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THE INFLUENCE OF *Rhizobium* INOCULATION AND NITROGEN/MOLYBDENUM FERTILIZATION ON THE GROWTH CHARACTERISTICS OF RED CLOVER

Niculae DINCĂ[1], Daniel DUNEA[2]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd., District 1, 011464, Bucharest, Romania
[2]Valahia University of Targoviste, Campus, Aleea Sinaia no.13, RO-130004, Targoviste, Romania

Corresponding author email: dan.dunea@valahia.ro

Abstract

This paper presents the individual and combined effects of inoculation and nitrogen (N)/molybdenum (Mo) fertilization (8 treatments) on the biological efficiency of red clover in controlled conditions. The rationale of the study was to establish the influence of Mo on the biological efficiency of red clover in the presence or the absence of N fertilization and inoculation of Rhizobium leguminosarum biovar. trifolii, and whether the results can lead to the improvement of classical crop technologies and the fertilization plan for obtaining superior forage yields. The effects of the treatments were evaluated using 5 replicatesbased on the average height, the dry weight, the density and the number of nodules on the roots of the red clover plants. Diploid cultivar Violettawas seeded in vegetation pots with the same amount of seeds and maintained 60 days in controlled conditions of a growth chamber. In the inoculation variants (5-8), the seeds were bacterized with R. trifolii-treated strains using the Nitragin® product (B type) using 7.5 g Nitragin/kg clover seed, dissolved in about 25 mL cold water. After pre-homogenization of the seed in the resulting solution, it was sown with the same quantity in all the repetitions of the variants. The bacterial solution contains at least 100 million viable cells g^{-1} of R. trifolii. Nitric fertilization was done with ammonium nitrate (34.5% N) supplied prior to sowing in an equivalent dose of 100 kg N ha^{-1}. Molybdenum (0.02 Mo) was supplied using a uniform foliar spraying with three applications. Descending ranking and multiple comparisons (Duncan test) of treatments were performed. The seed inoculation, without nitrogen fertilization provided relatively equivalent biomass yieldsto fertilized variants. Foliar fertilization with molybdenum did not determine higher dry weight amounts, but when combined with nitrogen established significant amounts of dry matter per plant. From the yield formation point of view, the variants in which a large number of nodules were formed did not correlate positively with dry matter accumulation; however, they can have a particular importance in improving the reserves of assimilable nitrogen available in the soil.

Key words*: red clover, inoculation, molybdenum fertilization, morphological traits, controlled conditions.*

INTRODUCTION

Red clover (*Trifolium pratense* L.) is one of the most valuable forage species from temperate regions providing benefits to the soil improving characteristics. It is well known that the symbiosis of red clover and various *Rhizobium* strains produces large quantities of nitrogen (N) in soil (Bărbulescu et al., 1991). The approximated amount of fixed N varied between 76 and 389 kg ha^{-1} in the various locations at global level (Taylor and Quesenberry, 1996). Detailed discussions regarding the *Rhizobium* relationships in red clover can be found in Burton (1985) and Taylor and Quesenberry (1996).

Experimental research has shown that the formation of root nodules and the symbiotic activity of molecular N fixation are negatively influenced when N is available in large quantities for leguminous plants (Streeter, 1988; Motcă et al., 1994; Dunea, 2006).

Experiences in controlled environments have pointed out that N reduces both the biomass of nodules on the plant and N fixation rates in relation with plant biomass or its roots (Boller and Nosberger, 1987; Hellsten and Huss-Danell, 2001; Carlsson, 2005).

In contrast to N fertilization, several researchers (Fedorova, 1984; Collins and Lang, 1985; Chalamet et al., 1987, cited by Taylor and Quesenberry, 1996) have reported the beneficial effect of molybdenum (Mo) on nitrogenase activity and the growth of forage yield. Mo is important in the metabolism of N in legumes. By ensuring Mo for the plant

growth process is often disproportionate to plant requirements for this microelement. Of the essential microelements, except for copper (Cu), Mo is found in most plant tissues in very small quantities. To compensate for Mo deficiency and to ensure effective Mo enzyme activity, foliar fertilization with this mineral element is often recommended (Dunea and Dincă, 2014).

