DOI: 10.22620/agrisci.2009.01.004

ВЛИЯНИЕ НА МУТАГЕННИ ТРЕТИРАНИЯ ВЪРХУ РАСТЕЖА НА КАЛУСА И РЕГЕНЕРАЦИЯТА ОТ ЛИСТНИ ДРЪЖКИ И КОРЕНОВИ ЕКСПЛАНТИ НА ФАСУЛ
INFLUENCE OF MUTAGENIC TREATMENTS ON THE CALLUS GROWTH AND REGENERATION BY LEAF PETIOLES AND ROOT EXPLANTS OF THE COMMON BEAN

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Резюме

Изследвано е влиянието на мутагенното третиране с етилметан сулфонат (ЕМС) и N-нитрозо-Nґ-етил карбамид (НЕК) върху три прехвърляния на хранителна среда на калус и регенерирали прораствци от експланти на корени и листни дръжки на 7-дневни растения. Калибрирани стерилни семена на българския сорт фасул Пловдив 11М са култивирани на основна MS среда, допълнена с 1 μM BAP. Третирането на експлантите с мутагените е извършено за 60 min в различни концентрации ($2.5 \cdot 10^{-2}$, $1.25 \cdot 10^{-2}$, $6.2 \cdot 10^{-3}$ М за ЕМС, и $6.2 \cdot 10^{-3}$, $3.1 \cdot 10^{-3}$, $1.55 \cdot 10^{-3}$ М за НЕК).

Мутагенните концентрации влияят върху растежа на калуса и регенерацията. При прилагане на най-ниските концентрации тези показатели се повишават. Третирането с НЕК, в сравнение с ЕМС, проявява по-силен ефект върху двата процеса. Прилагането на най-ниската концентрация на ЕМС ($6,2 \cdot 10^{-3}$ М) стимулира образуването на прораствци и растителната регенерация.

Установени са морфологични и хлорофилни промени (от типа *chlorina* и *viridissima*) в прорастъците и регенерантите, но не се развиват цели растения от тях. Ефектът на прехвърлянията на хранителна среда върху растежа на калуса е по-силно от това на мутагенните третирания. Взаимодействието между тези фактори е сравнително ниско.

Abstract

The influence of ethyl methanesulfonate (EMS) and N-nitrose-Nґ-ethyl urea (ENU) mutagenic treatments was investigated on three time sub-cultured calli and on regenerating shoots coming from roots and leaf petiole explants of 7-day old sterile plants respectively. Calibrated sterile seeds of Bulgarian the common bean variety “Plovdiv 11M” were pre-cultivated on MS basal medium supplemented with 1 μM BAP. Different concentrations of mutagens ($2.5 \cdot 10^{-2}$, $1.25 \cdot 10^{-2}$, $6.2 \cdot 10^{-3}$ M for EMS, and $6.2 \cdot 10^{-3}$, $3.1 \cdot 10^{-3}$, $1.55 \cdot 10^{-3}$ M for ENU) were applied for 60 min to the treated explants.

Mutagenic concentrations influenced both the callus growth and regeneration, these increasing at the lowest concentrations. ENU showed a stronger effect than EMS in both processes, while the lowest EMS concentrations ($6,2 \cdot 10^{-3}$ M) stimulated significantly shoot formation and plant regeneration.

Morphological and chlorophyll changes (*chlorina* and *viridissima* types) in shoots and regenerates were found but whole plants did not develop from them. The effect of subcultures on callus growth was higher than that of mutagenic treatments. Interactions between these factors were quite low.

Ключови думи: етилметан сулфонат (ЕМС), N-нитрозо-Nґ-етил карбамид (НЕК), *in vitro* култивиране, мутагени, *Phaseolus vulgaris* L.
Key words: Ethyl methanesulfonate (EMS), *in vitro* cultivation, mutagens, N-nitrose-Nґ-ethyl urea (ENU), *Phaseolus vulgaris* L.

