

Research article

Open access

Chromosome stability of *in vitro* propagated *Cucurbita* cultivars

Buse Dursun¹, Ahu Altinkut Uncuoğlu², Yıldız Aydın^{1*}

¹Marmara University, Faculty of Science and Arts, Department of Biology, Göztepe Campus, Istanbul, Turkey

²Marmara University, Faculty of Engineering, Department of Bioengineering, Göztepe Campus, Istanbul, Turkey

DOI: 10.31383/ga.vol3iss3pp25-32

Abstract

Cucurbita pepo L., a member of Cucurbitaceae family, is an annual plant with herbaceous stems, broad leaves and superficial scattered roots. Monoecious flower structure in the Cucurbitaceae family and the differences in the maturing time of male and female organs in flowers cause an increase in the foreign fertilization rate. Therefore, there may be positive or negative changes in the existing characteristics of the species. Micropropagation method can be performed in pumpkin species for clonal propagation, but their genetic stability in tissue culture is an important consideration. Chromosome number and morphology are primary cytogenetic parameters that must remain stable after *in vitro* propagation. We performed cytogenetic analysis of different hybrid pumpkin genotypes cultivated in our country (Ardendo, Angelina, Torpido, Roni, Sena Hanım) in order to determine their chromosome stability level. Cotyledon nodes, nodes, shoot apex, hypocotyl and internode explants were prepared from the 4-week old *C. pepo* seedlings by making a horizontal slice through the hypocotyl region. The highest shoot and callus regeneration was obtained in Torpido genotypes in cotyledonary node explants produced multiple shoots placed in tissue culture media MS+1 mg/l N6-benzylamino-purine BA. The chromosome number and karyotype analysis were determined in control and *in vitro* propagated *Cucurbita pepo* L. plants and ploidy levels were confirmed to be $2n = 40$.

*Correspondence

E-mail:
ayildiz@marmara.edu.tr

Received

November, 2019

Accepted

December, 2019

Published

December, 2019

Copyright: ©2019 Genetics & Applications, The Official Publication of the Institute for Genetic Engineering and Biotechnology, University of Sarajevo

Keywords

Cucurbita pepo L., *in vitro* micropropagation, karyotype analysis

Introduction

The Cucurbitaceae family, also referred as cucurbits, forms a very large group with approximately 130 genera and 800 species. Cucurbits plants can be

cultivated worldwide in warmer region and are popular food crop plants. Some of these species include squashes, pumpkins, melons and gourds (Perez Gutierrez, 2016). The cultivated *Cucurbita* species are: *C. argyrosperma*, *C. maxima*, *C.*

moschata, and *C. pepo*, which include both summer squash and winter squash as well as ornamental gourds. The most important species in terms of agricultural production worldwide are *C. maxima*, *C. moschata* and *C. pepo*. Turkey is one of the world's leading cucurbit producers by taking the 7th place in cucurbit production in the world (Turkish Statistical Institute, 2018 <http://www.tuik.gov.tr>). The most commonly grown species in Turkey are *Cucurbita pepo* L., *Cucurbita maxima* Duch. and *Cucurbita moschata* Pour. including summer squash and winter squash (Güner et al., 2012). *Cucurbita pepo* L. was found in summer grown squashes while *Cucurbita maxima* Duch. and *Cucurbita moschata* Pour. among the winter grown squashes. Among these species *C. pepo* is the most commonly cultivated pumpkin (Sunulu & Yağcıoğlu, 2014). The leaves of *C. pepo* contain 43.8% protein (Oloyede, 2012), the fruits are characterized by low fat content (2.3%), high carbohydrates (66%) and low proteins (3%) and very high carotenoids contents (171.9 to 461.9 µg/g) (Adedayo et al., 2013). Seeds are used as an antidiabetic, antihypertensive, antitumor, antimutagenic, immunomodulatory, antibacterial, anti-hypercholesterolemic, intestinal antiparasitic, analgesic, and anti-inflammatory agent (Kostalova et al., 2009) *C. pepo* has high agricultural properties and it is included in the fruit-eaten vegetables class. Its fruits can be used in food and cosmetic industry while seeds are used as snack foods (Sağlam & Çetin, 2018).

