EFFECT OF ETHYL METHANESULFONATE (EMS) AND N-NITROSE-N´-ETHYL UREA (ENU) ON CALLUS GROWTH OF COMMON BEAN

ВЛИЯНИЕ НА ЕТИЛМЕТАН СУЛФОНАТА (ЕМС) И N-НИТРОЗО-N´-ЕТИЛ КАРБАМИДА (НЕК) ВЪРХУ РАСТЕЖА НА КАЛУС ОТ ФАСУЛ

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ABSTRACT

Influence of ethyl methanesulfonate (EMS) and N-nitrose-N´-ethyl urea (ENU) mutagenic treatments was investigated on three time sub-cultured calli obtained from leaf petiole explants of 7-day old sterile plants. Calibrated sterile seeds of the common bean Bulgarian variety Plovdiv 11M were pre-cultivated on MS basal medium supplemented with 1 µmM BAP. Then, both mutagens EMS and ENU were applied for different times such as 15, 30, 60 and 90 min on the explants at the concentrations of: $2.5 \cdot 10^{-2}$ M and $6.2 \cdot 10^{-3}$ M, respectively. Times of the mutagenic treatments influenced callus growth, calli from 30-min treatment with both mutagens showing the highest weights. In both cases, the 90-min mutagen application caused a too relevant effect either on callus browning or growth inhibition. In general, ENU showed a stronger effect than EMS. The effect of subcultures on callus growth was higher than mutagenic treatments. Interactions between these factors checked by by correlation ratio (η%) were quite low.

KEY WORDS: Ethyl methanesulfonate (EMS), in vitro cultivation, mutagens, N-nitrose-N´-ethyl urea (ENU), Phaseolus vulgaris L.

Abreviations: BAP: 6-Benzyl-Amino-Purine; EMS: ethyl methanesulfonate; IBA: Indole-Butiric-Acide; NAA: Naphtyl-Acetic-Acide; ENU: N-nitrose-N´-ethyl urea; TDZ: N-phenyl-N`-1,2,3-thiadiazol-5-urea [thidiazuron]

РЕЗЮМЕ

Изследвано е влиянието на третирането с етилметан сулфонат (ЕМС) и N-нитрозо-N´-етил карбамид (НЕК) върху трикратно прехвърлен на свежа среда калус получен от експланти от листни дръжки на 7-дневни стерилни растения. Калибрирани стерилни семена от българския сорт фасул Пловдив 11М са предкултивирани на основна MS среда допълнена с 1 µmM BAP. След това, двата мутагена ЕМС и НЕК, са приложени в концентрации съответно: $2.5 \cdot 10^{-2}$ М и $6.2 \cdot 10^{-3}$ М за различно време 15, 30, 60 и 90 min. Времето на мутагенното третиране влияе върху растежа на калуса като калусът получен след 30-min третиране с двата мутагена има най-високи тегла. 90-min третиране причинява подобен ефект от двата мутагена – покафеняване на калуса или инхибиране на калусния растеж. НЕК показва по-силен ефект от ЕМС. Ефектът от прехвърлянето на свежа среда върху растежа на калуса е по-силен от мутагенните третирания. Взаимодействията между тези фактори, отчетени чрез корелационното съотношение (η%), са сравнително ниски.

KEY WORDS: Етилметан сулфонат (ЕМС), in vitro култивиране, мутагени, N-нитрозо-N´-етил карбамид (НЕК), Phaseolus vulgaris L.

Abreviations: BAP: 6-бензил аминопурин; EMS: етилметан сулфонат; IBA: индол-бутирова киселина; NAA: нафтил-оцетна киселина; ENU: N-нитрозо-N´-етил карбамид; TDZ: N-фенил-N`-1,2,3-тидиазол-5-карбамид [тидиазурон]

DETAILED ABSTRACT

Common bean (Phaseolus vulgaris L.) is an important crop legume in Bulgaria and in many countries of the world. However, there is evidence that its domestication has induced a strong reduction in diversity at the molecular level [14]. This reduction contrasts with the increase in diversity observed for morphological traits during and after domestication [7], because of different consumer and breeding purposes. Different ways were chosen to investigate common bean diversity.