The nitrogenase of the symbiotic bacteria enzyme is composed of two subunits, of which the MoFe protein is directly involved in the reduction of N_2 to NH_3 (Kaiser et al., 2005). In order to maintain the symbiotic activity in these species, the provision of Mo and iron (Fe) in bacteroids is a key regulatory component. The molybdates provided by the plant must cross the cell membranes of the node as well as the bacteroid membranes (external and internal) to reach the bacterial nitrogenous complex (Kaiser et al., 2005).

The control mechanism of the molybdates transport in the nodules is mostly unknown. However, a positive correlation was found between the development of nodules and Mo availability (Anderson, 1956). The tendency of legumes to maintain a high concentration of Mo in the nodules was experimentally determined (Hewitt and Bolle-Jones, 1952). Mo partitioning was predominantly directed towards the nodules and reproductive organs compared to othertissues of the plant (Gurley and Giddens, 1969, Franco and Munns, 1981, Ishizuka 1982; Giller, 1991, cited by Kaiser et al., 2005).

Nodules are believed to act as "substantial" reservoirs of Mo, but it is not known whether this is a consequence of the nitrogenase activity. Experiments on various species of legumes have shown that Mo fertilization substantially improves the symbiotic mechanism of molecular N fixation by forming larger nodules and increased nitrogenase activity (Kaiser et al., 2005).

The rationale of the study was to establish the influence of Mo on the biological efficiency of red clover in the presence or the absence of N fertilization and inoculation of *Rhizobium leguminosarum* biovar *trifolii*, and whether the results can lead to the improvement of classical crop technologies and the fertilization plan for obtaining superior forage yields. In this regard, some preliminary results were obtained in an experiment in which red clover plants were maintained for 60 days in agrowth chamber in order to determine the individual and combined effect of inoculation with *Rhizobium trifolii*, N and Mo fertilization on the morphological characteristics and the formation of nodulesin red clover.

There is a growing interest in reducing the quantities of chemical fertilizers as inputs in cropping systems (Dunea et al., 2014). The use of seed inoculation with strains has become a preferred practice especially in organic farming. Nowadays, there are numerous commercial products e.g., Nitragin Gold pre-inoculant from Monsanto BioAg.

The aim of the study was to determine the effect of applied treatments in controlled conditions on the following characteristics of red clover: *average plant height, average plant weight, average plant density and number of nodules on roots*. These variables have been considered as indicators of biological efficiency for yield formation.

MATERIALS AND METHODS

The experiments were carried out in controlled environment (Conviron plant growth chamber) using the Violetta diploid red clover cultivar. In order to assess the effects of treatments on the observed biological traits, the treatments were ranked in descending order, followed by the multiple comparison method i.e., the Duncan's multiple range test (DMRT).

The testing of the biological response of the plants comprised 8 variants showed in Table 1. The sowing was carried out with the same amount of seed in 5 L vegetation pots filled with sterilized perlite using 5 replicates. To ensure the nutrients needed for plants after emergence, the same amount of mineral fertilizer NPK (5-15-15) was supplied in all variants.

In the inoculation variants (5-8), the seeds were bacterized with *R. trifolii*-treated strains using the Nitragin® product (B type) using 7.5 g Nitragin/kg clover seed, dissolved in about 25 mL cold water.

After pre-homogenization of the seed in the resulting solution, it was sown with the same quantity in all the repetitions of the variants.

The bacterial solution contains at least 100 million viable cells g^{-1} of *R. trifolii*. Nitric fertilization was done with ammonium nitrate (33.5% N) supplied prior to sowing in an equivalent dose of 100 kg N ha^{-1}.

