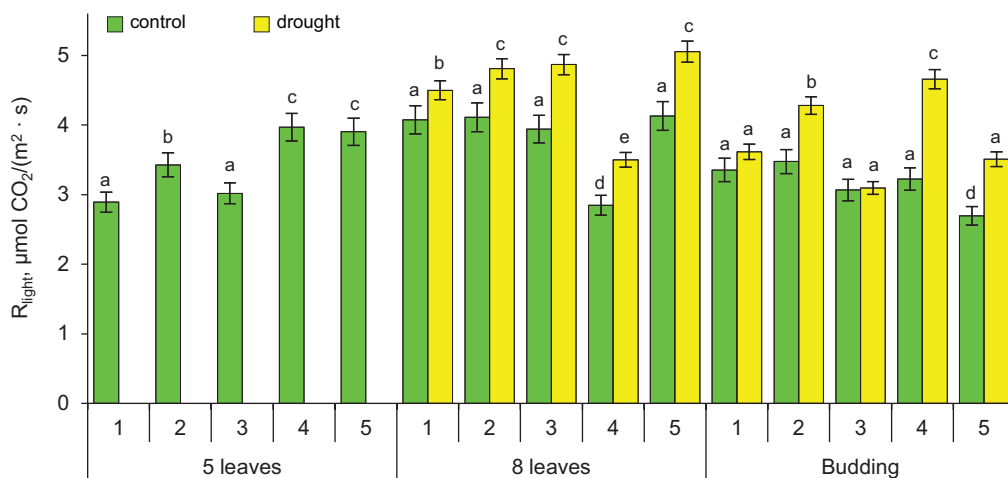


Fig. 4. Photorespiration (R_{light}) rate in leaves of clover plants inoculated with different combinations of nitrogen-fixing microorganisms under optimal substrate moisture (70 % FC, control) and drought-treated at the stage of 8 true leaves (40 % FC, drought): 1 – without inoculation; 2 – inoculation with *R. leguminosarum* bv. *trifolii* 348a; 3 – *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79; 4 – *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33; 5 – *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC 1 (values are means $\pm$ SE ($n = 4$))

For a long time, photorespiration was considered a harmful process that reduces the productivity of C_3 plants. However, recent studies suggest that under stress conditions, particularly drought, it may play a beneficial role (Broncano *et al.*, 2023). When CO_2 entry into the leaf is limited due to stomatal closure, photorespiration can serve as an internal source of CO_2 and as an alternative sink for electrons from the chloroplast electron transport chain. This reduces the risk of excessive ROS formation, which can damage the photosynthetic apparatus in mesophyll cells. In addition, photorespiratory metabolism produces the amino acids glycine and serine, thereby contributing to plant nitrogen metabolism.

Although the post-illumination CO_2 burst method used to estimate photorespiration provides only an approximation, our data indicate that in the drought-treated clover plants, the leaf photorespiration rate was significantly higher than in the control plants (Fig. 4). This pattern was observed both during the direct action of the stress factor and after the restoration of optimal watering in previously drought-exposed plants. Since this increase in photorespiration coincided with a decrease in A_N , the R_{light}/A_N ratio increased significantly compared with the control across all inoculation treatments. Thus, at the eight-leaf stage, this index averaged 0.20 in the control plants and 0.31 in the drought-treated plants. At the budding stage, after cessation of the stress factor, the corresponding values were 0.15 and 0.22, respectively.

Under drought conditions, the lowest photorespiration rate in the drought-treated plants was observed under inoculation with *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33, whereas under the other inoculation treatments, this parameter was significantly higher and remained at a similar level. At the budding stage, in contrast, the highest photorespiration rate was recorded under inoculation with *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33, while under all other treatments it was lower.

Since each measurement of NFA and A_N was accompanied by sampling to determine the dry weight of the entire clover plant, including the root system, we examined the relationships between these parameters. For the entire dataset, including all inoculation treatments, watering regimes, and developmental stages, a fairly strong correlation ($r = 0.84$) was observed between A_N and whole-plant dry weight (Fig. 5). In contrast, the relationship between NFA and plant dry weight was weaker ($r = 0.71$).

