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Improvement of pomegranate (punica granatum l.) to cold resistant through in vitro mutation

Annotation. *In Kazakhstan, a large pool of fruit trees is grown, one of which is the pomegranate (Punica granatum L.). The most significant limiting factor of pomegranate cultivation in Kazakhstan is the frosty winter, as well as low temperatures in spring and autumn. In order to obtain mutants resistant to low positive temperatures, chemical mutagenesis was used, with ethyl methanesulfonate (EMS) as the mutagen. Callus was treated with mutagen in vitro culture. In the Akdona cultivar, the survival rate of callus was 25.45% at the concentration of 3 μM of EMS mutagen, 31.67% at the concentration of 6 μM, and 24.35% at 9 μM. Plant regeneration was induced from mutagen-treated calluses. The resulting plants were exposed to low positive temperatures (4°C, for 30 days). After exposure to cold, the maximum survival rate of mutant plants of the Akdona cultivar was 58%, and the minimum survival rate was 3% for plants of the Pg1 line. The resulting mutant pomegranate plants will be used for breeding for cold resistance.*
Key words: *Pomegranate, mutagenesis, ethyl methanesulfonate (EMS), cold-resistance.*

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Introduction. Pomegranate *(Punica granatum)* is an economically important plant species from tropical and subtropical regions of the world, because of its delicious edible fruit, which also has pharmaceutical and decorative uses [1]. Fruit juice is a good source of sugar, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols, and is a source of iron. Some parts of the pomegranate tree (leaves, unripe fruit, fruit crust and flower buds) were traditionally used as medicinal products, as well as for tanning leather. The juice is considered useful for patients suffering from leprosy. The rind of the fruit is usually used for dysentery and diarrhea. Polyphenols have been found to inhibit the growth of cancer cells. Coumarins have hypertonic, anticoagulant, anabolic bactericidal and antitumor activity. Therefore, it is important for Kazakhstan to industrially cultivate pomegranate as a source of healthy nutrition.

The most significant limiting factor of pomegranate cultivation in Kazakhstan is the frosty winter, as well as low temperatures in spring and autumn. Even in areas of intensive cultivation, the b - 21°C cold pomegranate destroys up to 8,000 hectares of pomegranate orchards within three days [2]. In this regard, for the sustainable cultivation of pomegranate in Kazakhstan, it is necessary to conduct intensive selection to obtain lines of pomegranate that are resistant to cold. In our opinion, the most effective way to develop cold-resistant cultivars is mutagenesis using *in vitro* tissue culture.

As a chemical mutagen, ethyl methanesulfonate (EMS) is most interesting, since it induces more mutations at certain loci than physical ones, such as x-rays or gamma rays [3,4]. The chemical mutagen must penetrate the meristem zones of the wood crop sprouts, and the excess chemical must be removed after treatment. Previously, it was more difficult to obtain mutagenic plants using chemical mutagens than to obtain mutagens using physical methods [5,6,7,8]. However, with the development of the latest technology for culture of plant tissue cells, chemical mutagens have become more widely used to produce useful mutations *in vitro* culture [9].

Many studies have been carried out by the introduction of pomegranate (P. granatum L.) into the culture of tissues. Protocols have been developed for plant regeneration *in vitro* through organogenesis from callus derived from nodular segments, cotyledons [10,11,12], anthers [13], or by embryogenesis from various seedling explants, petals, and immature zygotic embryos. Almost all studies have determined that the regeneration potential depends on the genotype of the explant donor [12].

Broad genetic variability is necessary for crop selection. It can be induced using physical or chemical mutagens. Induced mutations are highly effective for improving natural genetic resources and have helped create new cultivars of many crops, including fruit [12].…

Since pomegranate is very susceptible to frost, there is a need to create frost-resistant mutant pomegranate plants. In this experiment, the authors attempted to mass-produce mutant plants by induced mutagenesis using ethyl methanesulfonate (EMS) *in vitro* culture.

Materials and methods. The objects of research were: cultivars of pomegranate Kazake anar, Nardan, Shahrisabzs and Akdona, lines Pg.1.