Table 1. Variants of the experiment with/without seed inoculation and fertilization with Nitrogen (N) and Molybdenum (Mo) in Violetta diploid cultivar

Variants without **Nitragin**® treatment	Variants with **Nitragin**®treatment
1 - no *Rhizobium* inoculation; Fertilization N + Mo	5 - *Rhizobium* inoculation; Fertilization N + Mo
2 - no *Rhizobium* inoculation; Fertilization Mo without N	6 - *Rhizobium* inoculation; Fertilization Mo without N
3 - no *Rhizobium* inoculation; Fertilization N without Mo	7 - *Rhizobium* inoculation; Fertilization N without Mo
4 - no *Rhizobium* inoculation; no fertilization	8 - *Rhizobium* inoculation; no fertilization

Molybdenum (0.02 Mo) was supplied in a single-dose using a uniform foliar sprayingwith three applications. Observations regarding the formation of nodules were performed after 6 weeks from plants' emergence, by immersing the roots in water followed by the estimation of the number of formed nodules. Proper nodulation involves the existence of specific formations on the pivotal root and lateral ramifications. After cutting the nodules, the color was assessed visually appreciating the effectiveness of the inoculation: white-green: inefficient; pink-red: efficient. The aboveground part of the harvested plants was dried in the oven at 80° C for 24 hours to determine the accumulated aerial dry matter (DM). The dried material was weighed with a Sartorius precision electronic balance.

The setup of the controlled conditions in the growth chamber was as follow:

Light - the light intensity was pre-selected on the cyclic control system providing 1200 µmoles/m^2/s by using PAR lamps; Temperature: +25° C ± 0.5°C in lights on period; Relative Humidity (RH): 85% on lights on ±5% RH.

StatGraphics Plus program was used to perform the statistical analysis of the results. We used the Duncan's multiple range test (DMRT), which is a multiple comparison procedure that uses the studentized range statistic q_r to compare sets of means.

The result of the test is a set of subsets of means, where in each subset means have been found not to be significantly different from one another (Duncan, 1955). DMRT is commonly used in agricultural research as a multiple comparison procedure. With this method, at the 99.0% confidence level, there is a 1.0% risk of calling one or more pairs significantly different when their actual difference equals 0. The response of the test is presented in box-and-whisker plots for each morphological trait. A plot is constructed in the following manner: the box is drawn extending from the lower quartile of the sample to the upper quartile; a vertical line is drawn at the median; a plus sign represents the location of the sample mean.

RESULTS AND DISCUSSIONS

The experimental results presented contrasting aspects regarding the correlation of the yield formation elements (weight, height, and density of clover plants) with the dynamics of the symbiotic nodule formation.

The classification of the variants in decreasing order (Table 2) allowed a preliminary overview of the effects of treatment on the considered biological indicators. The coefficients of variation (CV%) and the average of the five replicates of the indicators are presented within the group of treatments.

Depending on the ranking results, the positioning of each variant within the treatment group has provided useful information on the:

- yield elements (height and weight of clover plants): the variants 1, 7 and 5 were located at the upper end, in which nitrogen was supplied;
- nodule formation: variants 6 and 8 in which the seeds were bacterized and no nitrogen was applied;
- density: variants 4 and 1 without inoculation, without fertilization with nitrogen.

Table 2. Descending ranking of the variants after 60 days in controlled conditions (1-4 variants without *Rhizobium* inoculation; 5-8 variants with *Rhizobium* inoculation); CV – Coefficient of Variation

Number of variant	**1**	**7**	**5**	**2**	**6**	**8**	**3**	**4**
Average plant height(cm) CV = 30.79%	25.4	23.82	22.4	14.68	14.61	14.52	13.97	11.07
Number of variant	**1**	**7**	**5**	**8**	**3**	**2**	**4**	**6**
Average dry weight (g plant^{-1}) CV = 57.43%	10.43	7.79	6.22	4.58	3.21	2.87	2.79	2.36
Number of variant	**4**	**1**	**6**	**3**	**5**	**8**	**7**	**2**
Plant density (plants per pot) CV =13.68%	14.6	14	13.6	12.8	12.4	12.4	10.8	9.4
Number of variant	**6**	**8**	**7**	**4**	**1**	**2**	**3**	**5**
Number of nodules CV = 167.49%	22.72	21	3.33	2.3	0	0	0	0

Overall, the variants presented different values of the CVs, depending on the measured characteristic. If, in terms of plant density and average plant height, no significant amplitudes of CV% were found, at the level of nodule formation (167.49%) and dry matter accumulation (57.43%), a larger variability was noticed.

