

## EFFECT OF ADENINE SULFATE ( $\text{AdSO}_4$ ) ON THE IN VITRO EVOLUTION OF WHITE CLOVER VARIETY (*TRIFOLIUM REPENS* L.)

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### Abstract

We tested the effect of adenine sulfate on the in vitro behavior of the explants from *Trifolium repens* L bud. After 40 days of in vitro culture observations were made on the regeneration percentage of the explant, on the number of generated neoplantlets and on their length, on the differentiation of the root system and last, but not least on the formation of nodules on the roots. Adenine sulfate in the medium has generated the formation of a greater number of neoplantlets, when it is associated in the medium with cytokinine. The experiment has shown the fact that from cytokinine, benzyl adenine (BA) with adenine sulfate in low dose of 1 mg/l has stimulated a good regeneration percentage (80%), with a balanced number of plants (about 8 plants/explant), but also the formation of nodules along the root system, much thickened as in the absence of adenine sulfate. The best variant proved to be  $V_6$  ( $\text{MS}+1.0\text{mg/l Z} +0.1\text{mg/lAIA}+40\text{mg/lAdSO}_4$ ) containing zeatine which stimulated the parameters under the most balanced values. Noteworthy is the effect of adenine sulfate on the callus and nodules differentiation, in the presence of 1mg/l dose of zeatine or benzyl adenine.

**Key words:** *Trifolium repens* L, adenine sulfate, explants, flower bud, viability, multiplication, organogenesis, acclimatization.

### INTRODUCTION

Researchers in biotechnology and related improvements have manifested great interest over the time for the in vitro behavior of some species of economic value and for some species of perennial forage legumes (Varga, et al., 1998, Savatti et al., 2006), and also for obtaining mutations in vitro by using some chemical mutagens (Zăpârțan M., et al., 2006), useful for agricultural production. Of great interest and applicability is the using of chemical mutagens in cultures of perennial forage legumes and especially the white clover (Gliga, G., et al 1993). *Trifolium repens* L (white clover) through its fodder qualities, through its agro biological particularity and through the high degree of using in all stages of development, is a valuable specie of great economical interest (N. Dragomir, 1997). Over time, breeders have been interested in the in vitro behavior of the white clover variety and in its response to certain hormonal compounds, following its capacity of regeneration, multiplication and of obtaining new in vitro mutations, in controlled conditions (Phillips, G. C., G.B. Collins, 1984).

The use of additional additives in the culture medium of forage legume species has proved to be beneficial especially at the species that have a difficult reproduction in vitro, in different doses stimulating the growth and the development of some plant tissues, with results dependent on the substance concentration, on the nature of the specie and on the presence of some phytohormones in the medium. Additional additives used in culture media have proved their efficiency in the regeneration of some species of plants reluctant or non-responsive in the aseptic medium of life (M., Ziv., and Halevy, A. H., 1983; V.M Kulcarni, et al., 2007). The implications of those additives have also been followed at the red clover (Vicaş, 2009), either alone or in combination with some phytohormones, in a much smaller dose, in order to highlight the effect of the supplemented substance (Vicaş, 2010).

The effect of adenine sulfate is known in the tissue cultures at many plant species and types of vegetal tissues, effect that is superior in combination with a balanced dose of cytokinin and auxin (M. Zăpârţan, 2001). Also the introduction of adenine sulfate in the culture medium at flower bulb species has stimulated callus differentiation in some combinations and of bulbs in other (M. Zăpârţan, et al., 1999-2000). The present paper has followed the effect of adenine sulfate on the culture in vitro of the explant of *Trifolium repens* L bud, in combination with a cytokinin and an auxin.

#### **MATERIAL AND METHODS**

We have followed the in vitro reaction of *Trifolium repens* L bud, on media with adenine sulfate, supplemented in the basic medium (MS), with the following composition: macro elements, microelements and FeEDTA – MS; mesoinositol – 100mg/l; thiamine HCl, pyridoxine HCl and nicotinic acid 1 mg/l; sucrose – 30 g/l; agar – 7 g/l; pH=6,1. To this medium considered basic medium (MS), were added phytohormones: benzyl adenine, zeatin (in two concentrations), indolil acetic acid (one concentration) and adenine sulfate in a concentration of 40mg/l.

Medium pH was adjusted to 5,7 before its autoclaving at 1 atmosphere, at a temperature of 120°C, for 20 minutes. Medium variants conceived for this experiment are presented in table 1.