Development of *in vitro* regeneration technology is one of the solutions for crop improvement (Kurozawa et al., 1997; Ananthakrishnan et al., 2003). Since the first experiments of Gottlieb Haberlandt in the early 1900 on the *in vitro* cultivation of plant tissue, the fields of application have expanded from research of plant physiology to applications in breeding, molecular and microbiology and it became also an important tool for commercial plant production (Laimer & Rücker, 2003). Plant regeneration via shoot organogenesis is a more suitable and rapid approach (Obembe et al., 2011) in comparison to traditional *in situ* cultivation. Plant population produced by direct organogenesis from shoot meristem and leaf explants are homogenous. Therefore, genetically identical plants could be provided via regeneration in large scales

(Seyis et al., 2017). Some factors such as Plant Growth Regulator (PGR) balance, culture conditions, genotype and explant type are important for successful plant regeneration. Ananthakrishnan et al. (2003) reported the regeneration of *C. pepo* from seedling-derived cotyledon explant through direct organogenesis. Schroeder (1968) documented the regeneration of zucchini squash (*C. pepo*) from flesh pericarp wall-derived callus through somatic embryogenesis. Carol et al. (1995) in their work reported the initiation of somatic embryos via cotyledon explant in six squash cultivars (*C. pepo*). Tissue culture techniques, organogenesis and micropropagation enable clonal production by performing *in vitro* regeneration.

This clonal production enables the stability of the desired ploidy levels in plant species. Lee et al. (2003) reported that they obtained 82 and 92% shoot regeneration ratio in *C. maxima* for two cultivars using cotyledon explants through effective plant regeneration protocol via organogenesis. Their flow cytometric analysis revealed that most of the regenerated plants were diploid (45.8-95.0%), tetraploid (0-4.2%) and mixoploid (5.0-50.0%) using different BA concentrations and cotyledon sizes Obembe et al. (2017) cultured hypocotyl, cotyledonary node and cotyledon explants derived from 4-week old seedlings on MS medium fortified with 0.00, 1.00, 2.00 and 3.00 mg/l of BAP in combination with 0.00 or 0.05 mg/l of 2,4-D and investigated for callus, shoot and root induction. They reported that when the different explant types were cultured on MS media amended with the different concentrations of BAP in combination with 2,4-D, neither shoot nor root induction was observed.

The aim of the present study was to develop a highly repetitive protocol for the *in vitro* regeneration of an indigenous Turkish pumpkin from seedling derived different explants and to determine their chromosome stability.

Material and methods

Different hybrid squash varieties (Ardendo, Angelina, Sena Hanım, Roni, Torpido) seeds ($2n=2x=40$) were obtained from Thrace Agricultural Research Institute (TARI).

In vitro regeneration

Seed surface sterilization were performed by immersing seeds in 70% ethanol for 3 min followed by treating with 15% commercial bleaching solution for 15 min. Rinse (each 5 min) in sterile distilled water was repeated for three time (Kurtar et al., 1999). Sterilized seeds were transferred to magenta (tissue culture container) containing pure $\frac{1}{2}$ MS media (Murashige & Skoog, 1962) containing 30 g/l sucrose and 1,1 g/l gelrite. pH value of all media was adjusted to 5.8 before autoclaving.