From the ANOVA test regarding the dry matter accumulation observations, it was found that the F value (20.16) showed a statistically significant difference ($p< 0.05$) between the results of the 8 treatments. The Duncan test at 99% confidence level was applied to highlight the significant differences between variants (Figure1). For plant height, the F value (31.71) of the sample showed a statistically significant difference ($p< 0.05$) between the averages of the 8 treatments. There is a close relationship between plant height and accumulation of dry matter, expressed by a statistically significant Pearson correlation coefficient (R = 0.9289; $p< 0.01$).

The 99%-level Duncan test determined statistically significant differences ($p<0.01$) in the case of the average height of red clover plants (Figure 2).

In terms of plant density as a result of application of treatments, no statistically significant differences were found using the Duncan test at 95%-confidence level (Figure 3), compared to variants 1-2 (+4.6 plants); 2-4 (-5.2 plants) and 2-6 (-4.2 plants).

The potential of symbiotic activity expressed by the *mean number of formed nodules* on the roots of individual plants was observed in variants 6, 7, 8 and very poor in variant 4. The other variants (1, 2, 3 and 5) did not present these formations specific to the symbiosis (Figure 4).

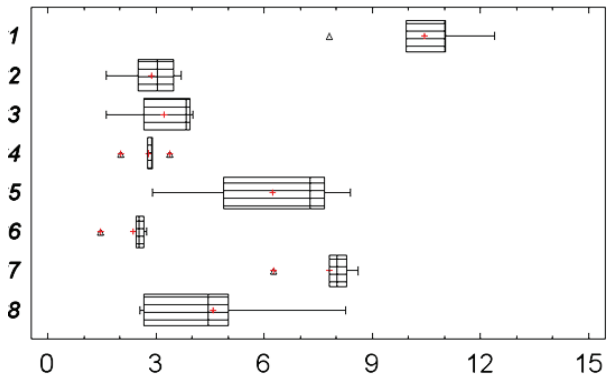

Figure 1. Results of Duncan test (99% confidence level) applied to the variant averages of dry weight showing the distribution on each variant among five repetitions (Y axe: 1-8 number of variant; X axe – grams/plant); vertical line of the box represent the median; the plus sign represents the location of the sample mean; * shows the significant differences between treatments

The response of the red clover plants to the inoculation with bacterial product was affected by the nitrogen fertilization. Consequently, the process of nodules' development was favorably influenced by the foliar fertilization with Mo in the absence of N (22.72 nodules) or in the absence of any fertilization (21 nodules).

The differences between these values were not statistically significant ($p> 0.05$). The use of nitrogen in the variants where the seeds have been bacterized caused a negative influence,

resulting the obstruction or elimination of the process of nodules formation. In variant (4) - without inoculation, and without fertilization there were nodules, although the seeds were not bacterized.

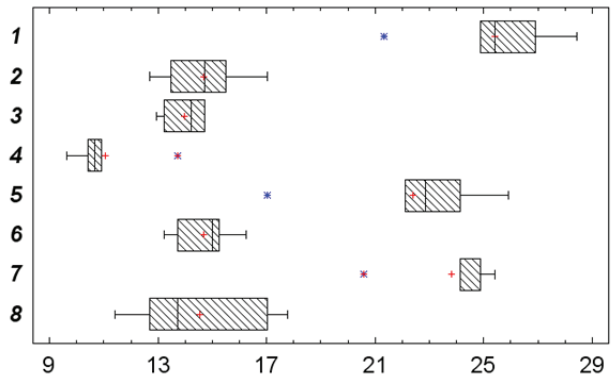

Figure 2. Results of Duncan test (99% confidence level) applied to the variant averages of plant height showing the distribution on each variant among five repetitions (Y axe: 1-8 number of variant; X axe – cm); vertical line of the box represent the median; the plus sign represents the location of the sample mean; * shows the significant differences between treatments