Table 1

The culture medium used to the *in vitro* culture of the *Trifolium repens* L specie

No. crt.	Var.	The basic medium	BA mgs/l	Z mgs/l	AIA mgs/l	AdSO <sub>4</sub> mgs/l
1.	V <sub>0</sub>	MS	-	-	-	-
2.	V <sub>1</sub>	MS	1.0	-	0.1	-
3.	V <sub>2</sub>	MS	1.0	-	0.1	40mgs/l
4.	V <sub>3</sub>	MS	2.0	-	0.1	-
5.	V <sub>4</sub>	MS	2.0	-	0.1	40mgs/l
6.	V <sub>5</sub>	MS	-	1.0	0.1	-
7.	V <sub>6</sub>	MS	-	1.0	0.1	40mgs/l
8.	V <sub>7</sub>	MS	-	2.0	0.1	-
9.	V <sub>8</sub>	MS	-	2.0	0.1	40mgs/l

(MS = the medium after Murashige-Skoog - 1962; Z. = zeatine; BA = benzyl adenine; AIA = indolylacetic acid; AdSO<sub>4</sub> = adenine sulfate)

## RESULTS AND DISCUSSIONS

The observations were made after 40 days of *in vitro* culture, following the evolution of the bud *in vitro* on media with or without adenine sulfate, following: the regeneration percentage of explants *in vitro*, the neoformation of plants (their number and their length), the differentiation of the root system (number, length, thickness), the formation of nodules on roots and callus differentiation. The results of the analyzed parameters after 40 days from inoculation on the 8th variants of medium are included in table 2.

Table 2

Effect of adenine sulfate on *Trifolium repens* L specie cultivated *in vitro*

Var.	% of regeneration	Neoplantlets		Root system		Observations (nodules/callus)
		No.	L(cm)	No. of roots	L (cm)	
V <sub>0</sub>	35	2,5	8,5	2,5	0,9	-
V <sub>1</sub>	52	5,2	4,2	3,5	1,9	-
V <sub>2</sub>	<b>80</b>	8,0	4,0	4,0	1,5	3-4 nodules
V <sub>3</sub>	50	17	4,0	5,0	2,8	-
V <sub>4</sub>	<b>90</b>	<b>20</b>	3,0	3,0	2,0	Callus with Ø=4mm + small nodules
V <sub>5</sub>	<b>95</b>	25	2,5	5,0	3,0	3 small nodules
V <sub>6</sub>	65	28	2,8	4,0	4,0	<b>7 - 8 nodules</b> Well consisted
V <sub>7</sub>	<b>100</b>	33	3,2	5,5	3,5	Small callus + Small nodules
V <sub>8</sub>	70	<b>38</b>	2,0	-	-	Callus of Ø=7mm

Regeneration percentage has a value between 35 and 100%, depending on the medium variant and its composition. Witness sample reaches only 35%, and the one containing BA and AIB, 52%, while variants with BA

and a plus of adenine sulfate stimulates a regeneration of 80 – 90%. The highest percentage of 100% is obtained on media with zeatine in concentration of 2 mg/l (V<sub>7</sub>), without adenine sulfate, and 95% on medium with a concentration of 1 mg/l zeatine (V<sub>5</sub>), but on this variant the nodules and the callus differentiated at the level of roots are very small, in their diameter. From fig. 1 can also be seen the corresponding values of the regeneration percentage on the other experimental variants. The capacity of regeneration of *Trifolium repens* L specie and of the bud explant is remarkable, superior on media with phytohormones and adenine sulfate.

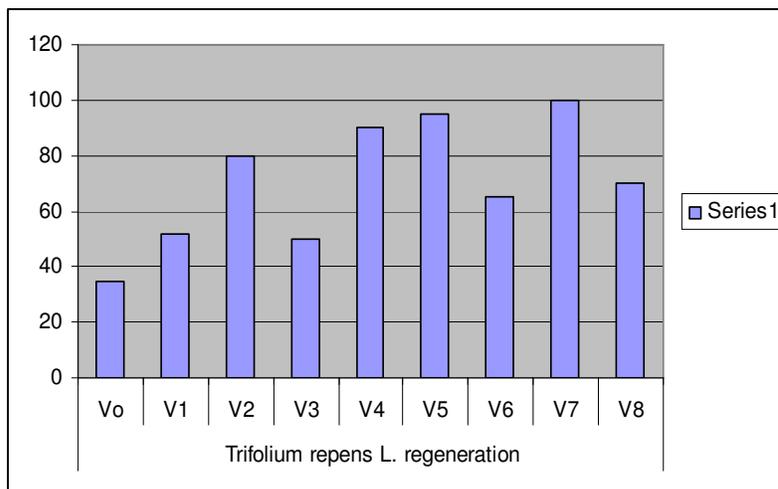


Fig. 1 The percentage of regeneration of the white clover bud cultivated on media with adenine sulfate

The number and the length of the new plants on variants with zeatine has reached an impressive number of about 25 – 38 neoplantlets/explant of 2,0-3,2 cm length. The number of plantlets on the witness sample (V<sub>0</sub>) is of only 2-3 plantlets/explants (due to the absence of phytohormones), very elongated (over 8 cm height) and fully compliant, being known the effect that a medium without hormones has on the elongation of in vitro differentiated plants' inter-nodules ( M. Savati et al., 2006).