Method for obtaining callus from nodal segments. The young nodes were collected from annual pomegranate plants grown in a greenhouse. The tops of the shoots were cut off, washed under running water for 20 minutes, and sterilized with 50% sodium hypochlorite solution for 5-10 minutes, then in 70% alcohol for 3-5 seconds, inside a laminar box. Then the treated explants were washed 4-5 times with sterile distilled water. Optimization was performed on nodal segments at MS and WPM in test tubes with the addition of NAA 1 mg/l, BAP 1 mg/l, adenine 40 mg/l, coal 200 mg/l, ascorbic acid 150 mg/l.

Method of proliferation of shoots from nodal segment callus. One-month nodal segments on which the callus was formed were transferred *in vitro* to a complete MS culture medium in Petri dishes with the addition of NAA 0.46 mg/l, BAP 2 mg/l, adenine sulfate 16 mg/l, silver nitrate 4 mg/l, ascorbic acid 150 mg/l. The material was cultured to produce shoots with 3, 4 leaves for up to 4 weeks, at a light of 5000 lux, a temperature of 25 ° C and a light period of 16/8 (day/night). The environment was changed every 7 days. During regeneration of shoots from nodal segments, the hormones kinetin 1 mg/l, 3 mg/l, 5 mg/l, gibberelin 0.1 mg/l, ascorbic acid 150 mg/l, and adenine 40 mg/l were added to the WPM and MS media.

Method of treatment of calluses with EMS mutagen. The callus was transferred to banks for a complete culture medium of MS, NAA 0.3 mg/l, BAP 0.5 mg/l, activated carbon 500 mg/l, adenine sulfate 8 mg/l 25 ml each with an EMS mutagen. The mutagen concentrations were: 3 μM, 6 μM, 9 μM. After that, the cans with callus were put on a shaker at 60 rpm for 1 hour. Then the DB was washed 3 times with water. The callus was transferred to jars for a full culture medium of MS, NAA 0.3 mg/l, BAP 0.5 mg/l, coal 500 mg/l, adenine sulfate 8 mg/l 25 ml and cultured on a 120 rpm shaker for 3 days. After three days, the callus was transferred to Petri dishes on a full nutrient medium MS NUC 0.3 mg/l, BAP 0.5 mg/l, activated carbon 500 mg/l, adenine sulfate 8 mg/l.

Method of testing pomegranate plantlets for cold resistance. Testing for cold resistance of pomegranate plant clones in vitro was performed in a44Pozis XK-400-1 refrigerator. On the nutrient medium MS with half the salt concentration activated carbon 500 mg/l, adenine sulfate 16 mg/l, silver nitrate 4 mg/l, ascorbic acid 75 mg/l. With lighting of 5000 Lux, a temperature of 4°C for 30 days and a light period of 16/8 (day/night). The environment was changed every 2 weeks.

Results and discussion. At the first stage, we worked out methods for inducing calluses and regenerating shoots from them.

Callus cultures were obtained from the nodal segments of pomegranate using liquid nutrient media. Calluses from the nodal segments of the pomegranate were obtained from the cultivars Akdona, Nardan, Kazake anar, Shahrizabzs early and Pg 1 line.

Callus from nodal segments began to form in all cultivars after 7-10 days. At the same time, the most active callus formation was observed in the Akdona cultivar for 7-10 days. In the Kazake anar cultivar, callus formation begins very slowly within 30 days.

The maximum callus formation from the nodal segments of the pomegranate was obtained in the Akdona cultivar 85%, the minimum in the Kazake anar cultivar 62.5%. These calluses were later used for mutagenesis with EMS (table 1).

Table 1

Callusogenesis from nodal segments of pomegranate

The name of the genotype	Number of node segments, PCs.	Number of calluses, PCs/%
Kazake anar	240	150/62.5
Shahrizabzs	40	26/65
Akdona	20	17/85

Mass callus formation was obtained on the nodal segments of the garnet in a month (figure 1).

