Use of chemical mutagens for production of inactive pollen grains, embryo rescue, and morphological changes in cucumber

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Abstract: Application of chemical mutagens as important factors is very practical for successful haploidization techniques in Cucurbitaceae through pollen grain deactivation and then immature embryo rescue. The present research investigated the effects of genotype, male flower age, receptor plant, and chemical mutagenesis (NaN3 and colchicine) on pollen grain deactivation as well as seed production (number of total, full, half-full, and empty seeds) and morphological traits. Moreover, the effects of different plant growth regulators were tested on embryo cultures. The effects of different factors (mutagen, genotype, flower age) were investigated on plant regeneration from immature embryos that were inspected from half-full and empty seeds. Based on this study, some mutagenic treatments (0.005 colchicine and 0.0012 NaN3) led to the highest values of morphological and fruit yield traits in control plants, while 0.005 and 0.025 NaN3 led to reduced values of these traits. Among the genotypes, NBDC3 showed the highest number of different types of seeds. Among different chemical mutagens, NaN3 (0.0012 mg/L) and colchicine (0.005 mg/L) produced the greatest effects on seed production traits. Moreover, the highest values for all seed-related traits were recorded for crossing with fresh male flowers, whereas crossing with old male flowers contributed to the decline of seed-related traits. Mutagen-treated plants showed the highest empty seed numbers. Furthermore, the highest total and half-full seed numbers were obtained using 0.0012 and 0.005 mg/L NaN3, and colchicine. Results revealed that the highest and lowest regeneration percentages (66.67% and 26.67%) belonged to the media containing BAP+Kin+IBA (2+1+0.5) mg/L and BAP+Kin+IBA+NAA (2+1+0.5+0.1) mg/L, respectively. These media were used for immature embryo culture. Finally, genotypes NBDC1 and NBDC3 treated with 0.005 colchicine and 0.012 NaN3 showed higher frequencies of plant regeneration from immature embryos extracted from half-full and empty seeds.

Key words: Embryo, flower age, seed, sodium azide

1. Introduction
Horticultural plants consisting of fruits and vegetables are genetically very diverse and are consumed by people as edible products, culinary ingredients, and medicinal items. Consumption of them plays a key role in human nutrition and health as they are sources of vitamins, minerals, antioxidants, dietary fiber, and phytonutrients (plant-derived micronutrients) (Benjak et al., 2005; Ercisli et al., 2010; Canan et al., 2016; Hricova et al., 2016; Zorenc et al., 2016). Different genotypes including landraces, open-pollinated, and hybrid cucumbers are globally popular cultivars. While it seems impossible to produce 100% homozygote plants by open pollination, in vitro techniques have marked a period of reform in obtaining pure lines over short spans of time. One of the fastest and most effective methods is the production of double-haploid plants (Baktemur et al., 2013), for which two laboratory procedures have been developed and are now used commonly. The first consists of using either an ovary or an ovule culture. The second method, called haploid parthenogenesis, based on pollination attributes, consists of pollination with irradiated or inactive pollen grains followed by embryo rescue conducted in an in vitro culture in order to prevent embryo abortion (Gemes-Juhasz et al., 2002; Reed 2005). Nunez Palenius et al. (2011) found that in vitro melon embryo rescue played an important role in obtaining viable and functional plantlets.

The effects of mutagenic factors on pollen sterility have been studied in some plants in order to evaluate dependency of pollen sterility on mutagen dose/concentration (Satpute and Fultambkar, 2012). However, these factors may affect ovule and ovary structure. The use of irradiated pollen has proved to be the most successful haploidization technique in Cucurbitaceae (Baktemur et al., 2013). After harvesting of fruits that were pollinated with irradiated pollen, the
classical method of “inspecting the seeds one by one” is used to find haploid embryos in the seeds (Baktemur et al., 2013). In using this technique, flower age has also been investigated as a decisive factor affecting the longevity and viability of pollen grains. This technique resulted in haploid embryo production. Baktemur et al. (2013) compared the various techniques of separation of haploid embryos (inspecting the seeds one by one, sowing seeds directly in nutrient media, and inspecting seeds in a light source), which were subsequently optimized for cucurbits by Navratilova et al. (2011). Despite its limitations, parthenogenesis is routinely used in cucumber breeding procedures to achieve complete homozygosity in one generation (Claveria et al., 2005). Kurtar (2009) showed that enhanced irradiation or increasing dosage of or extended exposure to chemical agents and other pollen-related factors (age and viability) led to significant declines in germination ability and fruit and seed-set traits such that only a small percentage of the seeds from irradiated pollen grains included haploid embryos in the embryo rescue. The inspection of seeds one by one for rescuing the embryos is a tedious and time-consuming task, which declines haploid production efficiency for cucurbit plants.