Съкращения: БАП: 6-бензил-амино-пурин; ЕМС: етилметан сулфонат; ИБА: индолил-бутирова киселина; НОК: нафтил-оцетна киселина; НЕК: N-нитрозо-Nґ-етил карбамид; ТДЗ: N-фенил-N‘-1,2,3-тидиазол-5-карбамид [тидиазурон].
Abreviations: BAP: 6-Benzyl-Amino-Purine; *EMS:* ethyl methanesulfonate; *IBA:* Indole-Butiric-Acide; *NAA:* Naphtyl-Acetic-Acide; *ENU:* N-nitrose-Nґ-ethyl urea; *TDZ:* N-phenyl-N‘-1,2,3-thiadiazol-5-urea [thidiazuron].

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is one of the most important rich-protein legumes on which different breeding methods were applied to develop cultivars with improved traits. In the last years, scientific efforts were focussed on different aspects of investigations on common bean, such as seed hormonal balance [13], seed pre-cultivation on different *in vitro* culture media [5], study of the physiological status of the plant used as source of *in vitro* culture explants [19, 27], thin-cell-layer application on *in vitro* culture methods [5], etc. However, more efforts are still required to broaden genetic variability of the natural germplasm for stress resistance [25], adaptability to mechanical harvesting, earliness, and grain quality.

Mutagenesis combined with *in vitro* culture technique can provide a profitable methodology to increase the frequency of new genetic variations [3]. In this context, we aimed at performing our investigations.

Common bean Bulgarian variety Plovdiv 11M, comparing to other varieties, showed better abilities for *in vitro* cultivation (unpublished data). That is the reason why we choose it for our investigations.

Influence of ethyl methanesulfonate (EMS) and N-nitrose-Nr'-ethyl urea (ENU) mutagenic treatments was investigated either on three time sub-cultured calli or on regenerating shoots coming from roots and leaf petiole explants of 7-day old sterile plants, respectively. Mutagenic concentrations were applied for 60 min on the treated explants.

Treatment (mutagens or their concentrations) influenced either callus growth or regeneration. Morphological and chlorophyll changes in shoots and regenerates were found.

Combined with *in vitro* culture technique, mutagenesis can provide a profitable methodology to increase the frequency of new genetic variation [3], this including resistance to biotic and abiotic stresses. The following main advantages such as (i) production of large populations in a small space and in a short time; (ii) easy application of mutagens; (iii) facilitated identification of stress resistant mutants by sterile treatment procedures; (iv) increased chances to display mutants within regenerates [6], are accounted by using *in vitro* culture techniques. However, although several *in vitro* regeneration procedures were up now described [4, 8, 11, 12, 15, 16, 17, 18, 20, 26, 32, 33], their low efficiency still remains a problem limiting the use [2].

Exposition to the mutagenic treatment must be quite long on seeds [9, 24], while plant tissues (roots, steams or calli) have to be treated for a shorter time [22]. Mutant frequency differed also in dependence on the type of the material treated [24] but the key factor is mainly represented by the mutagen concentration or the irradiation dose, this latter being required quite low (2-5 Gy) for *in vitro* culture [1].

In literature, data concerning the effect of the mutagenic treatment on *in vitro* seeds or explants of common bean do not yet exist. Considering this aspect together with the possibility that mutagens can make genome more plastic after treatment, this also being positively reflected on plant regeneration, we aimed at studying influence of the mutagenic treatment either on callus growth or on regeneration of common bean genotypes. On this aspect, Svetleva *et al.* [30] established that 60-min may be considered as optimal time for the mutagenic application of EMS and ENU on leaf petiole and root explants of common bean.

