AMELIORATION AND CONSERVATION OF *LOTUS CORNICULATUS* L. SPECIE, "*ALINA*" VARIETY THROUGH UNCONVENTIONAL TECHNIQUES IN ORDER TO OBTAIN VALUABLE GENOTYPES

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Abstract

It is known the implication of plant biotechnology in the amelioration of the species with economic value, these unconventional methods proved they can ensure the obtaining of some genotypes with superior biological value and can improve or even remove some deficiencies due to the specie's biology. Lotus corniculatus L. specie has a high adaptive capacity to the climate conditions, but it is known as having reduced capacity to form seed. Hence was born the idea of finding the method of in vitro culture which to bring an added advantage to the culture of the bird's-foot trefoil. There was initiated a comparative study for a period of two years by testing three types of tissues: apex, nod and young bud harvested from plants in the field and cultivated on LS medium with a few variants. There were analyzed the in vitro evolution of the tissues in terms of organogenesis (regeneration, multiplication), the nodosities differentiation as well as the aspects related to the forcefulness and the physiological changes occurred at the plants' level. After 60 days the superiority of the apex was established, followed by the bud and then by the nod: the apex reaches a regeneration percentage of 95% on V_1 (LS + 0.5mg/l AIB + 2mg/l Z) and of 90-82% on the other variants and only 5% on the control sample (V_0); the bud regenerates 85% on medium V_2 (LS + 0.5mg/l AIB + 5mg/l EGP) and on V_1 80%; but the nod has an inferior evolution on all variants. Nodosities form only after 80 days and just from apical tissue and floral bud (only on V_1 and on V_2): in the presence of zeatin (V_1) are formed about 12nodosities/plantlet and in the presence of the corn extract (V_2) about 7 nodosities: from the bud is formed a small number of 3-4 nodosities/explant (on the same variants). The forcefulness of the new plantlets is superior on V_1 generated from the apex to which some changes occur concerning the number of the leaves and their size (Photo. 4-5). In the presence of EGP (V_2) the bud has a good forcefulness, the physiological changes noted are also related to the number of leaves, that is much higher in the presence of zeatin, but the surface of the leaves is smaller (Photo. 3). We can conclude that the technique of in vitro cultures ensures obtaining valuable genotypes at the bird's-foot trefoil through a number of differentiated nodosities and through a superior foliar mass, in the presence of zeatin and of the natural extract of corn germ.

Key words: *Lotus corniculatus* L., unconventional *in vitro* technique, apex, nod, bud

INTRODUCTION

Lotus corniculatus L. specie has its center of origin in the Mediterranean area, but the small quantity of seed obtained even in this area prevents the spread of the specie (Seaney et. al., 1975), still the bird's-foot trefoil is considered in some states in Europe the most important forage plant, in England it stands next to lucerne and red clover (Borsos, 1976). Here it was created the first Romanian variety of the bird's-foot trefoil in 1980s when the production technology of the seed with a superior biological value developed (Dragomir, 1982), being cultivated in pure culture and in mixture (Dragomir, 1992). It has the widest spreading areal, the bird's-foot trefoil being considered a cosmopolite plant (Varga et. al., 1998), whose importance consists in the fact that it can replace lucerne and clover in some areas less favorable (Savatti et. al., 2004). Even though less productive than lucerne, clover has some favorable features, being considered a less roughage forage, and due to the large amount of green mass and to its nutritive qualities it is similar to clover and lucerne (Teodorescu, 1987). It is considered to have the highest adapting capacity to different climate conditions (Dragomir, 1993) and a high capacity of self-insemination (Savatti et. al., 2003). Selection value of the bird's-foot trefoil features and traits is given by the degree of variability of the specie (Nedelea and Madoşa, 2004); hence some populations or varieties have a great importance, given by the capacity of sprouting, the biomass production, etc. (Savatti et al., 2004). *Alina* variety was created at USAMV Cluj-Napoca and it is a variety with special performances and resistance to the environmental factors, cultivated especially in the favorable areas from Transylvania (Savatti et. al., 1994). Ameliorators had different objectives for obtaining some varieties of the bird's-foot trefoil with high productions of biomass, using different classical methods (Dragomir, 1998), to this effect applying today unconventional methods too (Cachiță, 1987). Those objectives were at the basis of our experiments of testing *in vitro* culture for obtaining genotypes with superior qualities at some perennial legumes (Savatti and Zăpârțan, 1991; Savatti et. al., 2000; 2003). *In vitro* culture of some agricultural species of economical importance represents in fact the involvement of biology in plant amelioration (Cachiță and Ardeleanu 2009), from classical methods of selection of species, to the use of automated robotic devices (robots) in obtaining the biomass (Cachiță, 2007).

