IN VITRO CULTURE OF RED CLOVER (*TRIFOLIUM PRATENSE* L.) AND ALFALFA (*MEDICAGO SATIVA* L.) ON NITROGEN FREE MS MEDIUM

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Abstract: *Legume seed and forage species are valuable feed plants that have been providing significant contributions to agricultural and animal production. In vitro culture of plant cells, tissues or organs on a medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics. The technique has also been effectively utilized to induce tolerance which includes the use of some selective agents that permit the preferential survival and growth of desired phenotypes. For this study we chose two varieties of Trifolium pretense L. (Select 2 and Rotrif) and two of Medicago sativa L. (Mihaela and Mădălina). Healthy seeds were selected for in vitro germination on agar medium and then transferred to nitrogen-free MS (Murashige and Skoog, 1962) medium to compare the growth rate between clover and alfalfa and which species resisted better on this medium. The biological material taken under study consisted of four varieties and for each variety was analyzed 30 seeds and was transferred into tubes passed into the growth chamber. First, the seeds were sterilized and then the germination rate of the four varieties studied was monitored. Rotrif variety had the highest rate of germination followed by Mihaela alfalfa variety. After three days from the inoculation, the seedlings formed were transferred to MS medium without nitrogen and also the growth rates were analyzed and what variety resist better on this medium was established. Between the four varieties there is no difference, however, the best development during the entire period was in the case of Rotrif variety, followed by cultivar Select 2. A slight increase was observed in Mădălina variety but as a general conclusion both varieties of red clover have resisted better than those of alfalfa.*

Key words: *in vitro, red clover, alfalfa, seeds, germination, medium*

INTRODUCTION

Red clover (*Trifolium pretense* L.) is one of the most cultivated forage legumes in the world (BARRETO DIAS *et al*. 2008) and is a valuable feed plant, providing significant contributions to agricultural and animal production (ÇÖLGEÇEN *et al*. 2008). Leguminous plants have a reputation for maintaining soil fertility as assimilate atmospheric nitrogen through symbiotic bacteria at the nodules on the roots of these plants. The soil bacteria *Rhizobium leguminosarum* bv. *trifolii* is capable of symbiotic interaction with the host plant *Trifolium* spp. (clover) (WIELBO *et al*. 2010). Alfalfa (*Medicago sativa* L.) is a perennial forage legume of great agronomical interest that produces indeterminate nodules upon infection of roots with *Sinorhizobium meliloti* (NAYA *et al*. 2007). Alfalfa and clover are examples of important legumes which are used in pastures for forage production (DANSO AND ESKEW 1984) and through atmospheric nitrogen fixation they can increase the production in a natural way of plants included in crop rotation.

The production and productivity of leguminous plants continues to be adversely affected due to various biotic and abiotic stresses. Most common abiotic stresses that affect both legumes and bacteria are: salinity, extremes temperatures, drought, heavy metals and nutrient imbalance. Abiotic stresses can occur due to continue changes of environment and

inappropriate agricultural practices. Damages caused by these stresses are responsible for enormous economic losses worldwide (RAI *et al*. 2011).

Nitrogen (N) is an element essential for the support of all forms of life. It is found in amino acids and proteins and many other organic compounds are derived from the nitrogen fixation process (BAGALI SHRIDHAR 2012). Successional changes in the availability of nitrogen have received particular emphasis, in part because nitrogen often limits primary production in terrestrial ecosystems. Generalizations concerning successional patterns of nutrient availability or nitrification can be tested empirically and many measurements of nitrogen availability and transformations during ecological succession have been reported (VITOUSEK *et al*. 1989).

Nutrient imbalance may result from the effect of salinity on nutrient availability, uptake, or partitioning within the plant or may be caused by physiological inactivation of a given nutrient, resulting in an increase in the plant's internal requirement for that essential element (GRATTAN AND GRIEVE 1993). Different abiotic stress factors may provoke osmotic stress, oxidative stress and protein denaturation in plants, which lead to similar cellular adaptive responses such as accumulation of compatible solutes, induction of stress proteins, and acceleration of reactive oxygen species scavenging systems (HAMDIA AND SHADDAD 2010).

Plant breeding of perennial forage legumes allows the use of *in vitro* cultures for the selection and multiplication of productive genotypes resistant to stress (SAVATTI 2003). *In vitro* cultures have an important role in plant breeding programs, allowing selection and multiplication of productive lines resistant to biotic and abiotic (VICAȘ GABRIELA 2009). *In vitro* culture of cells, tissues or organs on a medium containing selective agents has the ability to select and regenerate plants with desired characteristics. The technique has been used effectively for the induction of tolerance which includes the use of substances that allow the selective survival and growth of desired phenotypes (RAI *et al*. 2011).

