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Determination of Effective Mutation Dose on Walnut (*Juglans regia* L. cv. Chandler) Budwoods

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Abstract: Walnut (*Juglans regia* L., $2n=2x=32$, Juglandaceae) is a deciduous temperate fruit species with an increasing economic importance and health benefits. The effective mutation dose (EMD) was not determined for 'Chandler' budwoods. The objective of this study was to determine the radiosensitivity of walnut budwoods to cobalt-60 gamma ray. One-year-old budwoods of 'Chandler' walnut cultivar carrying 4-5 buds in 20 cm in length were irradiated with cobalt-60. The gamma irradiation and chip budding were performed three different times. The budwoods were irradiated (1) with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 Gy gamma doses on Apr 2017, (2) with 0, 20, 25, 30, 35, 40 Gy gamma doses on Sep 2017, and (3) with only 42 Gy gamma dose on Apr 2018. Then, gamma irradiated budwoods were chip budded on seedling rootstocks. Mutation 1 Vegetation 1 (M1V1) plants were obtained. The humidity content of the budwoods was determined after gamma irradiation. The bud take ratio, shoot length, and chlorophyll density of M1V1 plants were measured. From the shoot length of the plants, EMD was calculated as 42.1 Gy after the first irradiation on Apr, 2017, and that was calculated as 20.9 Gy in the second irradiation on Sep, 2017. The main plant population was obtained by previously calculated 42.1 Gy dose in the third irradiation on Apr, 2018. The survival rates of budded plants in three irradiation experiments were 21.6%, 54.8%, and 32.0%, respectively. According to the results, the most suitable gamma ray dose is 42.1 Gy.

Keywords: dormant budding, budwood, radiosensitivity, fruit breeding, physical mutagen, gamma irradiation

Ceviz (*Juglans regia* L. cv. Chandler) Aşı Gözleri Üzerine Etkili Mutasyon Dozunun Belirlenmesi

Öz: *Ceviz (Juglans regia L., 2n=2x=32, Juglandaceae), ekonomik önemi ve sağlık açısından faydaları artan, yaprak döken ılıman iklim meyve türüdür. 'Chandler' aşı gözlerinin etkili mutasyon dozu (EMD) henüz belirlenmemiştir. Bu çalışmanın amacı, ceviz aşı gözlerinin kobalt-60 ışınlamasına hassasiyetini belirlemektir. Üzerinde 4-5 aşı gözü bulunan 20 cm uzunluğundaki 'Chandler' ceviz çeşidi aşı kalemleri kobalt-60 ışınlamasına maruz bırakılmıştır. Üç farklı dönemde gama ışınlaması ve yongalı göz aşısı yapılmıştır. Aşı kalemleri (1) Nisan 2017'de 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 Gy gama dozlarına, (2) Eylül 2017'de 0, 20, 25, 30, 35, 40 Gy gama dozlarına ve (3) Nisan 2018'de 42 Gy gama dozuna tabi tutulmuşlardır. Gama uygulaması yapılan aşı kalemleri çöğür anaçları üzerine yongalı göz aşısı ile aşılanmıştır. Mutasyon 1 Vejetasyon 1 (M1V1) bitkileri elde edilmiştir. Aşı kalemlerinin nem içeriği gama ışınlamasından sonra belirlenmiştir. M1V1 bitkilerinin aşı tutma oranı, sürgün boyu ve klorofil yoğunluğu ölçülmüştür. Aşılamadan elde edilen bitkilerin sürgün uzunluklarından, birinci ışınlamadaki EMD=42.1 Gy olarak ve ikinci ışınlamadaki EMD=20.9 Gy olarak belirlenmiştir. Üçüncü ışınlama döneminde sadece daha önce belirlenen 42.1 Gy dozu uygulanılarak ana populasyon bitkileri oluşturulmuştur. Üç ışınlama denemesinden elde edilen aşılı bitki yaşama oranları sırasıyla %21.6, %54.8 ve %32.0. Bu sonuçlara göre, 'Chandler' aşı kalemlerine yapılacak en uygun gama dozu 42.1 Gy'dir.*