Each magenta cap was wrapped with aluminum folia and incubed in a growth chamber programmed with an 16/8 h light/dark cycle and 25 ± 2 °C temperature. Explants (node, cotyledon node, shoot apex and internode) were obtained from four weeks old plantlets. Explants were transferred in tissue culture media containing 1, 2, and 3 mg/l BA for shoot and callus regeneration (Obembe, 2017) and regularly (every 4 weeks) subcultured on the same media. Developed shoots were transferred in tissue culture media containing 1 mg/l indole-3-butyric acid (IBA) or plant growth regulator free MS medium (Ananthakrishnan et al., 2003) for rooting. All cultures were incubated in a growth chamber at 25°C and 6000 lux fluorescent light under a 16/8-h photoperiod. Successfully rooted shoots were planted into the pots containing soil for acclimatization and plants were gradually acclimatized to dry air to keep the environment moist. All micropropagation studies were performed in 3 replicates and 3 subcultures were performed for each replicate.

The percentage of explants on which buds developed and the number of distinguishable shoot buds on each explant were recorded. Tukey test was performed by using single factor analysis of variance (ANOVA) for all micropropagation studies.

Cytogenetic analysis

Representative root samples (obtained from apical buds only) for all plant propagation categories h) were excised when they were about 1 cm in length. Pretreatment with distilled water at 4°C for 12 h was followed with root tips fixation in acetic acid: ethanol (1:3 v/v) solution for 1–2 days. The samples were then hydrolyzed in 1 N HCl for 5 min at 60°C. Afterward, squash mounts were prepared with

Feulgen in order to determine chromosome number and the ploidy levels (Metwally et al., 1998).

Results and Discussion

In vitro regeneration

Seeds of Ardendo, Roni, Sena Hanım, Torpido and Angelina genotypes were sterilized under sterile conditions and planted in MS medium. Seeds were kept in the dark for 24 hours and germination percentages were calculated after four weeks. The highest germination percentage (100%) was achieved in the Torpedo genotype. It has been well known that genotype, explants type and composition of the medium are important factors on organogenesis. Furthermore, the physiological conditions and hormonal contents of explants are crucial points for regeneration potential. To establish optimal conditions for adventitious shoot induction, a variety of explants (cotyledon node, shoot apex and node) were prepared from seedlings and were cultured using media containing different concentrations of BA (1mg/l (K1), 2mg/l (K2), 3mg/l (K3). Shoot regeneration was obtained from cotyledon node, shoot apex and node explants in all genotypes (Ardendo, Angelina, Roni, Sena Hanım, Torpido) and in all media (Figure 1). Shoot regeneration was not achieved in hypocotyl and internode explants. Callus regeneration was obtained in all genotypes and all explants (Cotyledon node, shoot tip, node, internode and hypocotyl). Hypocotyl and internode explants showed low callus regeneration in all genotypes compared to cotyledon node, shoot apex and node explants. In the hypocotyl explants, no regeneration was observed with callus formation on the parts of the explants touching the medium.

According to the data obtained, the highest shoot regeneration response (92.98%) was achieved in the cotyledon node explant of Ardendo genotype. (Figure 2). The highest callus regeneration response (92.18%) was achieved in the node explant of the Roni genotype (Figure 3). Under proper culture conditions, plant cells possess a capacity to regenerate organs from specialized somatic tissues through a process known as de novo organogenesis. The importance of genotype for shoot and callus regeneration was emphasized in different studies.



Figure 1. Shoot multiplication with non-significant callus formation in MS media supplemented with BA (2 mg/l) from cotyledone nod, shoot apex and nod segment in Angelina (I), Ardendo (II), Roni (III), Sena Hanım (IV), Torpedo (V) genotypes.