Sodium azide (NaN₃) is a chemical mutagen that creates point mutations in the genome of plants by producing metabolites and the proteins produced in the mutant plant assume functions different from those in normal plants (Al-Qurainy and Khan, 2009). Investigation of the effects of mutagenic NaN₃ on in vitro development of four pea cultivars revealed that, based on LD₅₀ results, 0.001 M sodium azide was not highly lethal; however, it was the most appropriate for inducing mutagenesis (Divanli-Türkan et al., 2006).

The present study was designed and implemented to: 1) investigate the effects of flower age (fresh and old), receptor plants (mutagen-treated and nontreated), and chemical mutagenesis on deactivation of pollen grains; 2) produce different types of seeds in fruits obtained from deactivated pollen grains pollination; 3) rescue immature embryos from half and empty seeds and study the effects of different plant growth regulators (PGRs) on regeneration rates; and 4) evaluate variation in morphological traits in mutagen-treated plants in a greenhouse.

2. Materials and methods

2.1. Plant preparation

The present experiment was carried out in 2014 and 2015 at Islamic Azad University, Isfahan (Khorasgan) Branch, on the four greenhouse cucumber genotypes of NBDC1, NBDC2, NBDC3, and NBDC4 as the breeding lines developed at this university. Initially, in autumn 2014, 200 seeds of each cucumber genotype were presoaked in mutagen solutions including three levels of NaN₃ (0.0012, 0.002, and 0.005 mg/L), one level of colchicine (0.005 mg/L), and a control (water) for 24 h. The seeds were then rinsed in tap water before being immediately transferred to plastic pots containing a coco-peat and peat-moss substrate (1:1). Finally, 150 seedlings (treated and nontreated) of each cucumber genotype were planted in greenhouse soil (25–30/19–21 °C day/night at a relative humidity of about 60%). Various fertilizers were applied based on the results of greenhouse soil analysis, and irrigation regimes were adjusted according to crop water requirements. After about 45 days, two types of male flowers from mutagen-treated plants (one at the day of anthesis and the other one day after the anthesis stage) were used for crossing with female flowers in both control and mutagen-treated plants; hence, the receptor plants were of two types, mutagen-treated and control. The number of crossings in every plant was 5 with 75% to 95% success in every plant. All fruits obtained in every treatment were harvested 23 to 25 days after pollination. This was enough time for immature embryo production. Before extracting the seeds, harvested fruits were treated with 70% ethanol on the surface. At the end, seed production traits including numbers of total, full, half-full, and empty seeds were recorded. For cytological study of pollen grains, the fresh pollen grains of control and mutagen-treated plants and also pollen grains from fresh and old male flowers were assayed. Aceto-carmen (1%) was applied for pollen grain dyeing and then the percentages of deactivated pollens were measured based on the dye.

2.2. Tissue culture and embryo rescue

In early 2015, for the developmental growth of immature embryos, only sterilized half-full and empty seeds were transferred into an E₂₀ liquid medium (Sauton and Dumas de Vaulx, 1987). Full seeds were omitted from the experiment because these seeds were a consequence of crossing between active pollen grains and ovules; as a result, they were not haploid. After 10 days of E₂₀ treatment, the half-full seeds were carefully separated one by one and the embryos were neatly removed from the seeds. The sterilized embryos were then placed in MS basal medium (Murashige and Skoog, 1962) and solidified with 0.8% agar for regeneration in 200-mL culture bottles in each of which six immature embryos were cultured. The culture medium contained 3% sucrose and hormonal combinations including BAP+Kin+IBA (2+1+0.5) mg/L. To obtain this medium for immature embryo culture, initially an experiment was performed with different combinations of cytokinins BAP (1, 2 mg/L) and Kin (1, 3 mg/L) or in combination with auxins IBA (0.5 mg/L), IAA (1 mg/L), and NAA (0.1 mg/L) at 5 different concentrations and combinations of PGRs. Regeneration, shooting, and rooting rates were recorded in this experiment. All the immature embryo cultures were incubated in the dark at

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25 °C until they were swollen, then transferred to a cool room lit with fluorescent lights (16/8-h light/dark regime) at 25 °C and allowed to grow. Finally, the plantlets obtained from immature embryo culture were counted.

