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The Mutagenic Effect of the Diethyl Sulphate and Dimethyl Sulphate for in vitro regeneration to okra (*Abelmoschus Esculentus l. Moench*)

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ABSTRACT

The creation of genetic variability through the use of chemical mutagenic factors which increase the frequency of in vitro mutations to okra is not very frequently mentioned in scientific literature. This is why we have undertaken this study on the assessment of the in vitro regeneration in okra subject to the action of two chemical mutagens: Diethyl sulphate and Dimethyl sulphate. With a 0.1 ppm concentration, Diethyl sulphate had a low stimulative effect on the regeneration of okra in vitro, while this mutagen inhibited in vitro regeneration to some extent for 0.2 ppm and 2 ppm concentrations. On the other hand, the chemical mutagen Dimethyl sulphate resulted in an obvious decrease in the percentage of regenerated neoplantlet for all the three concentrations, but for the 2 ppm concentration in particular, and this negative effect was also seen in the case of the multiplication rate, through subculture. Moreover, the strongly mutagenic action of Dimethyl sulphate was also suggested by the appearance of a larger number of chromosome aberrations in the meristematic tissues of okra grown in vitro under the influence of this chemical mutagen, compared to Diethyl sulphate. Thus, in the case of okra culture in vitro, Dimethyl sulphate is a stronger mutagen compared to Diethyl sulphate and, hence, this one can be used to induce mutagenicity to okra, and any mutations may be speculated for the modern and quick improvement of this significant species, both at a nutritional and therapeutic level.

Keywords: Diethyl sulphate, Dimethyl sulphate, mutagen, okra.

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INTRODUCTION

Chemical mutagenic agents include a wide range of chemical substances such as: the analogues of nitrogenous bases, alkylating agents, intercalating substances, coloring agents, antibiotics, alkaloids, etc. The effects of these substances on the genetic material are diverse as well. The induction of in vitro mutations with the help of chemical mutagenic agents creates high genetic variability, which is highly useful in the improvement of plants needed for both human consumption and animal feed; however, many plants are also used in pharmaceutical industry.

In the last decades, mutagenesis has played a major part in the development of plants with higher productive qualities in the entire world, which is beneficial to ensure food safety at the world level[1,2,3]. Mutagenic treatment of cultivated plants is a useful tool for isolating desired variants and increasing resistance to biotic and abiotic factors, especially as mutagenic treatment is relatively simple and has low cost[4]. Some factors influence the effect of chemical mutagens in plants, as follows: the type of mutagen, concentration, exposure time and temperature[5].

Okra are annual plants with edible fruit from the *Malvaceae* family. Their scientific name is *Abelmoschus esculentus*; the synonym, i.e. *Hibiscus esculentus*, is more frequently used in Romania. Plants may grow up to 2 m high; leaves are 10-20 cm long and are wide, palmate leaves, with 5-7 lobes. Flowers have a 4-8 cm diameter, with 5 yellowish petals and a frequent red spot at the basis of each petal. The fruit is a hairy green capsule, 8-20 cm long, including many seeds.

The consumption of okra helps combat fatigue. In this regard, okra seeds are the anti-fatigue part of okra pods and polyphenols and flavonoids are active constituents[6]. The glycemic index (GI) is a measurement of how quickly carbohydrates in foods turn to sugar in your blood. Regularly consuming low GI foods like okra can help even out roller coaster blood sugar levels and aid in weight control. Okra has a GI below 20, which is considered a "low GI" food[7]. Also, the consumption of okra is an efficient method to manage the body's cholesterol levels; the soluble fiber content of the okra helps naturally to reduce cholesterol and, therefore, decreases the chance of cardiovascular disease[8].

Another benefit of consumption of okra is the content of the quality protein especially with regard to its content of essential amino acids (in its seeds) and this fact making okra one of the most important vegetable resource of food protein.

However, scientific literature does not include many data regarding the induction of mutagenesis in okra with the help of chemical mutagenic agents. This is why we have undertaken this study on

the assessment of the in vitro regeneration in okra subject to the action of two chemical mutagens: Diethyl sulphate (DES) and Dimethyl sulphate (DMS).

MATERIALS AND METHOD

The used biological material was represented by okra seeds (Clemson cultivar). The research was undertaken in the Biotechnology Laboratory of the Faculty of Agronomy in Craiova (Romania).

After they were washed and chemically disinfected, culture glassware was sterilized through hot air exposure (180^0C), for 2 hours, in an oven. The used surgical tools were disinfected with 70^0 sanitary alcohol, then sterilized through buckling. The sterile air flow hood was started about 20 minutes before beginning inoculation operations, so that the sterile air flow might reach the work area and vehiculate all the spores in the air outside the hood perimeter.