Mutagenesis combined with in vitro culture technique can provide a profitable methodology to increase the frequency of new genetic variations [4, 13]. 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.

In order to study the influence of EMS and ENU on growth of calli, leaf petiole explants of 7-day old sterile plants were used. Firstly, calibrated sterile seeds were pre-cultivated on MS basal medium supplemented with 1 μmM BAP. Both mutagens were applied in concentrations of: EMS $\Rightarrow$ $2.5 \cdot 10^{-2}$ M and ENU $\Rightarrow$ $6.2 \cdot 10^{-3}$ M for different times: 15, 30, 60 and 90 min on the explants. It was found that time of mutagenic treatments influenced callus formation and its growth. Calli from 30-min treatment with both mutagens showed the highest weights. In both cases, mutagen application of 90-min caused a too relevant effect either on callus browning or on growth inhibition. ENU showed a stronger effect than EMS. The effect of subcultures on callus growth was higher than mutagenic treatments while interactions between these factors were quite low. Sixty-minutes mutagenic treatment can be used as optimal for conduction of further in vitro investigations.

INTRODUCTION

Considering that common bean (Phaseolus vulgaris L.) is one of the most important rich-protein legumes, scientists focussed theirs investigations on different ways to broad genetic variability of the natural germplasm.

The role of mutagenesis in inducing desired attributes, which could not be expressed in natural germplasm or have been lost during evolution, was well recognized [1]. Combined with in vitro culture technique, it can provide a profitable methodology to increase the frequency of new genetic variations [4, 13] but low efficiency of in vitro regeneration systems still remains a problem limiting the use [3].

As for other crops, differences in the organogenetic response of genotypes must be considered [10]. Associated to mutation induction, molecular biology actually represents a further tool for studying gene expression and regulation [6], as already reported also in bean by Allavena [2].

Referring to the use of the mutagenic treatment additional to in vitro culture, its duration depends on the type of explants used (seeds, roots or steams) and has a great influence on the mutagenic effect recovered [8, 9]. Different ways of mutagenic treatments have been also reported [5].

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, we aimed at studying the influence of the mutagenic treatment on callus growth of Bulgarian common bean genotypes.

MATERIALS AND METHODS

Calibrated seeds of the common bean Bulgarian variety Plovdiv 11M were pre-cultivated on the basal MS medium [12] supplemented with 1μmM BAP. Leaf petioles from 7-day old sterile plants have been used as explants for in vitro culture techniques aimed at obtaining callus proliferating.

To study the effect of a mutagenic treatment on callus growth and to determine its optimal duration, high mutagen concentrations ($2.5 \cdot 10^{-2}$ M EMS and $6.2 \cdot 10^{-3}$ M ENU) were applied for different times (15, 30, 60, and 90 min) on leaf petiole explants.

Both mutagens, ENU and EMS, were dissolved in buffers at pH 6 and pH 7, respectively, and solutions were cold sterilized through 0.45 μmm Millipore filters. Then, explants were plunged under sterile conditions into the mutagen solutions. After mutagenic treatments leaf petiole explants were in vitro cultured on MSI_2 callus induction medium.

Hormonal composition of the media utilized is described in Table 1.

All treatments were performed in 5 replicates (50 explants per petri dish or 250 initial explants per each

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

Components	MSI_2
TDZ	2.640
NAA	0.372
BAP	-
IBA	-

variant). The first explant subcultures were 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 at each subculture.

Results were statistically elaborated by bi-factorial ANOVA analysis and the strength of influence of the factors studied was calculated by correlation ratio (η%).

RESULTS AND DISCUSSION

We have tested the influence of the chemical mutagenic treatment on the in vitro regeneration ability of the common bean Bulgarian variety Plovdiv 11M, which showed the best reaction respect to other genotypes analysed (unpublished data). After treatment of leaf petiole explants either with EMS or ENU for different times, mutagen effects were expressed by callus weight increases at each subculture on a fresh medium (Fig. 1).