2.3. Experimental design
There were three separate experimental designs in this study. In the greenhouse experiment, 8 plants in each replication and totally about 600 plants for all treatments and genotypes were planted. In this experiment, two factors, mutagen treatments and genotypes as a completely randomized factorial design, were analyzed. The effects of chemical mutagenic agents were investigated on fruit yield after about 50 days. Plant height (from the first node on the soil surface up to the tip), leaf number, and the number of deformed fruits were recorded. In the second greenhouse experiment, the four factors included genotype at 4 levels, mutagen treatment at 5 levels, flower age at 2 levels, and receptor plants at 2 levels with three replications applied in seed production studies. This experiment was also conducted in a completely randomized factorial design. In the third experiment, a completely randomized design was used for determining the best hormonal combination and concentrations for immature embryo culture. In the final experiment, only the frequency of plantlet production was reported for every medium culture. The data were analyzed by SAS software (Ver. 9.1) and the least significant difference (LSD) test was used at the 5% probability level (P ≤ 0.05) for mean comparisons.

3. Results and discussion
3.1. Greenhouse experiment
3.1.1. Morphological traits
Study of the effects of mutagenic treatments on morphological traits showed that the highest total fruit yield was obtained in control plants with no significant differences due to the application of either colchicine or sodium azide at 0.005 and 0.0012 mg/L, respectively (Table 1). The same was observed for other traits such as fruit yield per harvest and plant height. Moreover, the highest number of deformed fruits was observed with a NaN3 concentration of 0.025 mg/L, which had no significant difference from that obtained from a concentration of 0.005 mg/L (Table 1). Overall, the highest values of morphological and fruit yield traits were invariably achieved in the control, 0.005 colchicine, and 0.0012 mg/L NaN3 treatments; however, 0.005 and 0.025 mg/L concentrations of NaN3 led to reduced values of these traits. Thus, concentrations of 0.005 and 0.0012 mg/L of colchicine and NaN3 treatments, respectively, were considered as insufficient as they yielded results similar to the control. On the contrary, the 0.005 and 0.025 mg/L NaN3 treatments affected the studied traits; these concentrations could then be exploited for inducing modifications in plant structure (Table 1). Adebola (2013) used different concentrations of NaN3 on tomato seeds. The results showed that morphological growth parameters such as plant height and yield per plant decreased with increasing NaN3 concentration.

Table 1. Effects of different mutagen treatments and genotypes on growth and fruit yield traits in greenhouse experiment.

<table>
<thead>
<tr>
<th>Main factors</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagen (mg/L)</td>
<td>Total fruit yield (g)</td>
</tr>
<tr>
<td>Control</td>
<td>2374a</td>
</tr>
<tr>
<td>Colchicine (0.005)</td>
<td>2128.2ab</td>
</tr>
<tr>
<td>NaN3 (0.025)</td>
<td>1557.3bc</td>
</tr>
<tr>
<td>NaN3 (0.005)</td>
<td>940.1c</td>
</tr>
<tr>
<td>NaN3 (0.0012)</td>
<td>2087.4ab</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
</tr>
<tr>
<td>NBDC1</td>
<td>1982.4b</td>
</tr>
<tr>
<td>NBDC2</td>
<td>2183.6a</td>
</tr>
<tr>
<td>NBDC3</td>
<td>1769.8ab</td>
</tr>
<tr>
<td>NBDC4</td>
<td>1700.9ab</td>
</tr>
</tbody>
</table>

Traits with identical letters are compared with each other. Means followed by the same letters are not significantly different at the 5% level according to the LSD test.
Results of mean comparisons among the genotypes demonstrated that NBDC4 and NBDC1 had the highest and lowest fruit yield per harvest and leaf number, respectively. While NBDC2 had the highest fruit yield, it was not significantly different from those recorded for NBDC3 and NBDC4 (Table 1). Furthermore, the highest and lowest values of plant height belonged to NBDC3 and NBDC1, respectively. NBDC4 and NBDC1 had the highest and lowest numbers of deformed fruits, respectively. It may therefore be asserted that the genotypes treated with different NaN₃ concentrations exhibited a wide variation in terms of their plant growth and fruit yield up to 50 days after sowing.