A shoot regeneration protocol was developed for five cultivars of the Cucurbitaceae by Abrie and Van Staden (2001). They tested the effects of combinations of BA, kinetin, iP and TDZ with IAA in the culture medium on shoot regeneration of cotyledonary explants and showed that the cultivars *Cucurbita maxima* cv. A-line, *C. maxima* cv. Chicago Watted and *C. pepo* cv. Rolet, did not form shoots on any of the treatments. Although *Cucumis sativus* cv. Ashley responded poorly shoot development, *Cucumis melo* L. cv. Hales Best 36 variety regenerated successfully. In our case the

Torpedo genotype showed higher regeneration capacity compared with other genotypes. Cotyledons are frequently used as explants in shoot and callus regeneration in Cucurbitaceae family (Ananthakrishnan et al., 2003; Lee et al., 2003; Han et al., 2004; Kim et al., 2010; Obembe et al., 2017). After cotyledons, hypocotyls (Pal et al., 2007; Obembe et al., 2017) and shoot tips (Sarowar et al., 2003) are also preferably used as explants. It was observed that the highest shoot and callus regeneration rates were in cotyledon node explant (Obembe et al. 2017). However, in a few studies the

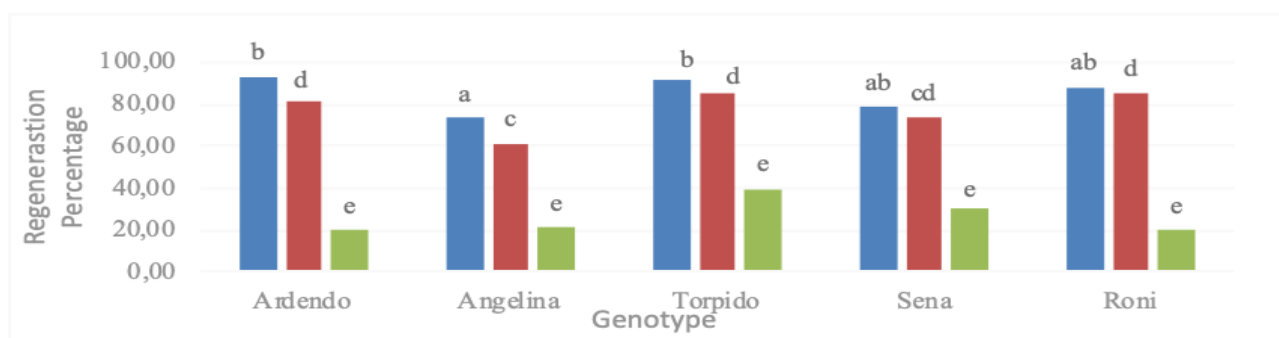


Figure 2. Shoot regeneration rates in MS media supplemented with 1mg/l (K1), 2mg/l (K2), 3mg/l (K3) BA from cotyledone nod, shoot apex and nod segment in all tested genotypes (three trials and three subculture results)

regeneration rate from the hypocotyl explant was reported to be higher than the regeneration rate from the cotyledon explant (Pal et al., 2007). Obembe et al., (2017) did not achieved shoot regeneration from the hypocotyl explants. In this study, the highest shoot regeneration rate, in all genotypes, were obtained from cotyledon nodes and shoot apex. Shoot regeneration was not obtained from hypocotyl and internode explants. Therefore, our findings are in concordance with other studies.

It is observed that cytokinins (BA, IP, Kinetin, Zeatin) and auxins (IAA, NAA, 2,4-D) are used alone or in combination for shoot and callus regeneration in Cucurbitaceae family (Abrie & Van Staden, 2001; Ananthakrishnan et al., 2003; Lee et al., 2003; Sarowar et al., 2003). According to these studies, BA has been reported to be more efficient in callus and shoot regeneration than other cytokinins (Abrie & Van Staden, 2001). Obembe et al. (2017) reported that a combination of 1.00 mg/l BAP with 0.05 mg/l 2,4-D was optimum for callus induction from hypocotyl and cotyledonary node explants, while for cotyledon explants, 2.00 mg/l BAP in combination with 0.05 mg/l 2,4-D was preferred. Cotyledonary node explants and cotyledonary node explant-derived callus responded with multiple shoots on full strength Murashige and Skoog (MS) medium (control) devoid of Plant Growth Regulators (PGRs). In this study, different concentrations of BA (1 mg/l, 2 mg/l BA, 3 mg/l) were found to decrease in stem and callus regeneration as the BA concentration increased.