Anahtar Kelimeler: *durgun yongalı göz aşısı, aşı gözü, radyasyon hassasiyeti, meyve ıslahı, fiziksel mutajen, gama ışınlaması*

INTRODUCTION

Juglans regia ($2n=2x=32$) is one of the oldest species in the Juglandaceae family for commercial nut production in the world (Bernard et al., 2018; Karadeniz, 2005; Şen, 2011; Tekintaş et al., 2014). Walnut is preferred by consumers for its health benefits of the omega-3 fatty acid. The major scion breeding objectives in walnut are late leafing out, lateral fruitfulness, low pistillate flower abscission, high yield and kernel quality, precocity, range of harvest season, and low blight scores. 'Chandler' is one of the most desired walnut cultivar in the world, and this cultivar is highly fruitful on lateral buds. Since it has a very vigorous branching habit under environmental conditions, it needs some pruning, particularly to avoid shoots with a narrow crotch angle (Tulecke and McGranahan, 1994). Tolerance to abiotic and biotic stress factors are the most important rootstock breeding aims in walnuts (Karimi et al., 2018; McGranahan and Leslie, 2012; Vahdati and Lotfi, 2013; Vahdati et al., 2009). Reducing plant height needs to be evaluated within *Juglans* spp. germplasm for the ease of cultural practices in high-density plantings (Vahdati et al., 2019).

When sufficient variation does not exist because of improvements in vegetative propagation of walnut (Ebrahimi and Vahdati, 2006; Rezaee et al., 2008), both natural and induced mutations can be used in plant

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breeding to obtain higher and healthier agricultural products of variant genotypes one or few characteristics differ from their respective progenitors (Jankowicz-Cieslak et al., 2017). Induced mutations can be achieved by either chemical or physical mutagens. Protocols and general considerations for induced mutations in seed and vegetatively propagated plants using gamma rays and EMS (ethyl methane sulfonate), the chemical mutagen, have been previously discussed (Bado et al., 2015; Lamo et al., 2017; Maluszynski et al., 2009; Oladosu et al., 2016; Saito, 2016; Tosri et al., 2019; Ulukapi and Nasircilar, 2015; Yadav et al., 2019). Physical mutagens are comprised of non-ionizing (microwave, ultraviolet light, ultra-high hydrostatic pressure, hydrothermal treatment) and ionizing (X-rays, gamma rays, alpha and beta particles, protons and neutrons) radiations (Jain, 2010; Piri et al., 2011). The advantages of using physical as compared to chemical mutagens are the precise dose determination, allowing for a sufficient reproducibility, and a high and uniform penetration in plant tissues, particularly for gamma rays (Piri et al., 2011). While major mutations were C/G to T/A transitions type in the EMS mutants, mutations were C/G to T/A and A/T to G/C transitions, and A/T to T/A transversions in the gamma-ray mutants (Shirasawa et al., 2016). Gamma radiation have provided a high number of useful mutants and is still showing an elevated potential for improving vegetative propagated plants. Gamma rays, since the first report in 1949, are the most used ionizing radiation of the physical mutagens (Suprasanna and Jain, 2017) for plant improvement in the past 70 years (Çelik and Atak, 2017). Seeds, pollen grains, embryogenic calli, mature stem segments, and budwoods can be used for irradiation treatments (Ollitrault and Navarro, 2012; Roose and Williams, 2007a).