The biological material was maintained in Berzelius glasses with tap water for 1 hour, for hydration purposes, then it was subject to a first sterilization in a solution of sodium hypochlorite and sterile distilled water (1:2 ratio), for 20 minutes. The second sterilization followed, in 70^0 sanitary alcohol, for 5 minutes. The last step in the preparation of biological material for inoculation was the immersion in 3 successive baths of sterile distilled water.

The used culture environment was Murashige-Skoog (MS), prepared in laboratory, following a concrete recipe and with the addition of vitamins (thiamine, pyridoxine and nicotinic acid). Table no. 1 presents the composition of the aseptic environment created in the laboratory for in vitro regeneration in okra.

Table 1: Composition of aseptic media for in vitro regeneration to okra

Elements	**Specification**
Macroelements	MS media protocol
Microelements	MS media protocol
FeEDTA	MS media protocol
Tiamine	0,5 mg/l
Pyridoxine	0,5 mg/l
Nicotinic Acid	0,5 mg/l
Sucrose	30 g/l
Agar	8 g/l
Adenin sulfat	40 g/l
Cytokinines (BAP)	2 mg/l
Auxines (ANA)	1 mg/l

MS - Basal media after Murashige-Skoog, 1962

Environmental pH was stabilized at 5.5 (with NaOH 2%); from this point of view, the pH value may be a limiting factor for the in vitro growth of explants, as the more recommended value is 5.5-5.8 (rooting is inhibited in medium culture with a pH lower than 5.4, and the multiplication of

meristematic cells is stopped by a pH higher than 6). Two chemical mutagens, i.e. diethyl sulphate (DES) and dimethyl sulphate (DMS) were added in the culture medium in order to induce in vitro mutations, in three concentrations: 0.1 ppm (variant V_1); 0.2 ppm (variant V_2) and 2 ppm (variant V_3) for each of the mutagenic agents. Each variant was compared with a control variant (V_0), inoculated on a standard MS culture medium and not treated with mutagenic agents.

Inoculation took place under the sterile air flow hood, with 100 seeds being inoculated for each variant of concentration and each chemical mutagenic agent used, along with the not treated control variant. Rules for maintaining laboratory and tool asepsis were observed during the entire inoculation phase (as the tip of the tweezers used for inoculation was buckled "until red"). After 24 hours, the biological material subject to the action of chemical mutagens was taken out from culture dishes, rinsed with sterile distilled water and transferred onto a fresh culture medium, identical to the one of the witness variant (control). The inoculation protocol was the same. After inoculation, culture dishes were transferred to the growth (incubation) room, with controlled lighting and temperature. Thus, inoculum vials were exposed to light (16 hours/day) on metal shelves, under fluorescent lighting (1000 lux), at a temperature of $\pm 25^0$C, where their growth continued for 30 days. After this period, two subcultures were performed for multiplication purposes, through the preservation of explants from the stem meristems of okra neoplantlets and their transfer onto the same MS nutritive medium used when initiating in vitro culture, with no added chemical mutagens. The chromosome coloring protocol through the Feulgen-Rossenbeck method was used for cytogenic determinations, with a laboratory prepared basic fuchsine solution, and the LCD Celestron Digital Microscope (model 44340) was used for the study of chromosome aberrations. After the two subcultures undertaken 30 days apart, some conclusions could be drawn up pursuant to morphological and cytogenic determinations.

RESULTS AND DISCUSSION

Okra culture in vitro is a mutagenic protocol, and the genetic variability it generates may be an additional source of valuable characters for the improvement of this species that is so useful for human health. The impact of the composition of the in vitro culture medium on the multiplication rate in okra is a very significant phase in the technological protocol, determining the quick achievement of a high number of neoplantlets, identical to the mother plant. Thus, after about four weeks of incubation and prior to the first subculture, the viability percentage of the initial biological material (okra seeds) was established, with the results being shown in table 2. Thus, pursuant to the exposure of the okra biological material to the action of the two chemical

mutagenic agents, it was seen that the DES mutagen slightly inhibited viability in the 0.1 ppm concentration, i.e. 9% of the okra seeds failed to germinate. However, the inhibition of germination increased as DES concentration increased, i.e. 25% non-viable seeds at a 0.2 ppm concentration, and 46% nonviable seeds at a 2.0 ppm concentration of the DES mutagen respectively. The average viability of the okra biological material, under the impact of the DES mutagen, was 73.3%.