The optimal concentration for shoot regeneration was 1 mg/l BA.

Root Regeneration

For root regeneration, basal MS medium and MS medium containing 1 mg/L IBA were used. Only regenerated shoots from the shoot apex and cotyledon node explants was used for rhizogenesis. Root regeneration was not achieved in the nod explants. The highest rooting rate (83.28%) in regenerated shoots was obtained in the Torpedo genotype. The root regeneration rate was 90.02% in basal MS medium and 49.63% in MS medium supplemented with 1 mg/L IBA.

In study conducted by Lee et al. (2003), regenerated cotyledons were successfully rooted in basal MS medium. Sarover et al. (2003), used the shoot apex in their study and regenerated shoots successfully rooted in MS medium containing 1 mg/ml IBA. In this study, cotyledon nodes and shoot apex were successfully rooted in MS medium containing 1 mg/ml IBA or without plant growth regulator. MS medium containing 1 mg/ml IBA or MS medium without plant growth regulator were used in different studies (Lee et al., 2003; Sarowar et al., 2003; Han et al., 2004; Pal et al. 2007; Mookhan, 2015) for rooting. On the other hand, Kim et al. (2010), preferred MS medium containing 0.1mg/l NAA for rooting. In our study on basal MS higher root regeneration was observed than on MS medium containing 1mg/l IBA and this result is compatible with recent findings (Obembe et al. 2017).

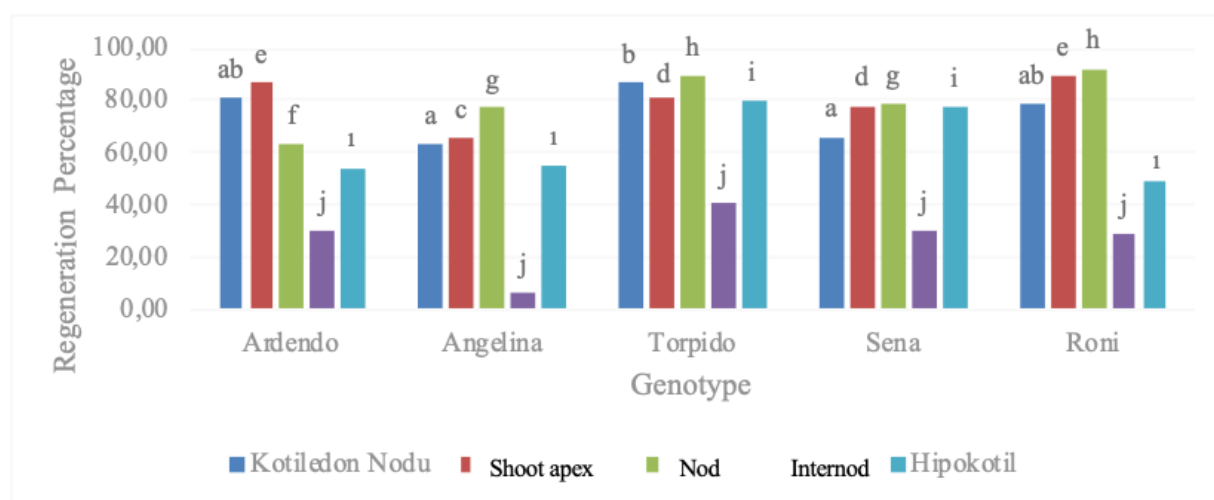


Figure 3. Callus regeneration rates of different explants from Ardendo, Angelina, Sena Hanım, Roni, Torpedo genotypes in MS medium containing 1mg/l (K1), 2mg/l (K2), 3mg/l (K3) BA (three trials and three subculture results)

Acclimatization

Regenerated shoots with healthy roots were planted in pots containing 1 kg of sterile soil. The plants in pots were covered with stretch film to keep the environment moist and the plants were gradually acclimated to dry air. The highest acclimatization percentage was achieved from the shoot apex explant of the Roni genotype with 98.88% acclimatized plants. In general, plants obtained from cotyledon nodes were able to survive in soil better than plants obtained from shoot apex.