NBDC1 and NBDC2 genotypes under control and NaN₃ (0.0012 mg/L) treatments showed the highest amount of total fruit yield and fruit yield per harvest. In addition, genotype NBDC1 showed the lowest amounts of all in Na₅ (0.005 mg/L) and NaN₃ (0.025 mg/L) treatments (Table 2). The least amounts of all was observed in NBDC1, NBDC2, and NBDC3 genotypes under NaN₃ (0.025 mg/L) treatment (Table 2). However, the NBDC4 genotype was not influenced under NaN₃ (0.025 mg/L) treatment.

3.1.2. Seed production experiment
In the second experiment, the effects of the four factors, including genotype, mutagenic agent, flower age, and receptor plants, were studied on the seed production traits.

3.1.2.1. Effect of genotype on seed production
The results of this experiment indicated that the highest and lowest full seed numbers belonged to the NBDC3 and NBDC1 genotypes, respectively (Figure 1A). It may thus be concluded that the genetic structures of different cucumber cultivars have functional effects on the seed production traits. These variations in genetic background might also play vital roles in seed traits. The significant effect of genotype on the different traits might also be attributed to genetic diversity since, for instance, the highest numbers of total and empty seeds were observed in NBDC3 and NBDC1, respectively. Compared to other genotypes, NBDC3 generally had the highest number of different kinds of seeds.

3.1.2.2. Effect of mutagen treatment on seed production
Another finding from analysis of variance indicated that mutagen treatment had significant effects on all the studied traits. Chemical mutagens seem to affect seed production traits through their effects on the molecular and cellular