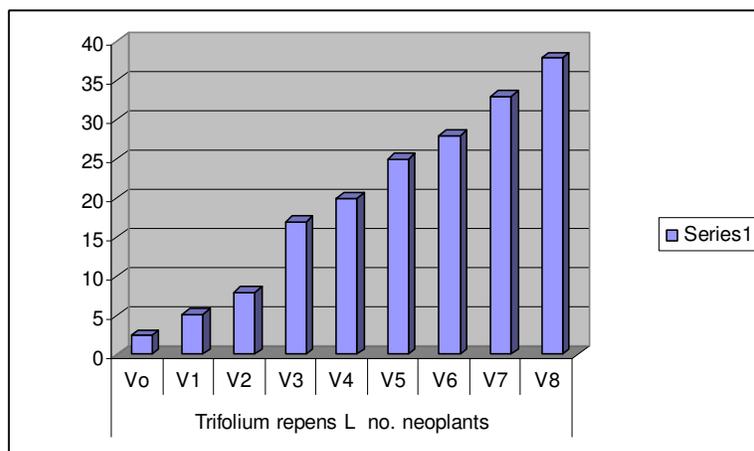


Fig. 2 The number of *Trifolium repens* L. neoplantlets differentiated from bud on experimented media

From fig. 2 and 3 we can see the number of differentiated neoplantlets and their length. We note the superior values of cytokinines in combination with adenine sulfate on the average number of clover plantlets. At a high dose of BA (2mg/l)+40mg/l Ad.SO<sub>4</sub>, there are formed about 20 neoplantlets/explant of about 3 cm length, and at the same dose of zeatine and adenine sulfate, the number is almost double (about 38 neoplantlets/explant), with a slightly smaller length.

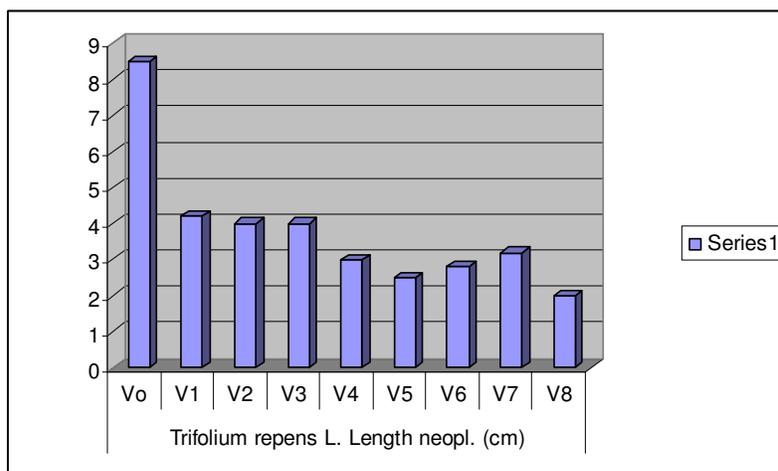


Fig. 3 The length of *trifolium repens* L. plantlets on media with AdSO<sub>4</sub>

The differentiated root system records a certain particularity. It is a first to see such an unprecedented thick of the roots, especially on variants with

adenine sulfate, thick that has stimulated the differentiation of callus and the appearance of nodules along the roots.

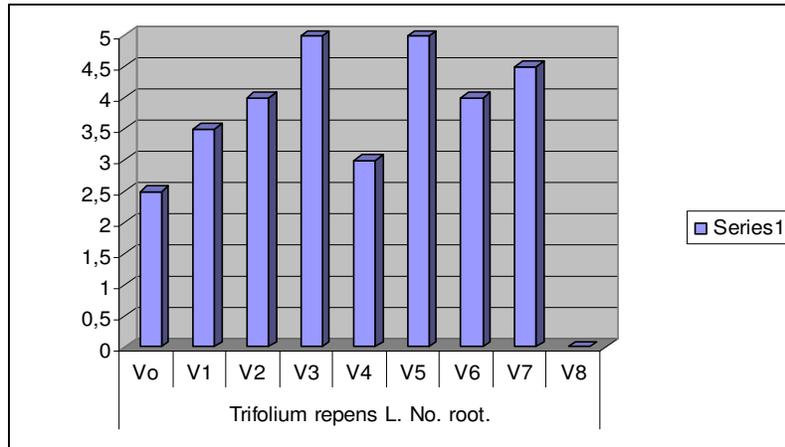


Fig. 4 The average number of differentiated roots on white clover plantlets cultivated in vitro