Ploidy Analysis

Determination of ploidy levels in regenerated *C. pepo* plants was achieved by chromosome counting in root tips of one-week old seedlings. Chromosome staining was obtained by Feulgen reaction and $2n = 40$ chromosomes were successfully counted in all genotypes by karyotype and idiogram analysis. The germinated seedlings control and the root tips taken from MS medium without plant growth regulator as a result of 3 subcultures in K1 (1 mg/l BA) medium. In both applications, $2n = 40$ chromosomes were successfully counted. This emphasizes that the

ploidy levels do not change after the root and shoot regeneration in Ardendo genotype and that the study is reliable (Figure 4).

The fact that the *Cucurbita* chromosomes are very small in the mitotic stage and that they do not show a good distribution in the cell, makes *Cucurbita* karyotype analysis quite difficult (Whitaker, 1930; Whitaker & Davis, 1962). Although it was previously stated that the number of chromosomes in *C. pepo* was $2n = 48$, it is now known that the number of chromosomes in *C. pepo* is $2n = 40$ (Whitaker, 1930; Tatum et al., 2006). In tissue culture studies, there is always a possibility of difference in ploidy levels in the control and regenerated plants. Lee et al. (2003) found that 73.7% of the regenerated plants were diploid, 25.8% were mixoploid and 0.5% were tetraploid. In another study, it was reported that all regenerated squash species are diploid (Ananthakrishnan et al., 2003). In this study, although small chromosomes in all pumpkin genotypes made karyotype analysis difficult, $2n = 40$ chromosomes were successfully counted and no changes in ploidy level in the control and *in vitro* regenerated plants.

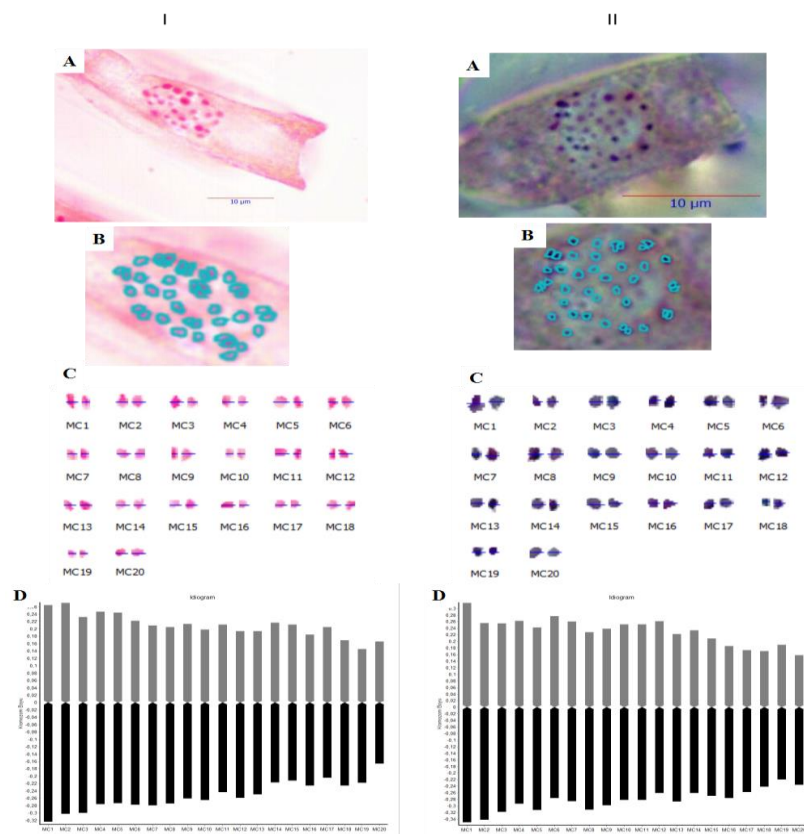


Figure 4. Analysis of the Ardendo genotype in the control group (I), 3 subculture results of Ardendo genotype in K1 (1 mg/l BA) medium (II). A) Chromosome photograph; B) Chromosome counting; C) Karyotype analysis; D) Idiogram