Plant biotechnologies have many applications, but the best known are in the field of amelioration of valuable genotypes (Laslo, 2013), being frequently used in the conservation and amelioration of some autochthonous species and varieties valuable for agriculture (e.g. potato) for obtaining *in vitro* plant material. At the species and varieties from the forage legumes group *in vitro* culture was used for following the behavior of some varieties newly ameliorated in controlled culture conditions (Zăpârțan et. al., 1990; Zăpârțan et. al., 2003), or in studies on the stimulation of *in vitro* regeneration and development of a corresponding Radicular System (Köteles, 2011), and also of induction of genetic variability after applying the mutagenic factors (Savatti et. al., 2006; Zăpârțan et. al., 2006, Vicaş. 2008).

MATERIAL AND METHOD

"Alina" variety of the bird's-foot trefoil ameliorated at USAMV Cluj represented for us a study object in the elaboration of the PhD. Thesis (Köteles, 2013), also following the *in vitro* germinative capacity of the seeds (Köteles and Pereş, 2011; 2013), being known the fact that bird's-foot trefoil forms few viable seeds (Photo. 1) this fact being considered a lack of the specie (Dragomir, 1992). The present study is a complex research which adds new concrete data, concerning the regenerative capacity of some types of tissues (Köteles and Pereş, 2013) and establishes the implications of the unconventional *in vitro* cultures at "Alina" variety of the bird's-foot trefoil, with an ameliorative purpose.

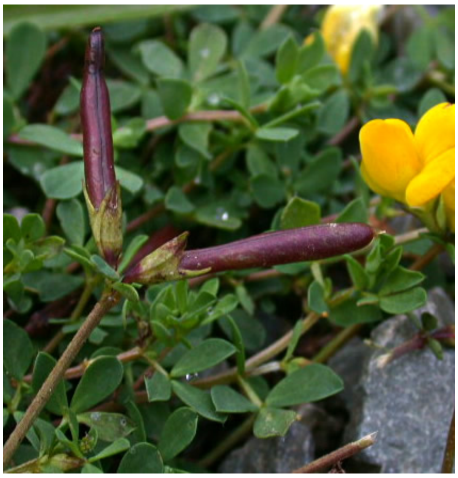

Photo. 1. Bird's-foot trefoil plant with seed

Photo. 2. Bird's-foot trefoil young plant donor of explants

The reaction of three types of explants was followed comparatively: *apex*, *nod* and *very young bud*, harvested from the field from plants of "Alina" variety of the bird's-foot trefoil (Photo.1) and cultivated on *Linsmaier-Skoog*, 1965 (LS) medium, with the following variants:

- **V_0** = LS (witness), macro, microelements and entirely added vitamins;
- **V_1** = LS + 0.5 mg/l AIB + 2 mg/l Z, with low dose of auxin and with high dose of zeatin;
- **V_2** = LS + 0.5 mg/l AIB + 5 mg/l EGP with corn germs without hormones;
- **V_3** = LS + 0.5 mg/l AIB + 80 mg/l $AdSO_4$ with sulfate adenine without hormones.

The mediums were conceived for testing other substances too, with stimulating effect, used alone (without phytohormones), being cheaper and handier than phytohormones, hereby attaining an economically advantageous culture. Concerning the results of the previous experiments (2010 – 2013) we tried to establish which is the best hormonal balance for the bird's-foot trefoil (the nature and concentration of the phytohormone), using other phytohormones (AIB and Zeatin), EGP and $AdSO_4$, alone into the composition of the medium.

RESULTS AND DISCUSSIONS

In vitro reaction of the bird's-foot tissue proved somewhat slower than to other species among forage legumes, being dependent on the type of explant and on the composition of the medium, this is why the effect of the culture was analyzed only after 80 days. *The followed parameters* were: differentiation of plantlets and roots (number/length); regeneration and multiplication percentage of the explants; differentiation of nodosities and observations concerning the forcefulness of the plantlets and of any physiological changes that occurred.