Modern biotechnologies represent a revolution regarding the improvement techniques, with their help new genotypes with high performance features, free of viruses, can be produced. This technique can improve the health of the culture and thus the agricultural environment, because there is no need for a series of treatments, moreover expensive, by means of which maintaining a healthy crop. Multiplication modern techniques helps preserve the biodiversity heritage of plant biology and conservation of germplasm.

Successful cultivation of *in vitro* explants depends on the composition of the culture medium and to achieve those nutrient mixtures, corresponding to the vital need of inoculated tissue, it is very important in order to compensate for the lack of the most important nutrients. Therefore, all culture media have a complex composition, an issue that was researched since the first studies on the cultivation of "*in vitro*" plant explants, of WHITE GAUTHERET, HELLER and continued nowadays by MURASHIGE, SKOOG, ERIKSSON, NITSCH, GAMBORG etc. *In vitro* practice implies an aseptic environment. Bacteria and fungi invades *in vitro* culture in a very short time if not ensure sterility space shuttle vessels culture, environment, tools and biological material (SAND CAMELIA 2007).

In vitro culture of plant cells, tissues or organs on a medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics. The technique has also been effectively utilized to induce tolerance which includes the use of some selective agents that permit the preferential survival and growth of desired phenotypes (RAI *et al*., 2011).

The objective of our study was to determine the effect of free nitrogen culture media on red clover and alfalfa but also to determine what specie is more resistant to the lack of nitrogen.

MATERIAL AND METHODS

Plant material

In this study, two species natural tetraploid *Trifolium pratense* L. with two varieties (Select 2 and Rotrif) and *Medicago sativa* L. also with two varieties (Mădălina and Mihaela) were used.

Sterilization and culture conditions

Seeds from clover and alfalfa were selected for this experiment. First the surface of the seeds was desinfected in a solution of 70% sodium hypochlorite and 30% sterile water for 15 minutes (commercial sodium hypochlorite was used in sterilization process). Then seeds were rinsed 3 times in sterilized water. After sterilization process seeds were germinated on agar medium and germination was controlled every day. The seeds were germinated aseptically on plain agar medium in screw-capped bottles. Three days after germination, seedlings were incubated on nitrogen-free MS medium (MURASHIGE AND SKOOG, 1962).

Statistical analysis

Differences among means were compared by Duncan's multiple-range test and Student test (option in Polifact soft).

RESULTS AND DISCUSSIONS

Seed germination

Healthy seeds (30 seeds/plant) were places on agar medium supplemented with sucrose (10 g agar, 4 g sucrose and distillate water 1000 ml). The medium was sterilized for 20 minutes at 121°C, after imbibition, germination started the next day. On the first day Rotrif variety had the highest rate of germination followed by Mihaela variety. In the second day Mădălina and Rotrif reach maximum germination capacity and the lowest germination was observed in Select 2. On the third day of observations all four varieties germinated fully (Figure 1).

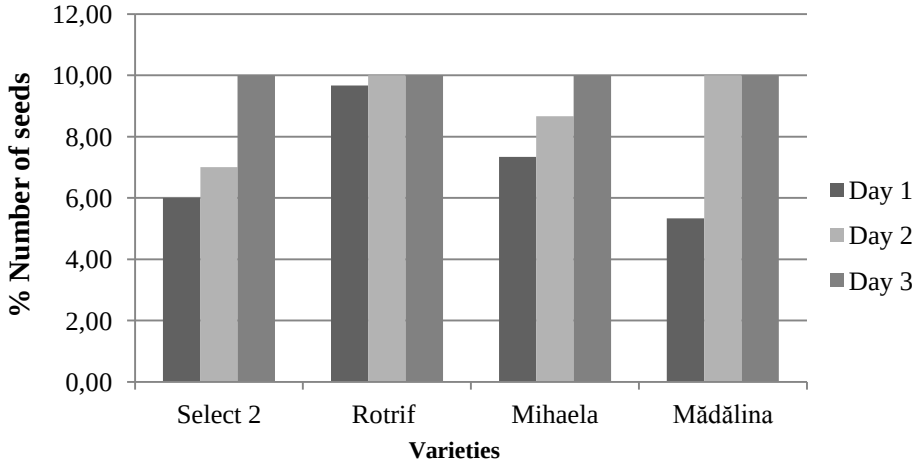

Figure 1. Seed germination on water agar medium

After third day of germination we transferred the seedling on nitrogen-free MS medium to compare the growth rate between clover and alfalfa and which species resisted better on this medium.

***In vitro* propagation**

From the data shown in table 1 the increase of seedlings did not record any difference between all four varieties taken under study, in the fifth day but at 10 days after the inoculation of the seedlings, only Rotrif (S2) variety showed significant positive differences to control.