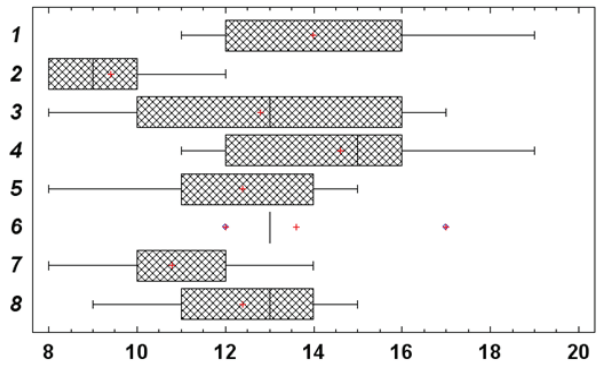

Figure 3. Results of Duncan test (95% confidence level) applied to the variant averages of plant density showing the distribution on each variant among five repetitions (Y axe: 1-8 number of variant; X axe – plants/pot); no significant differences between treatments were found

A possible explanation lies in the presence of native strains of the macrosymbiot existing on the seed coat that have somehow become active in the absence of fertilization.

The correlation test between the accumulated dry matter and the number of formed nodules indicated the lack of statistical significance (p> 0.05).

Mineral N treatment has provided a significant effect on the growth of red clover plants at the expense of the formation of symbiotic-specific nodules on the pivotal root and its lateral ramifications. The exception was variant 3, where nitrogen was supplied without foliar fertilization with Mo and without seed inoculation.

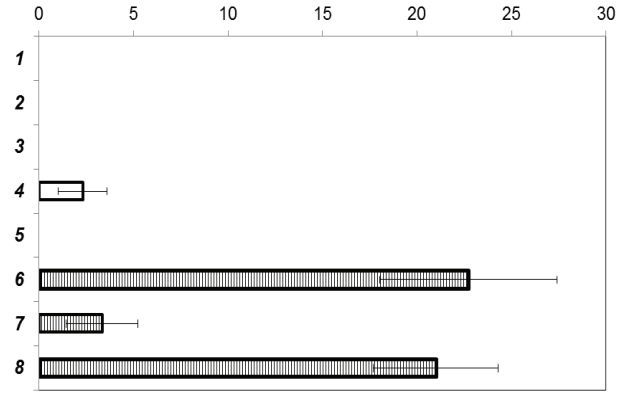

Figure 4. Effect of treatments on the number of nodules formed through symbiosis in each variant (variants 1, 2, 3 and 5 did not form nodules)

Compared to the results obtained by Fabian and Moga (1988), in which the mineral nitrogen variant (6.08 g DM pl^{-1}) exceeded the symbiotic-nitrogen variant (4.05 g DM pl^{-1}) by 50%, we have observed the dry matter accumulations presented in Table 3.

Our experimental results indicated values close to the previous experiment, namely 4.58 g DM pl^{-1} in the symbiotic-nitrogen variant and 10.43 g DM pl^{-1} in the variant of foliar fertilization with mineral nitrogen with molybdenum. The exclusive use of mineral N resulted in a low DM yield (3.21 g DM pl^{-1}).

Table 3.The influence of symbiotic and mineral nitrogen on red clover productivity in controlled conditions

Treatments	Inoculation with Nitragin®	
	g $pl.^{-1}$	%
Fertilization N + Mo	6.22	100
Fertilization Mo	2.36	100
Fertilization N	7.79	100
No fertilization	4.58	100
	Without Nitragin® inoculation	
Fertilization N + Mo	10.43	167.6
Fertilization Mo	2.87	121.2
Fertilization N	3.21	41.2
No fertilization	2.79	60.9

A non-productive consumption regime might occur in the N-fertilized variants, i.e., plants continue to absorb significant amounts of nitrogen over their requirements, quantities that were not reflected in yield increases.