Comparing to pH 7 and pH 6 controls, EMS and ENU 30-

Table 2: Evaluation of A (time of mutagenic treatment) and B (subculture on a fresh medium) factor's degrees on callus growth

Treatments	Average value of five replicates	Significance per P=0.05	Treatments	Average value of five replicates	Significance per P=0.05
(A_3) EMS – 30'	0.60	a	(A_3) ENU – 30'	0.53	a
(A_1) Control pH 7.0	0.56	a	(A_1) Control pH 6.0	0.49	a
(A_2) EMS – 15'	0.39	b	(A_4) ENU – 60'	0.28	b
(A_4) EMS – 60'	0.24	b	(A_2) ENU – 15'	0.21	b
(A_5) EMS – 90'	0.19	c	(A_5) ENU – 90'	0.19	c
(B_4) 3rd subculture	0.73	a	(B_4) 3rd subculture	0.60	a
(B_3) 2nd subculture	0.38	b	(B_3) 2nd subculture	0.30	b
(B_2) 1st subculture	0.31	b	(B_2) 1st subculture	0.18	c
(B_1) Fresh weight	0.09	c	(B_1) Fresh weight	0.06	d

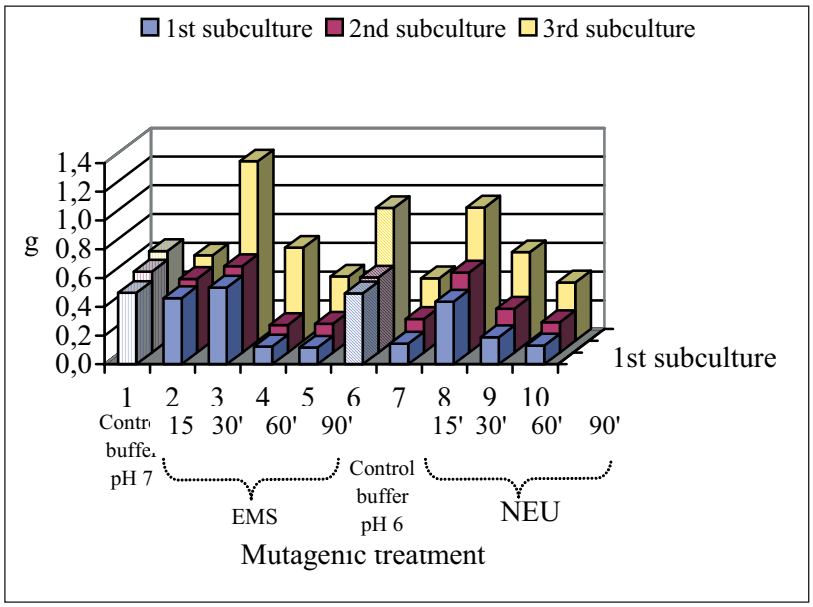

Figure 1: Influence of the duration of EMS and ENU treatments on the weight of three-time subcultured callus from leaf petiole explants

min treatments evidenced a stimulation effect on callus growth, while the 60 and 90-min ones have inhibited it, this trend being well expressed at the first subculture as well as at the subsequent ones. Nevertheless, a good correspondence among inhibitions caused by both mutagenic treatments was in general noticed, although the stimulation effect recovered on 15-min treated calli was evident only after EMS treatment. In both cases, mutagen application of 90-min caused a too relevant effect either on callus browning or growth inhibition. Significant differences among callus weights, all dependent on the mutagenic treatment duration (Factor A), as well as on the subcultures on a fresh medium (Factor B), are presented in Table 2.

Referring to the subcultures, the highest values of callus weight were recorded at the third subculture, independently from the mutagen treatment used. The average values and the level of significant differences within each factor and for both mutagens applied ranged similarly.

The combination between the 30-min mutagenic treatment and the third subculture showed the strongest significant influence on callus growth (Table 3). The second big group for the strength of influence incorporates the combination 60-min treatment + third subculture together with those of 30 min + second subculture.

Percentages of factors such as time of treatment (A), subculture of the explants on fresh medium (B), and interaction between them (AB), which are parts of total variation for the investigated callus weights, are reported in Figure 2.

On the basis of the ANOVA analysis, the factor B showed the strongest influence on callus growth ($\eta\%$=63 and 69) respect to the factor A, which is almost the half (31% for EMS and 23% for ENU). The interaction between factors (AB) expressed the poorest effect for both mutagens (6 and 8% respectively).