After the first five days of acclimatization, a very good development was seen in the case of Rotrif (S2) variety, this recording a 2.73 cm height were a large number of plants already having developed the first true leaf. The maximum growth for this variety was reached at day 25 of examination at this stage all the plants had developed one or two true leaves. On the last day (day 30) leaves began to turn yellow and dried very few compared to other varieties and differences between the intervals are not considered significant.

Table 1

In vitro cultivation of red clover and alfalfa

Period	Variety	Value (cm)	% to control	Difference to control	Significance of the difference	Duncan test
Day 5	Average	2.40	100.0	0.00	Mt.	
	S1	2.31	96.2	-0.09	-	A
	S2	2.73	113.4	0.32	-	AB
	S3	2.28	94.7	-0.13	-	A
	S4	2.30	95.7	-0.10	-	A
Day 10	Average	3.38	100.0	0.00	Mt.	
	S1	3.45	101.9	0.00	-	CDEF
	S2	4.13	122.2	0.06	*	FGH
	S3	3.04	90.0	0.75	-	BC
	S4	2.91	86.0	-0.34	-	ABC
Day15	Average	3.70	100.0	0.00	Mt.	
	S1	3.94	106.6	0.24	-	EFGH
	S2	4.54	122.9	0.85	**	HI
	S3	3.22	87.0	-0.48	-	BCD
	S4	3.09	83.6	-0.61	-	BCD
Day 20	Average	4.01	100.0	0.00	Mt.	
	S1	4.31	107.4	0.30	-	GHI
	S2	4.90	122.2	0.89	**	I
	S3	3.51	87.4	-0.51	-	CDEF
	S4	3.33	83.0	-0.68	0	BCDE
Day 25	Average	4.04	100.0	0.00	Mt.	
	S1	4.60	114.1	0.57	-	HI
	S2	4.93	122.2	0.89	**	I
	S3	3.79	93.8	-0.25	-	DEFG
	S4	2.82	70.0	-1.21	000	ABC
Day 30	Average	3.01	100.0	0.57	Mt.	
	S1	3.99	132.4	0.00	**	EFGH
	S2	3.46	115.0	0.98	-	CDEF
	S3	2.35	78.1	0.45	0	A
	S4	2.24	74.5	-0.77	0	A
LSD (p 5%)				0.61		
LSD (p 1%)				0.81		DS 0.62-0.77
LSD (p 0.1%)				1.03		

On the 15 th, 20 th and 25 th day from the transplant on the nitrogen free MS medium, S2 variety maintains the higher growth rate, the differences recorded being statistically assured as distinct significant positive to control. At the opposite pole the weakest development was observed in the case of S4 which in the 25th day showed very significant negative differences to control.

Alfalfa (Mihaela) variety (S3) obtained, generally, insignificant differences compared to the average of the experience in most observation data. The exception is day 30 after transplanting, when the variety recorded significantly lower values than average.

Compared with varieties of red clover, alfalfa variety Mihaela (S3) generally recorded much lower values throughout the entire experiment.

Examination of alfalfa variety Madalina (S4) indicated a weaker accommodation to experimental factors compared to other varieties studied. Although, the differences from the average of experience were insignificant at 5, 10 and 15 days after transplanting and increases in length were placed below this average. At 20 days from the beginning of experiment, the variety Mihaela recorded a significantly lower value than average of experience and the same statistical assurance was observed at 30 days. At 25 days, the variety Mihaela recorded the lowest value compared to the average, the differences being statistically assured as very significant negative.

Of all the four varieties studied, the variety Madalina (S4) had the lowest development in the established experimental conditions, with an almost constant decrease from average of the experiment over the 30 days studied.