MATERIALS AND METHODS

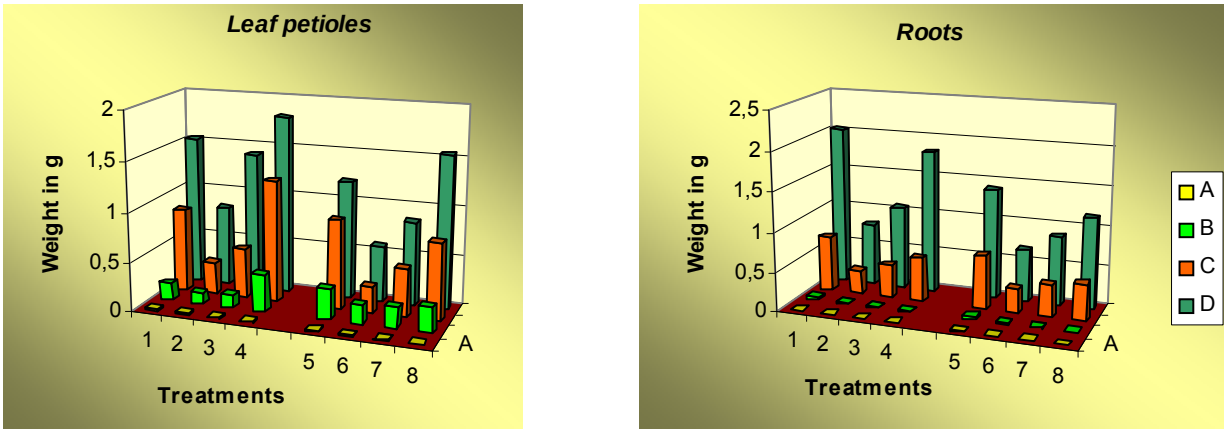
Calibrated seeds of Bulgarian common bean variety Plovdiv 11M were pre-cultivated on the basal MS medium [23] supplemented with 1mM BAP, according to the procedure proposed by Mok and Mok [21]. Roots and leaf petioles from 7-day old sterile plants have been used as explants for *in vitro* culture techniques aimed at obtaining proliferating and shoot regenerating callus, respectively.

To study the effect of a mutagenic treatment on callus growth as well as on regeneration ability, the mutagens EMS (ethyl methanesulfonate) and ENU (N-nitrose-Nr'-ethyl urea) were applied on root and leaf petiole explants for 60 min at the following concentrations: $2.5 \cdot 10^{-2}$, $1.25 \cdot 10^{-2}$, $6.2 \cdot 10^{-3}$ M for EMS and $6.2 \cdot 10^{-3}$, $3.1 \cdot 10^{-3}$, $1.55 \cdot 10^{-3}$ M for ENU. Both mutagens, ENU and EMS, were dissolved in buffers at pH 6 and pH 7, respectively, and solutions were cold sterilized through 0.45 mm Millipore filters. Then, explants were plunged under sterile conditions into the mutagen solutions. After mutagenic treatments, both root and leaf petiole explants were *in vitro* cultured on MSI_2 callus induction medium.

Proliferating calli from leaf petiole explants were then transferred on the media referred as MSE and MS_0 (MS medium without phytohormones). Shoot elongation was evidenced onto MSE medium in four weeks, after the third subculture, whereas both plant growth and rooting were established on MS_0 medium. Hormonal composition of the media utilized is described in Table 1.

Table 1. Hormonal composition of the media utilized ($mg \cdot l^{-1}$)

Components	Media	
	MSI_2	MSE
TDZ (N-phenyl-N`-1,2,3-thiadia-zol-5-urea [thidiazuron])	2.640	-
NAA Naphtyl-Acetic-Acide	0.372	-
BAP 6-Benzyl-Amino-Purine	-	0.700
IBA Indole-Butiric-Acide	-	0.001



Subcultures: ***A*** *– fresh weight;* ***B*** *– 1st subculture;* ***C*** *– 2nd subculture;* ***D*** *– 3rd subculture.*
Treatments: *1 - Control –buffer (pH 7); EMS ⟹ 2 - 2.5 ·10-2 M; 3 - 1.25 ·10-2 M; 4 - 6.2 ·10-3 M*
5 - Control –buffer (pH 6); ENU 6 - 6.2 ·10-3 M; 7 - 3.1 ·10-3 M; 8 - 1.55 ·10-3 M

Figure 1. *Influence of mutagenic concentrations on callus weight at different subcultures*

All treatments were performed in 5 replicates. The first explant subculture was done under dark conditions, while the second and the third ones were carried out under light conditions, at the temperature of 25+1°C, 8/16 hours photoperiod and 2500 Lx light intensity.

The effect of mutagenic treatments was studied by evaluating the callus weights and the regeneration ability at each subculture. Influence of different mutagenic treatments on the process of regeneration was estimated by counting the number of shoots per explant and the total number of shoots detected on the MSE medium, while the number of regenerates per explant and the total number of regenerates were recorded on MS_0 medium.

Chlorophyll changes were determined by classification of Lamprecht [14].

Results were statistically elaborated by bi-factorial ANOVA analysis or Student's "t"test, while the strength of influence of the studied factors was calculated by correlation ratio (η%).