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Total fruit yield (g)</th>
<th>Fruit yield per harvest (g)</th>
<th>Plant height (cm)</th>
<th>Deformed fruit number</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBDC1</td>
<td>Control</td>
<td>3773a</td>
<td>362.3abc</td>
<td>248d</td>
<td>3ob</td>
<td>32a</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.0012)</td>
<td>3997.5b</td>
<td>640.8ab</td>
<td>383c</td>
<td>5d</td>
<td>26abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.005)</td>
<td>453d</td>
<td>67dab</td>
<td>98d</td>
<td>0.75d</td>
<td>20d</td>
</tr>
<tr>
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<td>NaN₃ (0.025)</td>
<td>315d</td>
<td>59dab</td>
<td>83d</td>
<td>0.66d</td>
<td>17d</td>
</tr>
<tr>
<td></td>
<td>Colchicine (0.005)</td>
<td>2301.3bc</td>
<td>287.6abc</td>
<td>183d</td>
<td>3b</td>
<td>22c</td>
</tr>
<tr>
<td>NBDC2</td>
<td>Control</td>
<td>2814.4bc</td>
<td>303.6ab</td>
<td>276d</td>
<td>3.35d</td>
<td>26.5abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.0012)</td>
<td>3127.7bc</td>
<td>347.4abc</td>
<td>140f</td>
<td>1.36d</td>
<td>28abc</td>
</tr>
<tr>
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<td>NaN₃ (0.005)</td>
<td>2480.5abc</td>
<td>291.4abc</td>
<td>260d</td>
<td>3b</td>
<td>29.5abc</td>
</tr>
<tr>
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<td>NaN₃ (0.025)</td>
<td>865abc</td>
<td>198.3bc</td>
<td>173f</td>
<td>1.5d</td>
<td>21f</td>
</tr>
<tr>
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<td>Colchicine (0.005)</td>
<td>2103.3bc</td>
<td>327.3bc</td>
<td>224f</td>
<td>0.53d</td>
<td>26abc</td>
</tr>
<tr>
<td>NBDC3</td>
<td>Control</td>
<td>1898.3abc</td>
<td>287.4abc</td>
<td>270h</td>
<td>2.66abc</td>
<td>25.33abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.0012)</td>
<td>1616abc</td>
<td>261.5abc</td>
<td>261abc</td>
<td>1.75d</td>
<td>31h</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.005)</td>
<td>2920abc</td>
<td>486.6abc</td>
<td>210d</td>
<td>3b</td>
<td>26abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.025)</td>
<td>1105.3d</td>
<td>197.7d</td>
<td>244d</td>
<td>2dxc</td>
<td>27.33abc</td>
</tr>
<tr>
<td></td>
<td>Colchicine (0.005)</td>
<td>2127.3abc</td>
<td>319.8abc</td>
<td>262abc</td>
<td>1.66abc</td>
<td>27.33abc</td>
</tr>
<tr>
<td>NBDC4</td>
<td>Control</td>
<td>1813.2abc</td>
<td>312.4abc</td>
<td>236d</td>
<td>3.25ab</td>
<td>27abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.0012)</td>
<td>1232bc</td>
<td>253.7bc</td>
<td>261d</td>
<td>3.33b</td>
<td>29abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.005)</td>
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<td>365.7abc</td>
<td>214e</td>
<td>2dxc</td>
<td>30abc</td>
</tr>
<tr>
<td></td>
<td>NaN₃ (0.025)</td>
<td>1707.5abc</td>
<td>369.9abc</td>
<td>243d</td>
<td>3.5a</td>
<td>32abc</td>
</tr>
<tr>
<td></td>
<td>Colchicine (0.005)</td>
<td>1996.2abc</td>
<td>374abc</td>
<td>252d</td>
<td>2.75abc</td>
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</tr>
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Traits with identical letters are compared with each other. Means followed by the same letters are not significantly different at the 5% level according to the LSD test.
structures of pollen grains. Examination of the effects of different chemical mutagens on the seed production traits demonstrated that NaN₃ (0.0012 mg/L) and colchicine (0.005 mg/L) gave rise to the highest values of these traits (with the exception of full seed number), whereas the highest numbers of full seeds were observed in the control and 0.0012 mg/L sodium azide treatments (Figure 1B). Among mutagen treatments, 0.0012 mg/L sodium azide and 0.005 mg/L colchicine had positive effects on the number of half-full seeds (Figure 1B). In their assessment of mutagenic effects of sodium azide and fast neutron irradiation, Abubakar et al. (2015) reported that pollen production declined in all the treated plants when compared with the control. Their results indicated that the significant difference between the number of pollen grains per flower and anther might be attributed to the slight differences in the genetic composition of the plant.

3.1.2.3. Effect of flower age on seed production
It seems that flower age plays an important role in fertility and seed-related traits through its effects on molecular and cellular attributes. Comparison of means demonstrated that the highest values of all the studied traits (except for half-full seeds) belonged to the fresh flower treatment, whereas old flowers exhibited declining values for all the traits (except half-full seeds) (Figure 2A).

3.1.2.4. Effect of receptor plant on seed production
The results of variance analysis revealed that receptor plants had significant effects on all of the examined traits except for the number of half-full seeds. Mean comparisons demonstrated that neither the control nor the treated plants showed any significant differences in number of half-full seeds (Figure 2B), with the highest number of half-full seeds (42.65) recorded for treated plants (mean of all mutagen treatments in every genotype). The highest numbers of total and full seeds were observed in the control plants (Figure 2B). This result is logically expected because treated plants were exposed to chemical mutagenesis, which affected both the ovule structure and the pollen grains, thereby enhancing the probability of the formation of empty seeds.
3.1.2.5. Interaction assessment studied factors on seed production

Study of interaction effects revealed that application of 0.0012 mg/L sodium azide and 0.005 mg/L colchicine on NBDC1 and NBDC3, respectively, led to the highest levels of half-full seeds in these genotypes (Figure 3A). The lowest level of total seeds was obtained with 0.005 and 0.025 mg/L sodium azide applied to genotype NBDC4 (Figure 3B). On the other hand, this genotype showed the highest number of total seeds under control and 0.0012 mg/L sodium azide treatments.