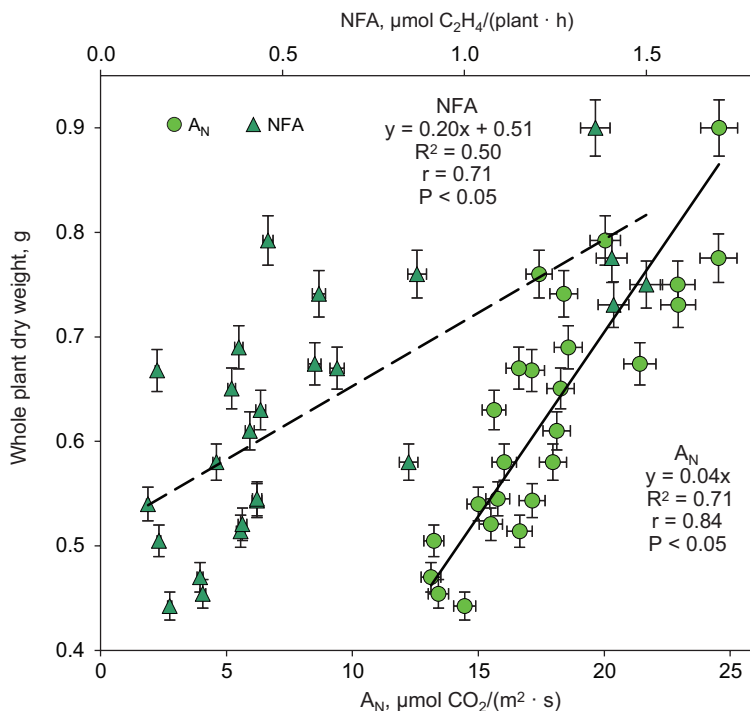


Fig. 5. Correlations between dry weight of whole clover plants and net CO_2 assimilation rate (A_N) or nitrogen-fixing activity (NFA), calculated for all inoculation and watering treatments and developmental stages (values are means $\pm$ SE ($n = 4$))

This may be explained by the presence of an initial dose of mineral nitrogen in the substrate, which was also utilized by clover plants to support the photosynthetic apparatus and partially compensated for the substantial decrease in NFA in the drought-treated plants. This does not contradict the relatively strong correlation between NFA and A_N (Fig. 3), since it was calculated over a limited range of variation in these parameters. In fact, extrapolation of the trend lines to zero NFA suggests that A_N in control plants would be approximately $14 \mu\text{mol CO}_2/(\text{m}^2 \cdot \text{s})$, while in the drought-treated plants it would be about $11 \mu\text{mol CO}_2/(\text{m}^2 \cdot \text{s})$. Thus, even in the absence of nitrogen fixation, photosynthesis in clover leaves would theoretically proceed due to the initial nitrogen supply, although at a reduced rate, resulting in lower plant productivity.

This is confirmed by the results from the second mowing of clover plants grown in pots under individual treatments, conducted six weeks after the restoration of optimal watering in previously drought-stressed plants. Figure 6 shows that this parameter was lowest in non-inoculated plants, which also exhibited the lowest A_N values.

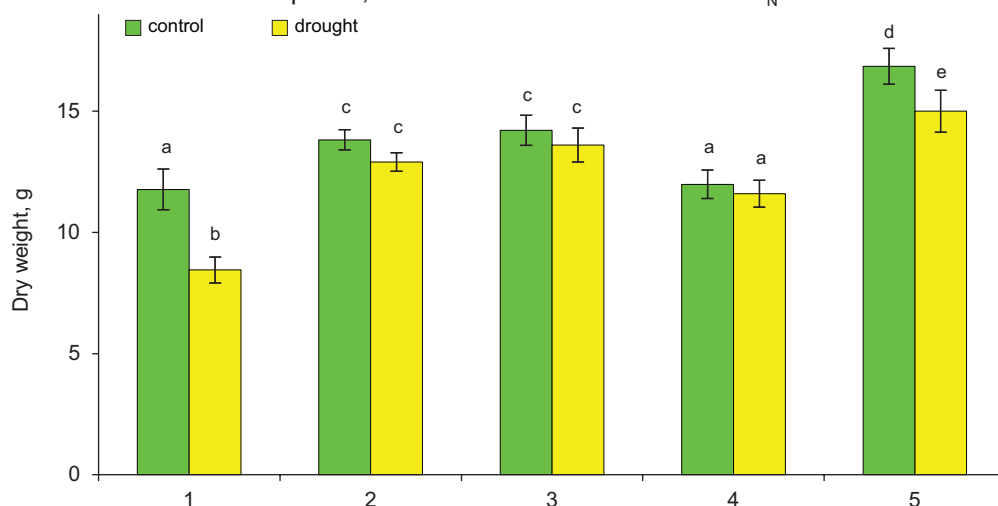


Fig. 6. Dry weight from the entire pot of the above-ground part of clover plants (at second harvest, six weeks after drought termination) inoculated with different combinations of nitrogen-fixing microorganisms under optimal substrate moisture (70 % FC, control) and drought-treated at the stage of 8 true leaves (40 % FC, drought): 1 – without inoculation; 2 – inoculation with *R. leguminosarum* bv. *trifolii* 348a; 3 – *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79; 4 – *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33; 5 – *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC 1 (values are means $\pm$ SE (n = 4))

The highest productivity of clover plants inoculated with different microbial combinations was observed in the treatment *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1, whereas the lowest was recorded in the treatment *R. leguminosarum* bv. *trifolii* 348a + *P. fluorescens* 33. Plants inoculated with *R. leguminosarum* bv. *trifolii* 348a alone and with *R. leguminosarum* bv. *trifolii* 348a + *A. chroococcum* 79 occupied an intermediate position and did not differ significantly from each other in this parameter.