CONCLUSIONS

The experiments performed in controlled conditions allowed the evaluation of the biological response of the red clover to various treatments considered to have a potential impact on the growth and development of the plants' canopy.
Seed inoculation, in the absence of fertilization, leads to relatively equivalent yields obtained from the mineral N fertilizer application.
Foliar fertilization exclusively using molybdenum did not provide significant yield increases, but when it was combined with nitrogen fertilization, it helped to achieve higher yields.
The "image" of the biological yield of plants excludes the aspects related to the nitrogenase activity that is responsible for the symbiotic nitrogen fixation.
From the yield formation point of view, the variants in which a large number of nodules were formed did not correlate positively with dry matter accumulation; however, they can have a particular importance in improving the reserves of assimilable nitrogen available in the soil.

REFERENCES

Anderson A.J., 1956. Molybdenum deficiencies in legumes in Australia. Soil Science81, p. 173-192.

Bărbulescu C., Puia I., Motcă Gh., Moisiuc Al., 1991. Cultura pajiştilor şi a plantelor furajere. Ed. Didactică şi Pedagogică, Bucureşti.

Boller B.C., Nosberger J., 1987. Symbiotically fixed nitrogen from field-grown white and red clover mixed with ryegrasses at low levels of ^{15}N fertilization. Plant and Soil. 104, p. 219-226.

Burton J.C., 1985. Rhizobium relationships. In: Taylor, N. L. ed. Clover science and technology. Madison, American Society of Agronomy, Inc. Crop Science Society of America, Inc., Soil Science Society of America, Inc.,p. 161-184.

Carlsson G., 2005. Input of Nitrogen from N_2 Fixation to Northern Grasslands. Doctoral thesis Swedish University of Agricultural Sciences Umeå.

Duncan D.B., 1955. Multiple range and multiple F tests. Biometrics 11, 1-42. doi:10.2307/3001478

Dunea D., 2006. Cercetări privind bioconversia energiei solare la *Trifolium pratense* L. în câmpia piemontană a Târgovişte. Teză de doctorat, Universitatea de Ştiinte Agronomice şi Medicină Veterinară din Bucureşti.

Dunea D., Iordache Ş., Pohoaţă A., Frăsin L.B.N., 2014. Investigation and Selection of Remediation Technologies for Petroleum-Contaminated Soils Using a Decision Support System. Water, Air& Soil Pollution, 225(7): p. 1-18.

Dunea D., Dincă N., 2014. Improving land utilization using intensive grass-clover mixtures in forage production systems. Romanian Agricultural Res., 31, p. 147-158.

Fabian I., Moga I., 1988. Influence of symbiotic and combined nitrogen on lucerne and red clover growth. Analele Institutului de Cercetări pentru Cereale şi Plante Tehnice Fundulea. 56, p. 391-400.

Hewitt E.J., Bolle-Jones E.W., 1952. Molybdenum as a plant nutrient. II. The effects of molybdenum deficiency on some horticultural and agricultural crop plants in sand culture. Journal of Horticultural Science, 27,p. 257-265.

Hellsten A, Huss-Danell K., 2001. Interaction effects of nitrogen and phosphorus on nodulation in red clover (*Trifolium pratense* L.). Acta Agriculturae Scandinavica. Section B, Soil and Plant Science, 50,p. 135-142.

Kaiser B.N., Kate L. Gridley, Joanne Ngaire Brady, Phillips T., Tyerman S.D., 2005. The Role of Molybdenum in Agricultural Plant Production. Annals of Botany, 96(5),p. 745-754.

Motcă Gh., Oancea I., Geamănu L.I., 1994. Pajiştile României, tipologie şi tehnologie. Ed. Tehnică Agricolă, Bucureşti.

Streeter J., 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. CRC Critical Reviews in Plant Sciences, 7, p. 1-23.

Taylor N.L., Quesenberry K.H., 1996. Red Clover Science. Kluwer Academic Publishers, Dordrecht.

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*** StatGraphics Plus: http://www.statgraphics.com/.