As of today, 3364 mutant varieties, approximately 80% of these obtained by gamma irradiation, have been registered in the mutant variety database (MVD) (Anonymous, 2019). A total of 58 mutant cultivars have been registered belonging to 33 fruit tree species such as apple (‘Super Compact’), almond (‘Supernova’), cherry (‘Aldamla’), fig (‘Bol (Abundant)’), grapefruit (‘Star Ruby’), lemons (‘Alata’, ‘Eylül’, Gülşen’, ‘Uzun’), mandarins (‘Mor’, ‘Moria’, ‘NIAB Kinnow’, ‘Orri’, ‘Tango’), olives (‘Briscola’), oranges (‘IAC 2014 Pera’), peaches (‘Fuku-ekubo’, ‘Shimizu Hakutou RS’), pears (‘Chaofu 1’, ‘James Grieve Double Red’) and plum (‘Spurdente-Ferco’) (Anonymous, 2019), a putative *Fusarium* wilt-resistant banana clone (Jain, 2010). In addition, the lethal dose 50 (LD_{50}) level of radiosensitivity of budwoods in some fruit tree species was determined such as apple (Atay et al, 2018), apricot (Legave and Garcia, 1988), mulberry (Nguyen, 2001), sweet cherry (Saamin and Thompson, 1998), grapefruit (Hearn, 1986), lemon (Spiegel-Roy et al., 2007; Uzun et al., 2008), mandarin (Roose and Williams, 2007a; Ballester, 2013; Bermejo et al, 2012; López-García et al., 2015; Roose and Williams, 2007b), and kumquat (Kara-Özbek and Dalkılıç, 2017). However, the radiosensitivity of different plant species and organs can show significant differences under different life-time and ecology (Caplin and Willey, 2018). During 16-h acute exposure to gamma rays, the predicted LD_{50} values for Persian walnut (*J.regia*) and eastern black walnut (*J.nigra*) were found as 48.0 and 38.3 Gy, respectively. These predicted values were based on interphase chromosome volume (ICV) from vegetatively active growing plants. If they were irradiated during their dormant stage, the LD_{50} values would be higher. In other words, the plants might be more resistant to gamma rays (Sparrow et al., 1971).

Radiosensitivity of white mulberry (*Morus alba*) was determined as 40 Gy (Nguyen, 2001) and that of apricot as 30 Gy (Legave and Garcia, 1988). Saamin and Thompson, (1998) suggested acute gamma irradiation for maximum mutation rate with adequate survival of accessory buds in air at dosages in ‘Bing’ sweet cherry was approximately LD_{50}=27.5-30.0 Gy. Also, recommended gamma ray exposure to *Citrus* spp. budwoods is between 30 and 50 Gy for seedlessness induction (Roose and Williams, 2007a). In budwoods of ‘Foster’ grapefruit were irradiated with 0-110 Gy cesium-137 gamma rays, the LD_{50} of the bud survival was 50 Gy and 90 Gy grown in the greenhouse and in the nursery, respectively; however, shoot growth was not observed above 50 Gy in neither of the environment (Hearn, 1986). Budwoods of ‘Villafranca’ and ‘Eureka’ lemons were exposed to gamma rays (13.3 Gy/min), and seedless ‘Ayelet’ and Galya’ mutants were obtained, respectively (Spiegel-Roy et al., 2007). Seedless mutants, namely ‘Orri’ from ‘Orah’ mandarin (Vardi et al., 2003a, 2003b) and ‘Moria’ from ‘Murcott’ mandarin, were obtained from budwoods irradiation of cobalt-60 gamma rays (3.5 kh). Budwoods of ‘Murcott’ mandarin were irradiated using with 50±10% Gy cobalt-60 source (Bermejo et al., 2012). Budwoods of ‘Clemenules’ mandarin was exposed to 50-75 Gy cobalt-60 gamma rays. Only 431(3.6%) plants gave fruit out of 12,000 buddings (Ballester, 2013). In *Ficus carica*, 0.0-67.0 Gy cobalt-60 doses were applied to budwoods in ‘Sarılop’ and ‘Bursa Siyahı’ in Aydın, Turkey. LD_{50} was found 50.7 Gy and 25.3 Gy in ‘Sarılop’ and ‘Bursa Siyahı’, respectively (Özen et al., 2017).