The highest levels of total and half-full seeds were obtained from 0.0012 and 0.005 mg/L sodium azide and colchicine, respectively, in treated-plants (Figures 4A and 4B). Although the control plants did not exhibit any significantly different responses from any of the mutagen treatments, genotypes had the lowest number of total seeds when 0.025 mg/L sodium azide was applied (Figure 4B).

Based on our results, the highest level of half-full seeds was recorded with 0.0012 mg/L sodium azide treatment plants as receptor plants in crossings with fresh male flowers, which varied significantly in response to other mutagen treatments (Figure 5A). The highest number of total seeds was obtained from fresh male flowers picked from plants treated with 0.005 mg/L colchicine (Figure 5B). Moreover, fresh male flowers showed no significant differences in their response to any of the mutagen treatments (with the exception of sodium azide at 0.025 mg/L) while old flowers treated with 0.005 and 0.025 mg/L mutagen treatments showed different responses (the lowest amount of total seeds). No significant differences were observed among the four genotypes in terms of their total seed number when crossed with fresh male flowers; however, the old flower treatment produced the lowest number of total seeds in NBDC2, NBDC3, and NBDC4 (Figure 6).

The results of variance analysis showed that none of the triple interactions had any significant effects on half-full and empty seed numbers. However, these interactions had positive and significant effects on the number of total seeds. This also held true for quadruple interactions.
3.2. Embryo culture experiment

This experiment was done for determining the best hormonal combination and concentrations for immature embryo culture. The ANOVA results demonstrated that addition of PGRs as hormonal treatment to the culture media had significant effects on regeneration, shooting, and rooting traits. The highest and lowest regeneration rates (66.67% and 26.67%) were obtained with the media containing BAP+Kin+IBA (2+1+0.5) mg/L and BAP+Kin+IBA+NAA (2+1+0.5+0.1) mg/L, respectively (Table 3). The highest shooting and rooting percentages were obtained with the BAP+Kin+IBA medium at a concentration of 2+1+0.5 mg/L. Cain et al. (1983) reported the clear influence of genotype on the capacity to form viable embryos in their cultures of seedless variants. It has been reported that many factors, including genotype, sampling/inoculation time, nutrient medium, culture methodology, and utilization of PGRs, might be involved in the success of the technique (Li et al., 2015).

Variation in BAP concentrations of the two observed culture media led to considerable reduction in shooting rates. Shooting rate increased when IBA was applied in combination with BAP+Kin. Grozeva and Velkov (2014) reported on in vitro plantlet regeneration obtained in hypocotyl explants with 1 and 2 mg/L BAP and induced regeneration in cotyledons established with BAP+IAA (3+0.5 mg/L). The types and concentrations of media supplements reportedly depend greatly on the development stage of the embryos (Li et al., 2014).

3.3. Regeneration frequency from immature embryo culture

Although plant regeneration frequencies from different treatments and genotypes were low, genotypes NBDC1 and NBDC3 when treated with 0.005 colchicine and 0.0012 mg/L NaN₃ showed higher frequencies of plant regeneration from immature embryos extracted from half-full and empty seeds (Table 4). The two mutagen treatments with 0.005 and 0.025 mg/L NaN₃ did not induce any
plantlets in empty and half-full seeds, seemingly due to the high concentrations of mutagens and death of pollen grains rather than the production of inactive pollen grains. Lotfi et al. (2003) noted that it was tedious and time-consuming to identify and excise induced embryos via opening and examining individual seeds. They therefore investigated different procedures for efficient haploid plant production in melon (*Cucumis melo* L.). Their results showed that the rate of embryos obtained through their procedures varied from 5% in Samsoori to 0.4% in the Talaie cultivar.

3.4. Cytological experiment
Cytological observations revealed greater numbers of inactive pollen grains in old flowers than in fresh ones. The number of inactive pollen grains was higher in mutagen-treated plants in contrast to old and fresh flowers. These results confirmed that the increased number of half-full seeds is due to the inactive pollen grains in old flowers. In our previous study (Hazem and Golabadi, 2016) the highest percentage of inactive pollen grains was obtained in mutagen-treated male flowers. Rosell et al. (2006) found no difference between the performance of pollen grains 90 min after their dehiscence and that of freshly dehisced ones; however, in vivo pollen grains showed a clear reduction in vigor and germinated much more slowly 120 min after dehiscence. In the current experiment, pollen grains were observed to lose their viability and function with positive effects due to molecular and cellular attributes of fertility and seed production.