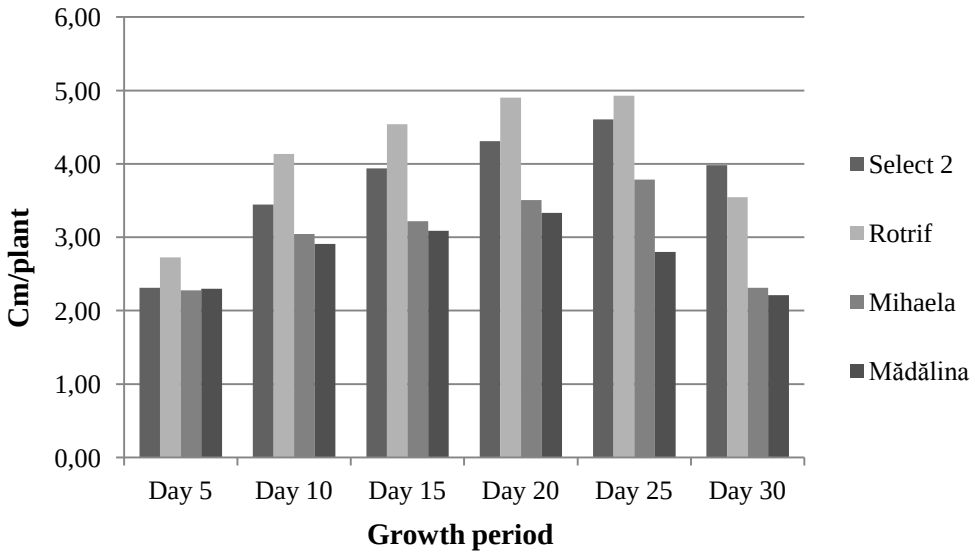

Figure 2. *In vitro* development of varieties Select 2, Rotrif, Mihaela and Mădălina

The Rotrif variety had a constant growth rate, the higher value was measured after 25 days from the inoculation on nitrogen free MS medium. This cultivar is followed by Select 2

and then Mihaela and Madalina (Figure 2). After 30 days from the inoculation we observed that the leaves were turning yellow and in the case of alfalfa even the stem was yellow to brown, which justifies the low values taken that day. In all the varieties taken under study after 30 days diseased plants were seen.

CONCLUSION

After analyzing the growth rates of the varieties studied *in vitro* on MS medium without nitrogen was observed that the variety with the best development was Rotrif, followed by variety Select 2. As a general conclusion, both varieties of red clover have held up better on this medium than those of alfalfa.

BIBLIOGRAPHY

1. BAGALI SHRIMANT SHRIDHAR, 2012: Review: Nitrogen fixing microorganisms, International Journal of Mucrobiological Research, 3(1), 46-52.
2. BARRETO DIAS PAULA, JULIER, BERNADETTE, SAMPOUX, J-P., BARRE, P., DALL'AGNOL, M., 2008: Genetic diversity in red clover (*Trifolium pretense* L.) revealed by morphological and microsatellite SSr (markers). Euphytica, 160, 189-205.
3. ÇÖLGEÇEN, H., BÜYÜKKARTAL, H. N., TOKER, M.C., 2008: In vitro germination and structure of hard seed testa of natural tetraploid *Trifolium pretense* L. African Journal of Biotechnology, 7(10), 1473-1478.
4. DANSO, S.K.A., ESKEW, D. I., 1984: Enhancing biological nitrogen fixation, IAEA Bulletin, 26(2), 29-34.
5. GRATTAN, S. R., GRIEVE M. CATHERINE, 1993: Mineral nutrient acquisition and response by plants grown in saline environments, Hanbook of plant and crop stress, 203-226.
6. HAMDIA, M. A., SHADDAD, M. A. K., 2010: Rewiew: Sapt tolerance of crop plants, Journal of Stress Physiology & Biochemistry, 6(3), 64-90.
7. NAYA, L., LADRERA, R., RAMOS, J., GONZALEZ, M. ESTHER, ARRES-IGOR, C., MINCHIN, R. F., BECANA, M., 2007: The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants, Plant Physiology, 144, 1104-1114.
8. RAI, M. K., RAJWANT, K. KALIA, SINGH, R., GANGOLA, M. P., DHAWAN, A. K., 2011: Developing stress tolerant plants through *in vitro* selection- An overview of the recent progress, Environmental and Experimental Botany, 71, 89-98.
9. SAND CAMELIA, 2007: Identificarea prin metode biotehnologice de noi resurse genetice rezistente la seceta si aciditate la plantele furajere perene de pajisti in scopul valorificarii suprafetelor afectate de aceste fenomene, Universitatea „Lucian Blaga" Sibiu, Contract de finanțare pentru executie proiecte.
10. SAVATTI, M., M. SAVATTI JR., MUNTEAN L., 2003: Ameliorarea plantelor- teorie și practică, Ed. AcademicPres, Cluj-Napoca.
11. VICAȘ GABRIELA, 2009: Cercetări privind influența culturii și mutagenezei *in vitro* în vederea obținerii de variații utile pentru procesul de ameliorare la trifoiul roșu (*Trifolium pretense* L.), PhD Thesis.
12. VITOUSEK, M. PETER, MATSON, PAMELA AND KEITH VAN CLEVE, 1989: Nitrogen availability and nitrification during succession: Primary, secondary and old-field seres, Plant and Soil, 115, 229-239.

13. WIELBO, J., MAREK-KOZACZUK MONIKA, MAZUR, A., KUBIK-KOMAR AGNIESZKA, SKORUPSKA ANNA, 2010: Genetic and metabolic divergence within a *Rhizobium leguminosarum* bv. *trifolii* population recovered from clover nodules, Applied and Environmental Microbiology, 76(14), 4593-4600.