Following Table 1 we see differences in the evolution depending on the type of explant and on the composition of the medium. *The apex* proves the best evolution: the average of the number of differentiated plantlets/apex is the highest, of over 28 plantlets/apex on V_1 (LS + 0.5 mg/l AIB + 2 mg/l Z), hence in the presence of zeatin in a dose of 2 mg/l, with an average of about 14 roots/apex; and on V_2 (LS + 0.5 mg/l AIB + 5 mg/l EGP) the apex records a good evolution, generating about 20 plantlets/tissue with about 10 afferent roots, hence the extract of corn germ can successfully replace cytokinins; on V_o (LS), evolution is weak, generating about 2 plantlets, of about 2 cm long, and frail, with 1 – 2 weak roots/plantlet. In the order of evolution, there is *the bud* which has the best reaction on V_2 (LS + 0.5 mg/l AIB + 5 mg/l EGP) on which it differentiates about 15 neoplantlets/bud of about 1 cm height with 5 – 6 afferent roots, on the other variants there is an inferior evolution (Table 1).

Table 1

The evolution of the explants of the bird's-foot trefoil, "Alina" variety, within the in vitro organogenesis (the formation of plantlets and of a Radicular System, after 60 days)

Explant	Var.	Average number of Plants/length (cm)	Average number of Roots/length (cm)	EVALUATION
Apex	Vo	2 / 2.0	2 / 1.5	x
	V_1	**28 / 1.0**	**14 / 1.8**	**xxxxxx**
	V_2	20 / 1.0	10 / 1.2	xxxxx
	V_3	14 / 1.7	6 / 1.5	xxxx
Nod	Vo	1 / 2.6	1 / 1.5	x
	V_1	**10 / 0.6**	**7 / 1.8**	**xxxx**
	V_2	5 / 1.0	5 / 1.0	xxx
	V_3	4 / 1.0	5 / 1.0	xxx
Bud	Vo	2 / 2.0	2 / 1.8	x
	V_1	10 / 1.0	5 / 1.0	xxx
	V_2	**15 / 0.8**	**6 / 1.4**	**xxxx**
	V_3	7 / 1.2	5 / 1.0	xxx

The nod records a more modest evolution, the best being also on V_1 in the presence of zeatin, forming 10 plantlets/nod, with about 7 roots/plantlet.

There were not recorded major differences concerning the evolution of the length of the plantlets and roots between variants, being of about 0.8 – 1.0 cm on the variants with phytohormones and with additional additives ($V_1 – V_3$), in exchange on the witness (V_o) the length of the plantlets is almost double, comprised between 2.0 – 2.6 cm, a phenomenon explained by the fact that simple mediums (without hormones) determine the elongation of the internodes of the plantlets generated *in vitro* (Cachiță, 1987). The average roots' length is situated between 1.0 – 1.8 cm, without significant differences between variants (Table 1). The average of the number of plantlets and roots/explants which recorded differences in evolution was graphically represented (Fig. 1), from which we highlight the superior evolution of the apical tissue in the variants with hormones and additional additives, followed by the bud and by the nod.

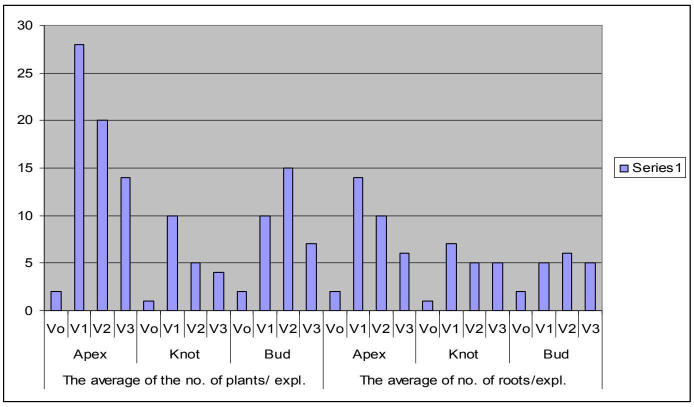

Fig. 1. *In vitro* organogenesis at the explants of *Lotus corniculatus*, "*Alina*" variety cultivated *in vitro* (after 80 days)

The capacity of regeneration, multiplication and differentiation of nodosities at the tissues of the bird's-foot trefoil cultivated *in vitro* is presented in Table 2 from which we can see the superiority of the apex on V_1 and V_2 on which regeneration is of 95% and 90%, and multiplication of 90% and 82%.