RESULTS AND DISCUSSION

a. Callus growth

Weight data of three-time subcultured calli, treated for 60 min with different EMS and ENU concentrations, are presented in Figure 1.

A hierarchic range of callus weights in dependence of the concentrations of both mutagens for leaf petiole and root explants.

Respect to the control buffer pH 7, in all subcultures, a stimulation effect of the lowest concentration 6.2 · 10^{-3}M on callus weight has been seen in the experiment involving EMS treatment on leaf petiole explants. The treatment with the lowest ENU concentration (1.55 · 10^{-3}M) also induced small stimulation, respect to the control buffer pH 6, only in the third subculture. Similar effects were not found when root explants were treated with both mutagens.

The results statistically evaluated by dispersion analysis and showing the degree of factor's influence, are presented in Table 2.

As the mutagenic treatments with both mutagens have to be compared with the pH 6 and pH 7 buffer controls, respectively, influence of the factor A (mutagen concentrations) on callus weights showed the highest significant values for the mutagen concentrations of 1.55·10^{-3} M ENU and 6.2·10^{-3} M EMS, mainly when leaf petioles were used as explants. Inhibition effects of the highest mutagen concentrations (6.2 ·10^{-3} M ENU and 2.5 ·10^{-2} M EMS) on callus weights were also evidenced. The same trend was noticed using the roots as explants. Referring to the influence of factor B (subculture on a fresh medium) on callus growth, the highest significant weight was found at the 3rd subculture for both types of explants , while the lowest one was recorded at the 1st subculture.

Referring to the interactions between both factors (A = mutagen concentrations and B = subculture) on callus weights, the first positions were determined by the influence of the lowest concentrations of both mutagens with the 3rd subculture on a fresh medium (Table 3).

b. Plant regeneration

Shoot formation and regeneration from root explants have been never expressed.

The effects of mutagenic treatments on shoot formation as well as on plant regeneration from leaf petiole explants have been reported in Table 4. For both mutagens applied, the number of shoots and regenerates per explant increased by decreasing the EMS and ENU concentrations.

All mutagenic treatments have inhibited the total number of shoots respect to both controls (buffers pH 7 and pH 6).

Table 2. Evaluation of significance between factor's differences

Leaf petioles			***Roots***		
Treatments	Average value of five replicates	Significance per P = 0.05	Treatments	Average value of five replicates	Significance per P = 0.05
Factor *A* = mutagen concentrations					
(***A*****4**) ENU 1.55 .10^{-3} M	0.86	a	(***A*****1**) Control-buffer (pH 6)	0.70	a
(***A*****8**) EMS 6.2 .10^{-3} M	0.65	b	(***A*****4**) ENU 1.55 .10^{-3} M	0.60	b
(***A*****1**) Control-buffer (pH 6)	0.64	b	(***A*****5**) Control-buffer (pH 7)	0.54	c
(***A*****5**) Control-buffer (pH 7)	0.61	bc	(***A*****8**) EMS 6.2 .10^{-3} M	0.41	d
(***A*****3**) ENU 3.1 .10^{-3} M	0.51	c	(***A*****3**) ENU 3.1 .10^{-3} M	0.37	d
(***A*****7**) EMS 1.25 .10^{-2} M	0.39	d	(***A*****7**) EMS 1.25 .10^{-2} M	0.33	de
(***A*****2**) ENU 6.2 .10^{-3} M	0.32	de	(***A*****2**) ENU 6.2 .10^{-3} M	0.28	e
(***A*****6**) EMS 2.5 .10^{-2} M	0.26	e	(***A*****6**) EMS 2.5 .10^{-2} M	0.25	e
Factor *B* = subculture on fresh medium (subculture)					
(***B*****4**) 3rd subculture	1.32	a	(***B*****4**) 3rd subculture	1.23	a
(***B*****3**) 2nd subculture	0.67	b	(***B*****3**) 2nd subculture	0.48	b
(***B*****2**)1st subculture	0.22	c	(***B*****2**) 1st subculture	0.02	c
(***B*****1**) Fresh weight	0.01	d	(***B*****1**) Fresh weight	0.01	c

Table 3. Evaluation of significance between the differences of factor's combinations degrees (AB=combinations; factor A=mutagen concentrations; factor B= subculture on fresh medium)