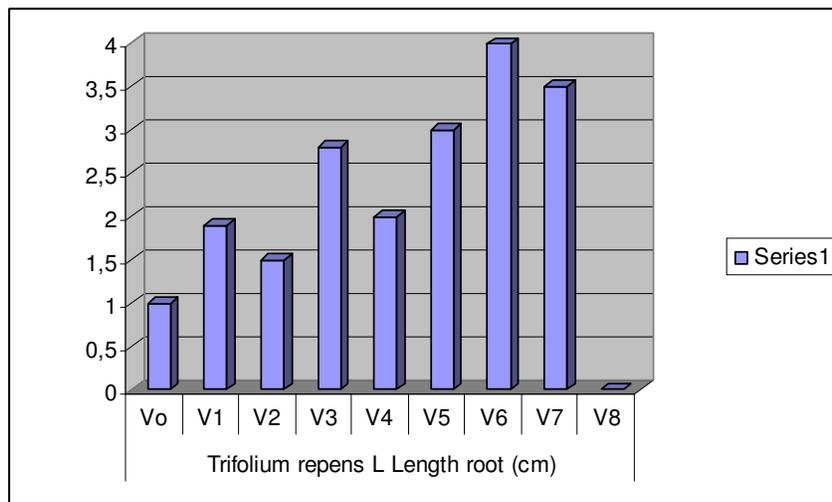


Fig. 5 The length of the root system at *Trifolium repens* L neoplantlets obtained in vitro

The highest number of roots and their length is recorded on the variants with cytokinin and adenine sulfate (Fig. 4). There were examples with a higher number of roots (see photo 1), but generally, the average was between 4 – roots/neoplantlet on the media with BA and adenine sulfate (of 1,5 – 2,8cm) and 4 – 5,5 roots/explant at the variants with zeatine and adenine sulfate and a length of about 4 cm (Fig. 5). Another absolute

premiere is that on V<sub>8</sub> with a high dose of zeatine and phytohormones the root system was inhibited, developing a mass of green callus with a diameter of over 7 mm.

The differentiation of nodules and callus. Table2 presents the values obtains concerning the differentiation of callus and the formation of the nodules. If on the witness sample and on the one with phytohormones there were no differentiated callus or nodules, on the other variants and especially in the presence of adenine sulfate there have formed 3 – 7 nodules (depending on the nature of the cytokinine) and callus of different diameters. The callus generated from the white clover explants represent a valuable material for the mutation induction at this specie, or in the works of transforming it into embriogenous callus, and finally in the plant regeneration from this tissue.

### CONCLUSIONS

1. Adenine sulfate introduced in the composition of the culture medium has a beneficial effect on the explant of white clover bud, in association with other phytohormones, especially cytokinine, obtaining superior values at the analyzed parameters.
2. The highest percentage of regeneration, of 100% was obtained on medium MS+2.0mg/l Z+1.0mg/l AIA (V<sub>7</sub>), in the absence of the adenine sulfate. But the higher dose of zeatine has stimulated the formation of a small callus on which there were differentiated only visible nodules.
3. The percentage of regeneration reaches values of 90% on medium with benzyl adenine, V<sub>4</sub>(2.0 mg/l BA + 0.1 mg/l AIA + 40 mg/l AdSO<sub>4</sub>). The high dose of BA associated with adenine sulfate has stimulated all parameters, but also the formation of callus at the bases of the explants.
4. The greatest number of nodules (7-8/explant) has formed on medium with 1.0mg/l Z + 0.1mg/l AIA + 40mg/l AdSO<sub>4</sub>(V<sub>6</sub>), proving to be the best, with a balanced evolution of the parameters: over 65% the regeneration, about 28 differentiated neoplantlets and a root system corresponding as a number, thickness and length.
5. We consider beneficial the presence of adenine sulfate in the culture medium of white clover, in combination with cytokinine (preferably zeatine) for the balanced and superior differentiation of the parameters.
6. The presence of adenine sulfate reduces the time of differentiation of the callus and of the nodules at half (they form only in 40 days), at other experiments over 70 days.
7. We recommend for the obtaining of callus at *Trifolium repens* L, a combination between a high dose of cytokinine (about 2 mg/l cytokinine + 0.1 mg/l auxine) and 40 mg/l adenine sulfate.

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