On the other hand, the DMS mutagen was seen to have an even stronger effect of inhibition of the viability of biological material, depending on the concentration, i.e. for a 0.1 ppm concentration, only 69% of the seeds maintained their viability, while 31% failed to germinate; for a 0.2 ppm concentration of the DMS mutagen, the viability of seeds decreased to 53% while the inhibition of germination was very strong at a 2.0 ppm concentration, resulting in the germination of only 42% out of the 100 seeds, and the remaining 58% losing their viability. The average viability of the okra biological material, under the impact of the DMS mutagen, was only 54.6%.

Table 2: The effect of chemical mutagenic factors on seeds viability to okra in vitro culture

Mutagenic factor	Variant/Concentration (ppm)	Number of treated seeds	Percent of viable seeds (%)	Percent of nonviable seeds (%)
DES	V_0=Control (untreated)	100	100	-
	V_1=0.1	100	91	9
	V_2=0.2	100	75	25
	V_3=2.0	100	54	46
Average	-	*100*	*73.3*	*26.6*
DMS	V_0=Control (untreated)	100	100	-
	V_1=0.1	100	69	31
	V_2=0.2	100	53	47
	V_3=2.0	100	42	58
Average	-	*100*	*54.6*	*45.3*

These first results suggest that the DMS mutagenic agent showed a higher capacity of inhibiting the viability of the okra biological material, compared to the DES mutagenic agent.

In order to assess how the DES and DMS mutagenic substances may impact the development and evolution of okra meristems in vitro, observations were performed on the percentage of regeneration of propagules from explants (proceeding from stem meristems) after two subcultures, performed every 30 days. The average results of these observations are presented in table no. 3. The results have shown a low proliferation of okra propagules, both for DES treatment and DMS treatment; however, the average was only 13.5% propagules for the latter, with a distinctly significant negative difference from the witness, at 0.2 ppm and 2.0 ppm concentrations.

Table 3: Number of regenerated propagules (%) to okra in vitro after two subculture

Mutagenic factor	Variant/Concentration (ppm)	$\bar{x}$	$\pm s_{\bar{x}}$	±d	Difference of signification
DES	V_0=Control (untreated)	17.2	-	-	-
	V_1=0.1	17.8	1.09	+0.6	-
	V_2=0.2	16.5	2.40	-0.7	-
	V_3=2.0	14.6	3.16	-2.6	0
Average		16.3	2.21		
DMS	V_0=Control (untreated)	17.5	-	-	-
	V_1=0.1	15.4	2.12	-2.1	-
	V_2=0.2	13.0	2.64	-4.2	00
	V_3=2.0	12.1	2.66	-5.4	00
Average		13.5	2.47		
LSD 5% = 2.46	LSD 1% = 3.84				LSD 0.1% = 5.53

Subcultures deriving from the initial culture showed some differences indicating the genotypic reactions of okra explants during the two subculture cycles. Thus, as it can be seen in figure 1, it was found that, out of the total of 100 explants (stem meristems) per variant, coming from the plantlets of the initial culture, 78 explants and 74 explants respectively (for variants V_1DES/V_1DMS), 75 explants and 70 explants respectively (for variants V_2DES/V_2DMS), and 70 explants and 67 explants respectively (for variants V_3DES/V_3DMS) proliferated sprouts after the first subculture. On the other hand, at the end of the second subculture the percentage of explants that proliferated new sprouts decreased even more dramatically, with only 57 explants with proliferation for variant V_3DES, and only 42 explants with proliferation for variant V_3DMS. The decreased percentage of sprout proliferation of explants, especially after the second subculture, suggests the tendency of senescence in okra, with the multiplications in subculture.

The obtained results suggest a stronger effect in terms of inducing mutations in the DMS mutagen, as a higher number of chromosome aberrations (*bridges* and *retarding* chromosomes) was seen in all three concentrations of the DMS, compared to the DES. Thus, 2 aberrant cells (out of the 100 studied cells) were recorded for the 0.1 DMS concentration, compared to 1 aberrant cell for the DES mutagen; 4 aberrant cells (out of the 100 studied cells) were recorded for the 0.2 DMS concentration, compared to 1 aberrant cell for the DES mutagen; 6 aberrant cells (out of the 100 studied cells) were recorded for the 2.0 DMS concentration, compared to 2 aberrant cells for the DES mutagen.