Vu Duc Quang et al. [15] studied the effect of mutagenic treatment of rice (Oryza sativa) panicles at the uninucleate pollen stage and found that the lowest concentrations of the mutagens stimulated callus induction and its growth. Moustafa et al. [11] also obtained dependence between applied doses of gamma irradiation and ENU on cultured maize callus growth and plant regeneration. In our

Table 3: Evaluation of significant differences of factor's combination degrees (AB=combinations; A=time of mutagenic treatment; B=subculture on a fresh medium)

E M S - treatment			N E U - treatment		
Combinations	Average value of five replicates	Significance per P=0.05	Combinations	Average value of five replicates	Significance per P=0.05
(A_2B_4) 30 min + 3rd subculture	1.25	a	(A_2B_4) 30 min + 3rd subculture	0.93	a
(A_3B_4) 60 min + 3rd subculture	0.65	b	(A_3B_4) 60 min + 3rd subculture	0.62	b
(A_2B_3) 30 min + 2nd subculture	0.60	b	(A_2B_3) 30 min + 2nd subculture	0.46	bc
(A_1B_4) 15 min + 3rd subculture	0.59	b	(A_1B_4) 15 min + 1st subculture	0.44	bc
(A_2B_2) 30 min + 1st subculture	0.53	bc	(A_4B_4) 90 min + 3rd subculture	0.41	bc
(A_1B_3) 15 min + 2nd subculture	0.51	c	(A_3B_3) 60 min + 2nd subculture	0.31	cd
(A_1B_2) 15 min + 1st subculture	0.46	c	(A_2B_2) 30 min + 1st subculture	0.24	d
(A_4B_4) 90 min + 3rd subculture	0.45	cd	(A_4B_3) 90 min + 2nd subculture	0.23	d
(A_4B_3) 90 min + 2nd subculture	0.20	cde	(A_4B_2) 90 min + 1st subculture	0.21	d
(A_3B_3) 60 min + 2nd subculture	0.19	de	(A_3B_2) 60 min + 1st subculture	0.19	d

investigations, ENU inhibited callus growth more than EMS, when the treatment was conducted for 15 and 30 min. Application of ENU and EMS for 60 and 90 min did not show any clear difference (Fig. 1 and Table 2).

CONCLUSION

On the basis of the conducted investigations, we can conclude that the treatment of leaf petioles explants by ethyl methanesulfonate (EMS) and N-nitrose-N´-ethyl urea (ENU) influenced callus growth of common bean. Treatment of plant explants with such chemical mutagens for 60 min can be optimal in case of investigations of the regeneration capacity in common bean (Phaseolus vulgaris L.). ENU evidenced an inhibition effect stronger than EMS.

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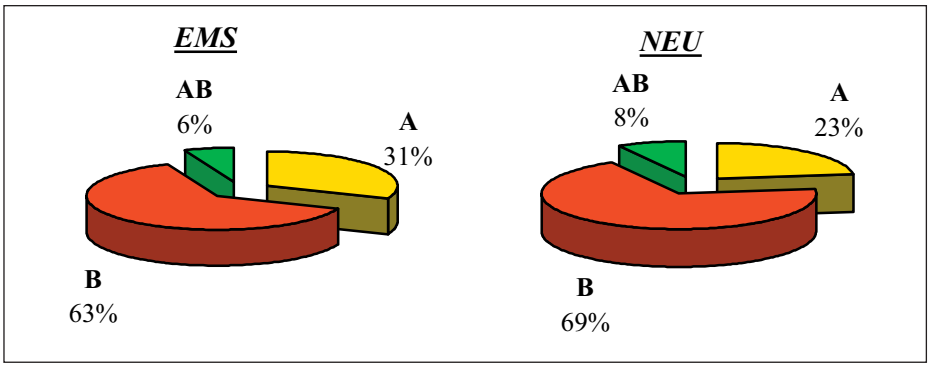

Figure 2: Strength of factor's influence and interactions between them (A=time of mutagen treatment; B=subcultures; AB=interaction between factors A and B showed by ηh% index)

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