Favourable effects were recorded both on the medium with cytokinins (V_1) and on the medium with EGP (V_2) with small differences between variants. *The bud* records the best values on the medium with EGP (V_2), on which we have a regenerative capacity of 85% and 80% multiplication; on the medium with zeatin (V_1) the values being slightly lower; on the other variants the capacity of regeneration and multiplication is of about 60% and 50%, and on the witness sample of only 4% and 2%. *Nodal* tissue has a slower evolution, of 80% regeneration and 70% multiplication on V_1 and of only 55 – 40% on the other variants, on the witness (V_o) is weak, similar to the other explants. Table 2 reproduces the forcefulness of the plantlets of the bird's-foot trefoil obtained *in vitro*, highlighting the superiority of the medium with zeatin (variant V_1) and of the apical tissue.

In evaluating the forcefulness we also centered on the observations made on some physiological features visible with the naked eye at the new plantlets of the bird's-foot trefoil generated *in vitro* (number of leaves and their diameter).

Table 2

The percentage of in vitro regenerated and acclimatized explants at *Lotus corniculatus* L specie, "Alina" variety (after 80 days)

Explant	Var.	Regeneration %	Multiplication %	Number of nodosities/explant	Evaluation of forcefulness
Apex	Vo	5	3	0	x
	V_1	**95**	90	12 (> 2mm ø)	**xxxxxx**
	V_2	**90**	82	7 (> 1mm ø)	xxxxx
	V_3	82	75	0	xxxx
Nod	Vo	4	2	0	x
	V_1	80	70	1 (v. v. small)	**xxxx**
	V_2	55	51	0	xxx
	V_3	40	28	0	xxx
Bud	Vo	5	2	0	x
	V_1	80	70	3 (< 1mm ø)	xxx
	V_2	85	80	4 (< 1mm ø)	**xxxx**
	V_3	60	50	0	xxx

In Figure 2 is suggestively reproduced the regenerative and multiplication capacity of the three types of tissue detached from the young plants of the bird's-foot trefoil, appreciating that the values obtained on V_1 and V_2 are superior both to the apex and to the bud, and inferior to the nod. We observe the frail evolution of the tissues on the witness variant V_o at all types of tissues, what makes us appreciate that the success of the *in vitro* culture depends on the presence of the phytohormones in the medium, especially of the cytokinin (zeatin), of the natural extract of corn germs (EGP) and of the surplus of sulfate adenine.

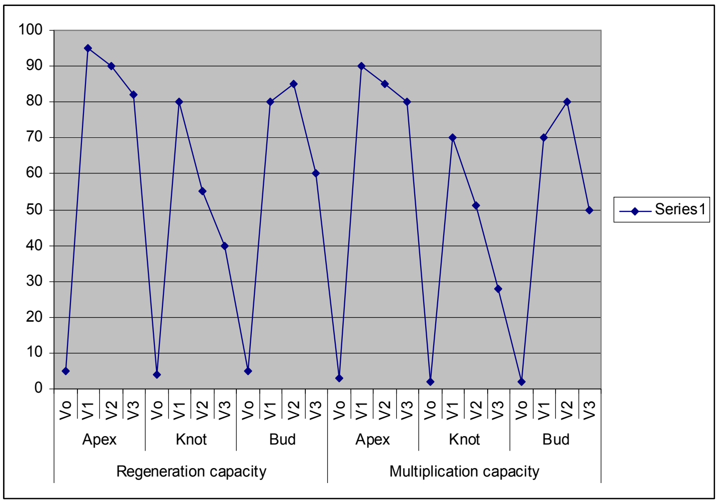

Fig. 2. *In vitro* regeneration and multiplication capacity of the explants of the bird's-foot trefoil, "Alina" variety (after 80 days)

It was followed the formation of nodosities after 80 days, appearance that was signalized only to the apical tissue and to the floral bud and just on V_1 and V_2: from the apex, in the presence of zeatin (V_1) there are forming about 12 nodosities and in the presence of EGP (V_2) about 7 nodosities which reach a diameter of about 12 mm/V_1 and 7 mm/V_2; from the bud there are forming a small number of nodosities 3 – 4 (on the same variants) with approximately the same diameter. From the nod there were no differentiated nodosities, but small lumps, barely perceptible, appeared on the roots on V_1 (Fig. 3).