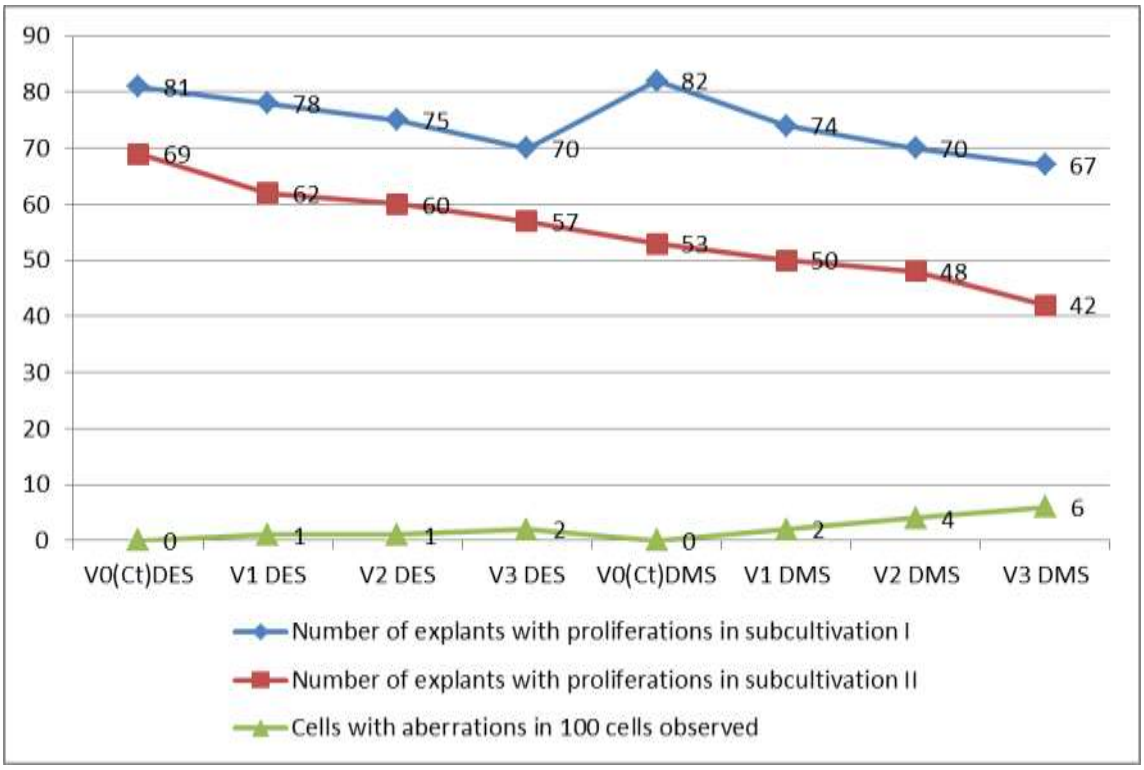

Figure 1: The evolution of multiplication in two subcultures for okra (100 explants) and the frequency of chromosomal aberrations following treatment with chemical mutagens DES and DMS

All these results show that DMS is a chemical mutagen stronger than DES and this is why it could be used in modern biotechnologies in order to induce mutations in okra, helping achieve plants with new, much improved features, in terms of productivity and, perhaps, nutritional and therapeutic quality.

Other authors have shown that the Dimethyl sulfate is a very powerful chemical mutagen, capable to induce mutations to other plants, like *Khaya senegalensis*[9] and *Vigna mungo*[10]. In the same vein, DES is a monofunctional alkylating agent capable to induce mutations, chromosomal aberrations, and other genetic alterations in plants, like *Cannabis sativa L.*[11] or other organisms, like *Bombyx mori L.*[12].

The in vitro reproduction of plants with a therapeutic effect has every opportunity of becoming the technology of future, since in vitro culture helps obtain a very large number of healthy plants in very short time. Compared to classical plant improvement methods, transformation through vegetal biotechnology (in vitro culture) provides the possibility of introducing a single feature in a variety that has already been assessed as over-performing; on the other hand, the transferred gene may proceed from any source, which maximizes the possibilities for modern plant improvement[13].

CONCLUSION

The achievement of genetic variability (useful for modern plant improvement) at the in vitro plant cultures is possibly by inducing mutations, using various mutagenic agents, including chemical ones. The introduction of the DMS mutagen in the culture medium negatively influences the new development of okra propagules in meristem cultures and the multiplication rate in subculture; simultaneously induce various chromosome aberrations. These results show that Dimethyl sulphate (DMS) is a stronger mutagen compared to Diethyl sulphate (DES), which is why it may cause possible mutations to a higher extent, making it suitable for modern biotechnologies, in order to obtain plants with improved productivity and nutritional and therapeutic quality. In vitro culture of okra under the influence of the DMS chemical mutagen may extend the possibilities of creating genetic variability in this species; however, the most suitable experimental system and the most adequate mutagens should be found in order to create valuable mutations in vitro. The purpose is both to establish the expression of the mutating character of neoplantlets and transmit and maintain this feature of interest in the descendency.

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