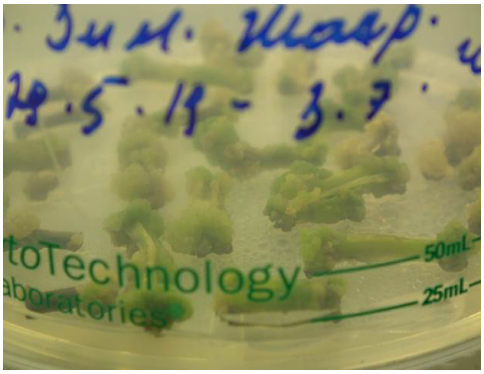

Figure 1. Regeneration of the garnet callus on the nodal segment

When cultivating shoots from nodal segments on WPM and MS media with the addition of the hormones kinetin and gibberellin, as well as ascorbic acid and adenine, plantlets were obtained (figure 2). 20% regeneration was obtained from the callus of Pg 1 cuttings on MS medium with the addition 3mg/l and 5mg/l of kinetin.

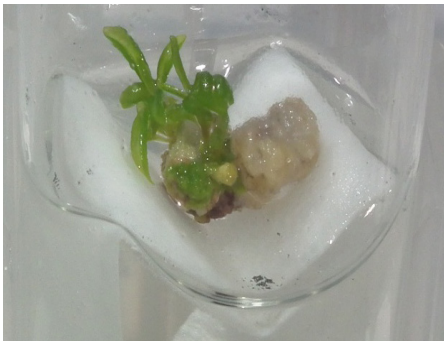

Figure 2. Regeneration from the callus of a pomegranate stalk

At the second stage, an experiment was conducted using the EMC mutagen.

After treatment with mutagen in various concentrations, calluses were obtained from the Akdona cultivar 131 PCs. (figure 3), callus survival at different mutagen concentrations is shown in table 2.

Table 2

Surviving garnet callus (%) after treatment with EMS mutagen in various concentrations

The name of the genotype	3 μM	6 μM	9 μM
Akdona	25,45	31,67	24,35

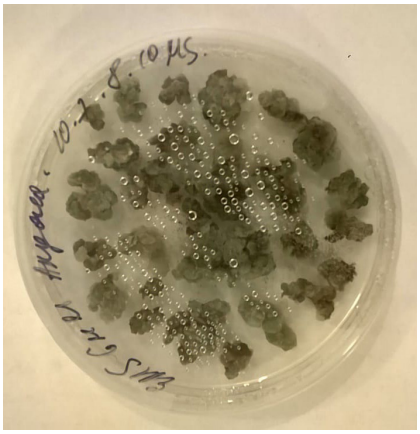

Figure 3. Garnet callus treated with EMS mutagen

In the future, was carried out regeneration, and then the cloning of plants from callus, treated with mutagens. Testing for cold resistance of pomegranate plant clones *in vitro* was performed in a Pozis XK-400-1 refrigerator for 30 days. Testing for cold resistance in the AK don cultivar treated with EMC mutagen revealed an increase in resistance with an increase in the mutagen concentration from 3 μM to 9 μM. The maximum survival rate of Akdona plantlets at an EMC concentration of 9 μM was 58% (55 out of 94 plantlets survived), and the minimum survival rate was 3% for Pg1 plantlets (8 out of 282 plantlets survived). The resulting plants of all genotypes were cloned (figure 4) and transplanted into the ground.

At the same time, control plants *in vitro* from calluses not treated with mutagen plants mostly die at 4°C after 20 - 30 days without recovery. (Table 3)

Table 3

Testing of pomegranate plantlets for cold resistance (4°C for 30 days)

The name of the genotype	Total number of plants tested	Plants survived after cold treatment	
	PCs	PCs	%
Kazeke anar (control)	197	8	4
Nardan(control)	337	13	4
Nardan, EMS (9μM)	79	24	31
Shahrizabzs (control)	211	8	4
Shahrizabzs, EMS (3μM)	46	13	29
Shahrizabzs, EMS (9μM)	15	5	36

Akdona (control)	245	12	5
Akdona, EMS (3μM)	42	10	23
Akdona, EMS (6 μM)	108	45	42
Akdona, EMS (9 μM)	94	55	58
Pg1 (control)	282	8	3
Pg1, EMS (3 μM)	17	1	7

Figure 4. Surviving pomegranate regenerant plants at 4°C

Thus, as a result of the research, morphogenic calluses were obtained and propagated. Conditions were created for processing callus cultures with the chemical mutagen EMS and mass cloning of plantlets to obtain full-fledged plants. Pomegranate plantlets were tested for cold resistance in vitro.