Conclusion

Although, different types of explants have been using by different researchers, it is clear that the influence of the genotype is often the parameter which will determine how well a cultivar will react to growth regulators in tissue culture studies. Therefore, it is necessary to test the response of specific explants from each cultivar to different levels of growth regulators to determine optimum culture conditions for shoot regeneration.

The success of *in vitro* culture depends mainly on the growth conditions of the source material, medium composition, culture conditions and on the genotypes of donor plants (Tiwari et al., 2013). In our case the Torpedo genotype showed higher regeneration capacity compared with the other. The most successful explant type for shoot and callus regeneration was the cotyledon node and the most successful culture medium for shoot and callus regeneration was K1 (MS + 1 mg/l BA). The best root regeneration was achieved in the torpedo genotype, cotyledon node giving the best root regeneration and MS growth medium without plant growth regulators was became the most successful nutrient medium for root regeneration. We established an *in vitro* propagation method for *Cucurbita* cultivars by culturing the meristem in MS medium containing different concentrations of BA. The resulting meristem-derived plants were cytogenetically stable, therefore, we expect that the *in vitro* propagation method implementing 1 mg/l BA will be applicable in commercial production of *Cucurbita* plants.

Acknowledgement

We gratefully acknowledge the support of the Marmara University Research Foundation BAPKO (Project No: FEN-C-YLP-110618-0343) for their financial support.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

- Abrie ALL, Van Staden J (2001) Development Of Regeneration Protocols For Selected *Cucurbita* Cultivars. *Plant Growth Regulation*, 35(3):263–267.
- Adedayo OR, Farombi AG, Oyekanmi AM (2013) Proximate, Mineral and Anti-Nutrient Evaluation of Pumpkin Pulp (*Cucurbita pepo*). *J Applied Chem*, 4:25-28.
- Ananthakrishnan G, Xia X, Elman C, Singer S, Paris HS, GalOn A, Gaba V (2003) Shoot Production in Squash *Cucurbita pepo* by *in vitro* Organogenesis. *Plant Cell Rep*, 21:739–746.
- Carol G, Baodi X, Dennis G (1995) Somatic Embryogenesis and Regeneration From Cotyledon Explants Of Six Squash Cultivars. *Hort Science*, 30:1295–1297.
- Güner A, & Aslan S (Eds.) (2012) Türkiye Bitkileri Listesi:(Damarlı Bitkiler). Nezahat Gökyiğit Botanik Bahçesi Yayınları.
- Han JSS, Oh DGG, Mok IGG, Park HGG, Kim CK, (2004) Efficient Plant Regeneration From Cotyledon Explants of Bottle Gourd (*Lagenaria siceraria* Standl.). *Plant Cell Reports*, 23(5):291–296.
- Kim KMM, Kim CK, Han JSS (2010) *In vitro* Regeneration From Cotyledon Explants İn Figleaf Gourd (*Cucurbita ficifolia* Bouch), a Root stock for Cucurbitaceae. *Plant Biotechnology Reports*, 4 (2):101–107.
- Kostalova Z, Hromadkova Z, Ebringerova A (2009) Chemical Evaluation of Seeded Fruit Biomass of Oil Pumpkin (*Cucurbita pepo* L. var. *Styriaca*). *Chem Pap*, 63:406- 413.
- Kurozawa C, Pavan MA (1997) Doenças das Cucurbitáceas. In: Kimathi H, Amorin L, Bergamin Filho A, Camargo L and Rezende J, Eds., *Manual de Fitopatologia, doenças das plantascultivadas*, 3rd Edition, São Paulo, Editora Agronômica Ceres, 2:325-337.
- Kurtar ES (1999) Research on Haploid Embryo Stimulation and Plant Formation in Pumpkin (*Cucurbita pepo* L.). Cukurova University Graduate School of Natural and Applied Sciences PhD Thesis, Adana, Turkey.
- Laimer M, Rücker W (2003) *Plant Tissue Culture – 100 years since Gottlieb Haberlandt*, Springer Verlag Wien.