From the literature search, no record could be found on the radiosensitivity of ‘Chandler’ walnut cultivar. Therefore, the objective of this study was to determine the radiosensitivity of ‘Chandler’ walnut budwoods using gamma ray. The scion breeding aim of this irradiation study was to expect a new genotype to improve low fill of the kernel/shell ratio (49%) of ‘Chandler’ cultivar. In order to accomplish that the best gamma ray dose used in mutation breeding studies was investigated. This is the first report on radiosensitivity of ‘Chandler’ budwood to gamma irradiation.

MATERIALS AND METHODS

One-year-old ‘Chandler’ (‘Pedro’ × UC56-224) walnut budwoods were used in the study. ‘Chandler’ was obtained from a cross made by Serr and Forde in 1963 and was released by the University of California in 1979 (Tulecke and McGranahan, 1994). Irradiation experiments were carried out at three different dates in a cobalt-60 gamma ray cell (Ø250 × 220 mm, 10 L) (Izotop, Ob-Servo Sanguis Co-60 Research Irradiator, Budapest, Hungary) located at Turkish Atomic Energy Authority (TAEK), Sarayköy Nuclear Research and Education Center (SANAEM), Ankara, Turkey. In the first treatment, the dormant budwoods were collected on March 16, 2017 and stored at +4 °C until used. The dormant budwoods were irradiated with 0, 10, 20, 30, and 40 with 36 Gy/h speed, and 50, 60, 70, 80, 90, and 100 Gy with 403 Gy/h speed cobalt-60 gamma rays on April 27, 2017. In the second treatment, the budwoods were collected from the lignified branches of the same growing season on September 26, 2017. These active season’s budwoods were irradiated with 0, 20, 25, 30, 35, and 40 Gy with 34 Gy/h speed on September 27, 2017. Because the bud-take ratio was too low at 50 Gy and higher gamma doses, 40 Gy and lower doses was used in the second treatment. In the third treatment, the dormant budwoods were collected during winter pruning period and stored at +4 °C until used. Because the calculated EMD was found as 42 Gy, the dormant budwoods were irradiated with only 42 Gy with 31 Gy/h speed on April 26, 2018. The budwoods were wrapped in moist towel contained in cryopreservation box while they were carried to and returned back to the gamma irradiation facility in Ankara. All irradiated budwoods were chip-budded on one-year-old *J.regia* seedling rootstocks in the field nursery one day after irradiation. The mean moisture ratio of the budwoods was recorded after cobalt-60 treatment. The budwoods which were not budded were used for the moisture ratio measurement. Five-cm-length pieces of budwoods were dried at 70 °C for 48 h in an incubator (JP Selecta, Digiheat, S. A., Spain) located at ADU-AgBioCenter for dry weight measurement. In the first treatment, the rootstocks were cut above the bud union 27 days (on May 25, 2017) after the treatment. The bud take was determined 42nd day on June 9, 2017. Then the number of bud taking was recorded every 10 days until September 15, 2017. In the second treatment, the bud take ratio and shoot length measurement were done eight months (on May 12, 2018) after the treatment. In the third treatment, the bud take ratio was recorded 75 days (on July 11, 2018) after the treatment. The M1V1 mutation screening was made by observing survival ratio (%) at the end of the first growing season in the juvenile nursery plants in 2017 and 2018. Shoot length (cm) was measured with a metal tape measure as a distance from the bud union to the tip of the shoot on September 15, 2017 in the first treatment, on August 25, 2018 in the second treatment, and on August 25, 2018 in the third treatment. Chlorophyll density was recorded as normalized difference vegetation index with PlantPen (NDVI 300, Photon System Instruments, Drasov, Czech Republic) instrument on the upper side of the leaves on August 14 and August 25, 2018 in three experiments. Effective mutation dose (EMD) was calculated using formula as follows: y = a + bx formula where y is 50% of the shoot length in control (0 Gy), a is the constant from the regression graph, b is the constant for x-values, and x is EMD predicted. Mutation frequency (MF) was calculated using formula as follows (Kunter et al., 2012): MF (%) = (abnormal plants / normal plants) × (100 / EMD).