CONCLUSIONS

Thus, drought suppressed both NFA in root nodules and A_N in clover leaves, although the effect on NFA was more pronounced than on A_N . This difference is likely attributable to distinct mechanisms by which this stress factor affects the respective processes, as well as the presence of an initial dose of nitrogen in the substrate. A significant increase in leaf photorespiration rate was also observed in the drought-treated plants.

It was shown that drought weakened the correlation between NFA and A_N , which was relatively strong under optimal plant water conditions. A strong correlation was also found between A_N and whole clover plant dry weight, whereas the relationship between plant dry weight and NFA was weaker.

Inoculation of clover plants with different combinations of nitrogen-fixing microorganisms increased their productivity and improved their performance under drought conditions, although to varying extents. The combination *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC-1 was the most effective in stimulating clover productivity under both optimal and stress conditions.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: the authors declare that research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Human Rights: this article does not contain any studies with human subjects performed by any of the authors.

Animal Studies: this article does not include animal studies.

AUTHOR CONTRIBUTIONS

Conceptualization, [S.K.; O.K.]; data collection [O.K.; D.K.]; analysis and interpretation of results [D.K.; S.K.; O.K.]; writing – original draft preparation, [O.K.; D.K.]; writing – review and editing, [D.K.; S.K.; O.K.]. All authors have read and agreed to the published version of the manuscript.

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ВПЛИВ ПОСУХИ НА АКТИВНІСТЬ СИМБІОТИЧНОГО І ФОТОСИНТЕТИЧНОГО АПАРАТІВ РОСЛИН КОНЮШИНИ ЗА КОІНОКУЛЯЦІЇ РІЗНИМИ ШТАМАМИ РИЗОБАКТЕРІЙ

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Обґрунтування. Конюшина лучна – важлива фуражна культура, яка забезпечує підвищення родючості ґрунтів. Передпосівна інокуляція насіння бобових рослин азотфіксувальними мікроорганізмами є ефективним засобом збільшення їхньої продуктивності. Відомо, що спільна інокуляція ризобіями і ростостимулювальними бактеріями є ефективним способом боротьби з абіотичними стресами, зокрема, з посухою. Метою роботи було дослідити вплив посухи на азотфіксувальну активність, інтенсивність фотосинтезу та продуктивність рослин конюшини, інокульованих мікробними комплексами на основі бульбочкових бактерій і вільно-існуючих ґрунтових мікроорганізмів.

Матеріали та методи. Об'єкт дослідження – симбіотичні системи, створені за участю рослин конюшини (*Trifolium pratense* L.) сорту Тіна і бульбочкових бактерій *Rhizobium leguminosarum* bv. *trifolii* 348a, а також у їхніх композиціях із *Azotobacter*

chroococcum T79, *Pseudomonas fluorescens* 33, *Neorhizobium galegae* MC-1. Методи дослідження – мікробіологічні, біохімічні та фізіологічні, статистичний аналіз.

Результати. Посуха пригнічувала як азотфіксувальну активність (АФА) корневих бульбочок, так й інтенсивність фотосинтезу листків рослин конюшини, хоча вплив на АФА був сильніший, ніж на фотосинтез. Виявлено істотне підвищення інтенсивності фотодихання листків у рослин в умовах посухи, що могло відігравати позитивну роль у захисті їхнього фотосинтетичного апарату. Показано, що посуха послаблювала кореляційний зв'язок між АФА і фотосинтезом, який за оптимального водного режиму рослин був досить сильним. Також виявлено сильний кореляційний зв'язок між інтенсивністю фотосинтезу та сухою масою цілих рослин конюшини.

Висновки. Отримані результати свідчать, що інокуляція рослин конюшини різними композиціями азотфіксувальних мікроорганізмів підвищувала їхню продуктивність і стійкість до посухи. Найефективнішою для стимуляції продуктивності рослин конюшини як за оптимальних, так і за стресових умов, виявилася композиція *R. leguminosarum* bv. *trifolii* 348a + *N. galegae* MC1.

Ключові слова: *Trifolium pratense* L., *Rhizobium leguminosarum* bv. *trifolii*, *Neorhizobium galegae*, *Azotobacter chroococcum*, *Pseudomonas fluorescens*, посуха, азотфіксувальна активність, фотосинтез, продуктивність