Leaf petloles			***Roots***		
Combinations	Average value of five replicates	Significance per P = 0.05	Combinations	Average value of five replicates	Significance per P = 0.05
(***A*****4*B*****4**) ENU 1.55 .10^{-3} M + 3rd subculture	1.81	a	(***A*****1*B*****4**) Control pH=6.0 + 3rd subculture	2.03	a
(***A*****8*B*****4**) EMS 6.2 .10^{-3} M + 3rd subculture	1.55	b	(***A*****4*B*****4**) ENU 1.55 .10^{-3} M + 3rd subculture	1.82	b
(***A*****1*B*****4**) Control pH=6.0 + 3rd subculture	1.52	b	(***A*****5*B*****4**) Control pH=7.0 + 3rd subculture	1.41	c
(***A*****3*B*****4**) ENU 3.1 .10^{-3} M + 3rd subculture	1.41	bc	(***A*****8*B*****4**) EMS 6.2 .10^{-3} M + 3rd subculture	1.17	d
(***A*****4*B*****3**) ENU 1.55 .10^{-3} M + 2nd subculture	1.23	c	(***A*****3*B*****4**) ENU 3.1 .10^{-3} M + 3rd subculture	1.06	d
(***A*****5*B*****4**) Control pH=7.0 + 3rd subculture	1.20	c	(***A*****7*B*****4**) EMS 1.25 .10^{-2} M + 3rd subculture	0.89	e
(***A*****5*B*****3**) Control pH=7.0 + 2nd subculture	0.90	d	(***A*****2*B*****4**) ENU 6.2 .10^{-3} M + 3rd subculture	0.79	e
(***A*****1*B*****3**) Control pH=6.0 + 2nd subculture	0.85	d	(***A*****1*B*****3**) Control pH=6.0 + 2nd subculture	0.71	e
(***A*****7*B*****4**) EMS 1.25 .10^{-2} M + 3rd subculture	0.85	d	(***A*****5*B*****3**) Control pH=7.0 + 2nd subculture	0.68	e
(***A*****2*B*****4**) ENU 6.2 .10^{-3} M + 3rd subculture	0.82	d	(***A*****6*B*****4**) EMS 2.5 .10^{-2} M + 3rd subculture	0.60	e

Influence of the degrees of both factors studied as well as that of the interaction between them (AB), expressed by η%, is reported in Figure 2. Influence of the factor B is almost three times higher (66 and 69 %) than the factor A (21 and 17 %) for callus coming both from leaf petiole and root explants.

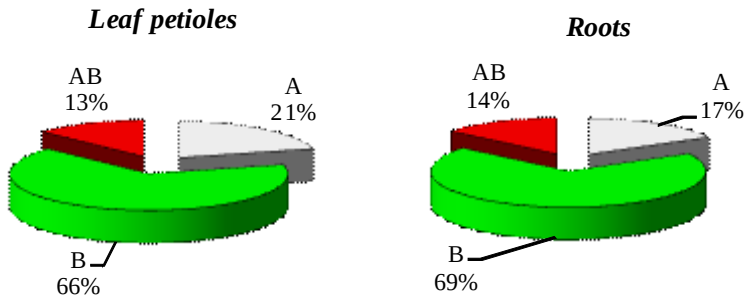


FIGURE 2. Strength of factor's influence and interactions between them showed by hh% index (A = mutagen concentrations; B = subcultures; AB = interaction between factors A and B

Table 4. Regenerative capabilities of common bean variety Plovdiv 11M after EMS and ENU treatments of leaf petiole explants

Treatments	No. of initial explants	Shoots		Regenerates	
		total number	no./explant	total number	no./explant
Control-buffer (pH 7)	250	48	8	6	6
EMS					
$2.5 \cdot 10^{-2}$ M	250	20 ---	6 --	4 --	1 ---
$1.25 \cdot 10^{-2}$ M	250	32 --	9 n.s.	9 ++	4 --
$6.2 \cdot 10^{-3}$ M	250	35 --	12 +++	10 +++	7 n.s.
Total:	1000	135	35	29	18
Control - buffer (pH 6)	250	50	7	7	5
ENU					
$6.2 \cdot 10^{-3}$ M	250	22 ---	6 n.s.	2 ---	0
$3.1 \cdot 10^{-3}$ M	250	29 --	8 n.s.	6 n.s.	3 --
$1.55 \cdot 10^{-3}$ M	250	33 --	10 ++	7 n.s.	5 n.s.
Total:	1000	134	31	22	13

Respect to the control (buffer pH 7), the lowest concentration of EMS ($6.2 \cdot 10^{-3}$ M) stimulated shoot formation and regeneration expressed as number of shoots per explant and total number of regenerates, respectively. Significant differences were found at the highest level (P = 0,1 %).