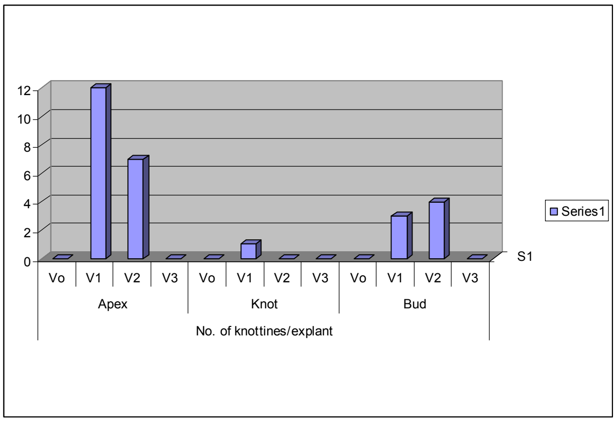

Fig. 3. Differentiation of nodosities on the roots of the bird's-foot trefoil, "Alina" variety obtained *in vitro*, depending on the explant and on the variant of medium (after 80 days)

Nodosities have a hoary color, are inserted on roots and have a diameter of ≥ 1 – 2 mm depending on the variant. Nodosities differentiation is graphically presented in fig. 3 from which we can also see, to this parameter, the superiority of the apex on the variants with zeatin (V_1) and with natural extract of corn germs (V_2), followed by the bud which generates nodosities on the same variants but fewer in number and smaller. Carefully observed, it was seen that neoplantlets with differentiated nodosities resisted better to acclimatization and without any losses.

The most *vigorous* plantlets formed *in vitro* are the ones on V_1, from apex on which there also appear some changes regarding the number of leaves and of their size (Photo. 4).

Photo. 3. Plantlets of the bird's-foot trefoil morphologically and physiologically unmodified

Photos 4 and 5 Morphological and physiological changes concerning the size of the foliar limb at the plants of the bird's-foot trefoil

In the presence of EGP (V_2) the bud has a good forcefulness; physiological changes signalized being related to the number of leaves, which is higher than in the presence of zeatin, but with the diameter of the foliar surface somewhat smaller (Photo. 5). These changes made possible the subsequent research comprised within a study concerning the quantitative and qualitative values of the fresh and dry biomass obtained on each variant.

CONCLUSIONS

The success of a classic amelioration program reports itself directly to the sources of the genetic variability that we have. An important step in the culture of the bird's-foot trefoil was realized through the creation of the genetic variability by using biotechnologies.

The techniques of *in vitro* induction and selection offer the possibility of achieving selection in controlled conditions and of isolating some new characteristics of the formed plantlets.

For the *in vitro* regeneration and multiplication there were used aseptic consecrated mediums, to which there were brought some corrections. The plant material used was *apex, nod* and *bud* harvested from the field from *Lothus corniculatus* L. specie, "Alina" variant; the evolution of the tissues depended on the nature of the explant, on the composition of the medium and on the followed objective.

The apex generates about 28 plants with about 14 roots/apex on V_1 (LS + 0.5 mg/l AIB + 2 mg/l Z) and 95% regeneration; a good evolution also takes place on V_2 (LS+ 0.5 mg/l AIB + 5 mg/l EGP), of about 20 plantlets with about 10 roots/explant and 90% regeneration; the other variants have an inferior reaction.

The bud evolves well in the presence of EGP (on V_2), differentiating about 15 plantlets with about 6 roots/bud and 85% regeneration; on the other variants evolution is slow; *the nod* also reaches a good evolution on the medium with zeatin (V_1) about 10 plantlets with about 7 roots, with the regeneration of 80% of the explants (an inferior evolution with regard to the other explants).

The presence of the extract of corn germs in the LS medium can substitute the phytohormones necessary for the *in vitro* regeneration: on V_2 (LS + 0.5 mg/l AIB + 5 mg/l GP), the analysed parameters reach good values.

Sulfate adenine in a concentration of 80 mg/l (V_3) has a beneficial effect similar to cytokinin, but inferior to the variants with zeatin (V_1) and EGP (V_2). We recommend economical mediums for the *in vitro* multiplication of the bird's-foot trefoil (with small doses of cytokinins or even without, but with additional additives and natural extracts).

In conclusion, the technique of the *in vitro* cultures of tissues ensures the obtaining of valuable genotypes at the bird's-foot trefoil by the number of differentiated nodosities and by a superior foliar mass, statement also proved by the values of the (fresh and dry) biomass.

The adaptation stage of the plantlets of the bird's-foot trefoil formed *in vitro*, at "Alina" variety is favorable, with differences depending on the presence of the nodosities and on the forcefulness of the new plantlets.

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