RESULTS

The first irradiation experiment: On April 27, 2017, the mean moisture ratio in budwoods was found 44.4% being lowest 42.9% (40 Gy) and highest 45.4% (10 Gy). In Table 1, the final surviving budded plants were presented in numbers and survival ratios. The bud taking ratio was drastically reduced to 64.6% (20 Gy) and 7.4% (50 Gy) on June 29, 2017 while the survival ratio obtained as 62.2% and 1.8%, respectively, at the end on the growing season. No plants survived above 60 Gy dose. The shoot length changed from 65.5 cm (0 Gy) to 26.0 cm (50 Gy) at the end of the growing season (Table 1). EMD was calculated as 42.1 Gy. MF values were 0.0% (0 and 10 Gy), 5.9% (20 Gy), 27.6% (30 Gy), 27.2% (40 Gy), and 50.0% (50 Gy). While chlorophyll density was 4268 in control (0 Gy), it changed from 3175 (50 Gy) to 4763 (10 Gy) in the upper side of the leaves. From this, while gamma dose was increased, chlorophyll density was decreased.

The second irradiation experiment: On Sep 27, 2017, the mean moisture ratio in budwoods was found 57.4% being lowest 55.0% (0 Gy) and highest 60.0% (40 Gy) (Table 1). The bud taking ratio changed from 37.7% (40 Gy) to 82.3% (20 Gy) on Aug 25, 2018. The shoot length changed from 31.6 cm (30 Gy) to 74.4 cm (25 Gy) at the end of the growing season. EMD was calculated as 20.9Gy. MF values were 0.0% (0 Gy), 4.7% (20 Gy), 7.5% (25 Gy), 20% (30 Gy), 25.0% (35 Gy), and 7.5% (40 Gy). While chlorophyll density was 3435 in control (0 Gy), it changed from 3097 (30 Gy) to 3756 (40 Gy) in the upper side of the leaves irradiated with gamma rays. From this, except 30 Gy gamma dose was increased chlorophyll density (Table 1).

Table 1. Effects of gamma ray irradiations in 'Chandler' walnut budwoods

Irradiation Application Time and Dose (Gy)	Chip Budding with Irradiated Budwoods (in numbers)	Moisture Ratio (%)	Survival of Budwoods (in numbers) (%)*	Shoot Length (cm)	Chlorophyll Density (upper side)
April 2017					
0	52	43.4	29 (55.8)	65.5	4268
10	85	45.4	40 (47.0)	54.1	4743
20	82	45.2	51 (62.2)	56.9	4640
30	103	45.1	47 (45.6)	37.6	4363
40	107	42.9	50 (51.4)	55.2	4195
50	108	45.3	2 (1.8)	26.0	3175
60	110	44.7	0 (0.0)	N.A.	N.A.
70	99	43.4	0 (0.0)	N.A.	N.A.
80	96	43.7	0 (0.0)	N.A.	N.A.
90	104	44.3	0 (0.0)	N.A.	N.A.
100	91	45.0	0 (0.0)	N.A.	N.A.
September 2017					
0	38	55.0	15 (39.5)	63.1	3435
20	51	55.9	42 (82.3)	60.4	3577
25	51	58.4	40 (78.4)	74.4	3727
30	50	57.4	20 (40.0)	31.6	3097
35	51	57.7	24 (47.0)	57.2	3726
40	53	60.0	20 (37.7)	48.1	3756
April 2018					
0	N.A.	N.A.	N.A.	N.A.	N.A.
42	303	50.7	97 (32.0)	27.0	5041

*Numbers in parenthesis present the ratios. MF: Mutation frequency. N.A.: not applicable

The third irradiation experiment: On Apr 26, 2018, EMD from the first experiment was used. The mean moisture ratio in budwoods was found 50.7% using only 42 Gy. The bud taking ratio was 32.0% (42 Gy). The survival ratio was 32.0% and the shoot length was 27.0 cm at the end of the growing season. MF value was 21.6. Chlorophyll density was 5041 in the upper side of the leaves (Table 1).