Comparing with ENU, more regenerates were obtained after EMS treatment.

Callus and shoot formations are represented in Figure 3.

After EMS and ENU mutagenic treatments, morphological changes of leaves and stems as well as chlorophyll changes mainly referred to *chlorina* and *viridissima* types (Figure 4) were induced. The number of the morphological changes was lower respect to that of the chlorophyll ones (Table 5). ENU treatment induced a number of changes higher than EMS.In general, whole plants with morphological or chlorophyll changes were not developed.

The explant age and the medium choice in *in vitro* culture of common bean are of great importance for both callus formation and its subsequent growth, as preliminary steps in developing an efficient regeneration procedure of whole plants [28]. According to our previous work [27], we have cultivated common bean seeds on MS-BAP medium to develop 7-day old plants as initial material for leaf petiole and root explants. Statistical analyses on the strength of factor's influence as well as on the interactions between them showed the highest effect of subcultures respect to that of mutagenic treatment on callus growth. Also the composition of the medium influenced strongly callus growth at the first subculture, while in the second and the third ones, the influence of both genotypes and explant age was more evident [28]. In the present study, decreases of callus weight under effect of the mutagen concentrations applied can be due to physiological disturbances expressed strongly at the first explant subculture on a fresh medium. This influence was lesser noticed at the third subculture because of the partial repairing of induced disturbances.

Both callus growth and plant regeneration capacity decreased by increasing the levels of mutagenic concentrations. Moustafa *et al.* [22] obtained similar results by studying the effect of gamma irradiation and ENU on cultured maize callus growth and plant regeneration. The lowest concentrations of the two mutagens stimulated callus induction and growth, similarly to the findings of Vu Duc

Quang *et al.* [31] on mutagenic treatment of rice (*Oryza sativa*) panicles at the uninucleate pollen stage.

The type of mutagen applied and its concentration influenced lesser the total number of shoots regenerated while the number of regenerates per explant as well as the total number of regenerates were strongly affected by the mutagenic concentrations. Only a few regenerates have shown morphological changes, such as plant size and leaf shape. Regenerates with morphological and chlorophyll changes did not develop whole plants.

A high number of shoots with chlorophyll chimerism (variegated forms) were also found after treatment of leaf explants of *Saintpaulia ionantha* Wendl. with N-methyl-Nr'-nitrosourea [10]. Treatments of inflorescence explants of *Brassica oleracea* with the same mutagen have induced a broad variability either in morphology or in fertility of regenerated plants [7].