4. Conclusions
In the current study, three main experiments were done on cucumber. In the first greenhouse assessment, among mutagen treatments, the 0.005 and 0.025 mg/L NaN₃ concentrations affected the studied traits, so these concentrations could then be exploited for inducing modifications in plant structure. In the second greenhouse experiment, seed production was investigated. Results illustrated that the genotype as a main factor had functional effects on the seed production traits and these effects were related to the genetic structure of studied cultivars. The mutagen treatments had some effects on the molecular and cellular structure of pollen grains and...
Figure 5. Interaction effects of flower age and mutagen treatments on total and half-full seed numbers. Means followed by the same letter are not significantly different.

Figure 6. Interaction effect of flower age and genotypes on total seed number. Means followed by a common letter are not significantly different.
showed that NaN₃ (0.0012 mg/L) and colchicine (0.005 mg/L) gave rise to the highest levels of seed production traits, except full seed numbers. Another factor, flower age, played an important role in fertility and seed-related traits, as the old flowers and mutagen-treated flowers caused the highest number of half-full seeds. The receptor plants had considerable effects on all the traits examined except for the number of empty seeds. These plants were exposed to chemical mutagenesis, which affected both the ovule structure and the pollen grains, thereby enhancing the probability of formation of half-full and empty seeds. In the third experiment, hormonal treatment had significant effects on regeneration, shooting, and rooting traits from embryo culture, and variation in BAP concentrations of the two culture media was observed to lead to considerable reduction in shooting levels. This experiment showed which of the culture media was suitable for immature culture extracted from empty and half-full seeds. Finally, the percentage of regeneration from empty seeds was low, some genotypes at some concentrations of mutagen showed better results.

### Table 3. Effects of different PGR combinations and concentrations on regeneration, shooting, and rooting traits in embryo culture.

<table>
<thead>
<tr>
<th>Plant growth regulators</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormonal combination</td>
<td>Concentration (mg/L)</td>
</tr>
<tr>
<td>BAP + Kin</td>
<td>1 + 3</td>
</tr>
<tr>
<td>BAP + Kin</td>
<td>2 + 1</td>
</tr>
<tr>
<td>BAP + Kin + IBA</td>
<td>2 + 1 + 0.5</td>
</tr>
<tr>
<td>BAP + Kin + IAA</td>
<td>2 + 1 + 1</td>
</tr>
<tr>
<td>BAP + Kin + IBA + NAA</td>
<td>2 + 1 + 0.5 + 0.1</td>
</tr>
</tbody>
</table>

Traits with identical letters are compared with each other. Means followed by the same letters are not significantly different.

### Table 4. Frequency of plantlets obtained from immature embryos extracted from half-full and empty seeds.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagens</td>
<td>Genotypes</td>
</tr>
<tr>
<td>Control</td>
<td>NBDC 1, 3, 4</td>
</tr>
<tr>
<td>Empty</td>
<td>-</td>
</tr>
<tr>
<td>Half-full</td>
<td>1.14</td>
</tr>
<tr>
<td>0.0012 mg/L NaN₃</td>
<td>NBDC 1, 3</td>
</tr>
<tr>
<td>Empty</td>
<td>1.06</td>
</tr>
<tr>
<td>Half-full</td>
<td>2.14</td>
</tr>
<tr>
<td>0.005 mg/L NaN₃</td>
<td>NBDC 1, 3</td>
</tr>
<tr>
<td>Empty</td>
<td>-</td>
</tr>
<tr>
<td>Half-full</td>
<td>0.92</td>
</tr>
<tr>
<td>0.025 mg/L NaN₃</td>
<td>NBDC 2</td>
</tr>
<tr>
<td>Empty</td>
<td>-</td>
</tr>
<tr>
<td>Half-full</td>
<td>0.87</td>
</tr>
<tr>
<td>0.005 mg/L Colchicine</td>
<td>NBDC 1, 3</td>
</tr>
<tr>
<td>Empty</td>
<td>2.56</td>
</tr>
<tr>
<td>Half-full</td>
<td>2.43</td>
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</tbody>
</table>
References