DISCUSSION

The radiosensitivity of different plant organs, namely buds, can show significant differences according to species and ecology (Caplin and Willey, 2018). In our experiments, the dormant 'Chandler' budwoods showed higher EMD values (42.1 Gy) than that in the active budwoods (20.9 Gy). These results could be related to the moisture content in the budwoods during the irradiation application (Kunter et al., 2012). The higher the moisture content of the plant materials is the higher the effectiveness of irradiation. The reason for that water is the most abundant molecule in living cells. Reactive oxygen species (ROS) are the primary radiation products. The indirect action of radiation is responsible for 99.9% of the protein damage. Gamma irradiation induces stress in the surviving plants. Therefore, it significantly affects physiological and biochemical processes (Lagoda, 2012). Ionizing radiation can involve oxidative stress by producing primary (•OH, H•) and secondary (H_2O_2, $O_2\bullet^-$) free radicals (Esnault et al., 2010). While minimum radiosensitivity was observed at intermediate moisture content, increased radiosensitivity was obtained at lower or higher moisture levels rice seeds (Bhattacharya and Joshi, 1977). It was observed that there was marked increase in mutation frequency as the moisture content decreases below 14% in barley seeds (Mba et al., 2010).

One of the first mutation studies was conducted in walnut seeds in Ukraine (Kudina, 1988), in that study 1-150 Gy (100-15000 R) of gamma rays applied to the walnut seeds. While fast-growing seedlings were obtained from gamma doses below 200 Gy, slow-growing (dwarf) seedlings were observed all, especially higher, doses.

Kudina (1988) reported when the irradiation dose increases, the bud take ratio decreases after a certain dose.

In our experiments, bud taking ratio was reduced after 40 Gy.

Haploid plants were obtained in ‘Hartley’, ‘Pedro’, Z_{63}, and Z_{67} whose female flowers were pollinated using 300 and 600 Gy gamma ray-irradiated pollen of walnut selections Z_{53} and Z_{30} in Iran. Diploid plants were obtained from pollen irradiated at 50 and 150 Gy. The results from molecular data showed parthenogenetic origin of the obtained haploid plants (Sadat Hosseini et al., 2011). A previously predicted LD_{50} gamma dose of 48.8 Gy for Persian walnut (Sparrow et al., 1971) is in very close proximity to our findings of 42.1 Gy with dormant budwoods. However, Sparrow et al. (1971) prediction was for actively growing trees. They expected the survival rate would be higher if irradiation was applied in the dormant stage.

Chlorophyll density was reduced on the upper side of the leaves irradiated with 50 Gy (3175) gamma rays in the first treatment. The reason for that might be the negative effect of irradiation doses (Kunter et al., 2012).

Our possible mutant plants will be observed in the field for any leaf or shoot growth aberrations. Leaf aberrations can be observed in natural plant populations (Babcock, 1915; Pennington and Beineke, 1977) or expected after exposure to irradiation (Opeke and Jacobs, 1973). Around 1900, some small-leaved-oak shape *J.californica* trees grown from seeds were appeared in California. The chromosome numbers (2*n*=2*x*=34) were not different than the normal seedlings (Babcock, 1915). In 1976, a leaf mutation was discovered in *J.nigra* in Indiana, and showed new form of leaf had a large round terminal leaflet (7 cm in diameter), indented apex, and small lateral leaflets (2.0-2.5 cm in diameter). It was speculated that a single recessive trait could be responsible for this mutation in both above observations (Pennington and Beineke, 1977).

In conclusion, recommended cobalt-60 doses for ‘Chandler’ budwoods are 42.1 Gy for dormant budwoods and 20.9 Gy for the same year’s lignified budwoods. Above mentioned gamma rays in ‘Chandler’ budwood can be used in mutation breeding applications to obtain a new cultivar which has higher than 49% fill of the kernel/shell ratio. The results obtained from this first report on EMD of ‘Chandler’ budwood to gamma irradiation might open avenues to other chemical and physical mutation studies in walnut.

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