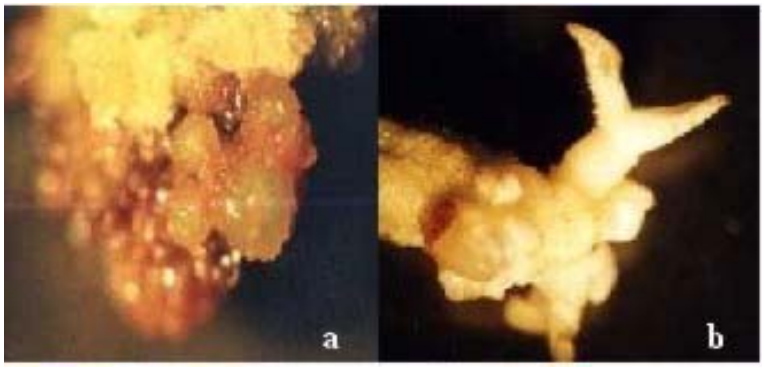


Figure 3. *Organogenetic callus (a) and shoot formation (b) in common bean*

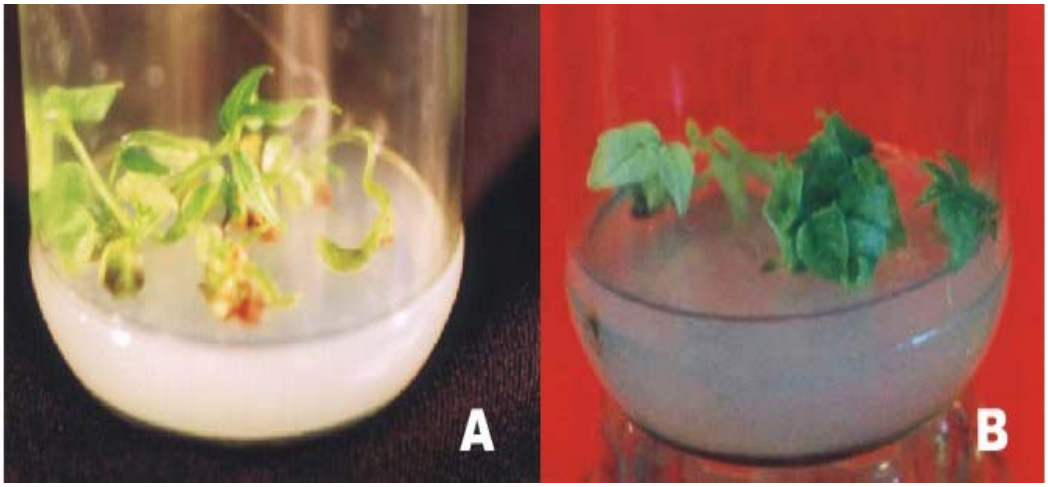


Figure 4. *Regenerates with chlorophyll changes (A = chlorina; B = viridissima)*

TABLE 5. Morphological and chlorophyll changes in shoots as well as in regenerates after EMS and ENU treatment on leaf petiole explants

Treatments	Total number	Morphological changes		Chlorophyll changes			
		irregular shape of leaves and stems		Chlorina type		Viridissima type	
		No.	%	No.	%	No.	%
Shoots							
Control-buffer (pH 7)	48						
2.5 $\cdot 10^{-2}$ M *EMS*	20	1	5.0	1	5.0	1	5.0
1.25 $\cdot 10^{-2}$ M *EMS*	32	1	3.1			2	6.2
6.2 $\cdot 10^{-3}$ M *EMS*	35						
Total:	135	2	1.5	1	0.7	3	2.2
Control-buffer (pH 6)	50						
6.2 $\cdot 10^{-3}$ M *ENU*	22	2	9.1	2	9.1	2	9.1
3.1 $\cdot 10^{-3}$ M *ENU*	29	1	3.4			2	6.8
1.55 $\cdot 10^{-3}$ M *ENU*	33						
Total:	134	3	2.2	2	1.5	4	3.0
Regenerates							
Control-buffer (pH 7)	6						
2.5 $\cdot 10^{-2}$ M *EMS*	4						
1.25 $\cdot 10^{-2}$ M *EMS*	9	1	11.1			1	11.1
6.2 $\cdot 10^{-3}$ M *EMS*	10						
Total:	29	1	3.4			1	3.4
Control-buffer (pH 6)	7						
6.2 $\cdot 10^{-3}$ M *ENU*	2	1	50.0	1	50.0	1	50.0
3.1 $\cdot 10^{-3}$ M *ENU*	6	1	16.7			1	16.7
1.55 $\cdot 10^{-3}$ M *ENU*	7						
Total:	22	2	9.1	1	4.5	2	9.1

CONCLUSION

On the basis of the conducted investigations, we can conclude that the treatment of leaf petiole explants by chemical mutagens such as EMS and ENU influenced both callus growth and regeneration of common bean, these decreasing with the highest mutagen concentrations. ENU evidenced an inhibition effect stronger than EMS on the traits investigated. Treatment of explants with 6.2 $\cdot$ 10^{-3} M EMS improved the efficiency of plant regeneration. This system could be useful to broaden genetic diversity of common bean that is quite narrow in the natural germplasm [29].

Plant regeneration from common bean root explants was not found.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Saccardo from Tuscia University (Viterbo, Italy) for the critical suggestions in improving the manuscript.

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Статията е приета на 20.02.2009 г.
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