Conclusions. In the course of this work, the selection of conditions for obtaining garnet calluses from nodal segments was carried out. Morphogenic callus capable of regenerating full-fledged plants were obtained on solid and liquid nutrient media. The maximum callus formation from nodal segments was observed in the Akdona cultivar and Pg 1 line, and the minimum in the Kazake anar cultivar.

In the Akdon cultivar, 25.45% of callus survival was detected when exposed to the 3 μM EMS mutagen, 31.67% when exposed to the 6 μM concentration, and 24.35% when exposed to the 9 μM concentration.

The maximum survival rate of mutated plantlets of the Akdon cultivar was 58%, and the minimum survival rate was 3% for control plantlets of the Pg1 line.

The mutant pomegranate plants obtained during the experiment were propagated and transplanted into the ground for further research on their resistance to abiotic stresses.

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Мутагенез арқылы суыққа төзімді анар (Punica granatum L.) линияларын алу

Аңдатпа. Қазақстанда жеміс ағаштарының көптеген түрлері өсіріледі, оның бірі - анар *(Punica granatum L.).* Анар өсіруді шектейтін фактор - бұл қыстың аязды болуы, сондай-ақ, көктем мен күздегі төмен температура. Төмен оң температураға төзімді мутанттарды алу үшін химиялық мутагенез, ал мутаген ретінде этилметансульфонат (ЭМС) қолданылды. Каллустар *in vitro* культурада мутагенмен өңделді. Ақдона сортында ЭМС мутаген концентрациясы 3 μМ болған кезде каллустардың тіршілік ету коэффициенті 25,45%, 6 μМ концентрацияда - 31,67%, 9 μМ-де тіршілік ету коэффициенті 24,35% құрады. Мутагенмен өңделген каллустардан өсімдік регенерациялану процесі пайда болды. Алынған өсімдіктер төмен оң температурада сыналды (40С, 30 күн). Суықпен әсер еткеннен соң Ақдона сортының мутантты регенеранттарының максималды тіршілік ету деңгейі 58%-ды құрады, Pg1линиясының регенеранттарының тіршілік ету көрсеткіші ең төменгі деңгейді 3%-ды құрады. Алынған мутантты алдағы уақытта суыққа төзімді анар өсімдіктерін сұрыптау жұмыстары үшін пайдаланылатын болады.

Түйін сөдер: анар, мутагенез, этилметансульфонат (ЭМС), суыққа төзімді.

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Получение холодоустойчивых линий граната (Punica granatum L.) путем мутагенеза

Аннотация. В Казахстане выращивается большой пул фруктовых деревьев, одним из которых является гранат *(Punica granatum L.).* Наиболее существенным лимитирующим фактором выращивания граната в Казахстане является морозная зима, а также низкие температуры весной и осенью. С целью получения мутантов, устойчивых к низким положительным температурам использовали химический мутагенез, в качестве мутагена – этилметансульфонат (ЭМС). Обработке мутагеном подвергались каллусы в культуре *in vitro.* У сорта Ақдона при концентрации мутагена ЭМС 3μМ выживаемость каллусов составила 25,45%, при концентации 6μМ – 31,67%, при 9μМ выживаемость составила 24,35%. Из обработанных мутагеном каллусов индуцировался процесс регенерации растений. Полученные растения подвергались воздействию низких положительных температур (40С, в течении 30 дней). После воздействия

холодом максимальная выживаемость мутантных регенерантов сорта Акдона составила 58%, минимальная выживаемость была у регенерантов линии Pg1 – 3%. Полученные мутантные растения граната будут использованы для селекции на устойчивость к холоду.

Ключевые слова: гранат, мутагенез, этилметансульфонат (ЭМС), холодоустойчивость.

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