- Lee YK, Chung WI, Ezura H, (2003) Efficient Plant Regeneration Via Organogenesis in Winter Squash (*Cucurbita maxima* Duch.). Plant Science, 164(3): 413–418.
- Metwally EI, Moustafa SA, El-Sawy BI, Haroun S A, Shalaby TA (1998) Production of Haploid Plants From *In Vitro* Culture of Unpollinated Ovules of *Cucurbita pepo*. Plant Cell Tissue And Organ Culture, 52(3):117-121.
- Mookhan M (2015) Direct Organogenesis from Cotyledonary Node Explants of *Cucurbita pepo* (L.) An Important Zucchini Type Vegetable Crop. American Journal of Plant Science, 6:157–162.
- Murashige T, Skoog F (1962) A Revised Medium For Rapid Growth And Bio-Assays With Tobacco Tissue Cultures. Physiol Plant, 15:473–497.
- Obembe OO, Aworunse OS, Bello OA, Ani AO (2017) Multiple Shoots Induction from Indigenous Nigerian Pumpkin (*Cucurbita pepo* L.). Annual Research & Review in Biology, 17(5):1-10.
- Obembe OO, Khan T, Popoola JO (2011) Use of Somatic Embryogenesis as A Vehicle for Cotton Transformation. J Med Plants Res, 5 (17):4009-4020.
- Oloyede FM (2012) Growth, Yield and Antioxidant Profile of Pumpkin (*Cucurbita pepo* L.) Leafy Vegetable as Affected by NPK Compound Fertilizer. J Soil Sci Plant Nutr, 12(3):379-388.
- Pal SP, Alam I, Anisuzzaman M, Sarker KK, Sharmin SA, (2007) Indirect Organogenesis in Summer Squash (*Cucurbita pepo* L.). Turkish Journal of Agriculture and Forestry, 31(1):63–70.
- Perez Gutierrez RM (2016) Review Of *Cucurbita Pepo* (Pumpkin) Its Phytochemistry And Pharmacology. Medicinal chemistry, 6(1):12-21.
- Sağlam C, Çetin N (2018) Çerezlik Kabak Üretiminde Kullanılan Ekim ve Hasat Makinalarının İşletme Performanslarının Belirlenmesi. International Eurasian Conference on Science, Engineering and Technology (EurasianSciEnTech), November 22-23, Ankara, Turkey www.EurasianSciEnTech.org, p. 1979 – 1986.
- Sarowar S, Oh HY, Hyung NI, Min BW, Harn CH (2003) *In vitro* Micropropagation of a *Cucurbita* Interspecific Hybrid Cultivar – A Root Stock Plant. Plant Cell Tissue and Organ Culture, 75(2):179–182.
- Schroeder CA (1968) Adventive Embryogenesis in Fruit Pericarp *in vitro*. Bot Gaz. 129 (4):374–376.
- Seyis F, Yurteri E, Özcan A (2017) *In Vitro* Multiplication of *Stevia rebaudiana* (Bertoni) Genotypes by Using Different Explants. International Journal of Crop Science and Technology, 3(2):36-41.
- Sunulu S, Yağcıoğlu M (2014) Kayseri’de Çerezlik Kabak (*Cucurbita pepo* L.) Üreticilerinin İşletme, Pazarlama ve Üretim Teknikleri Durumu. İl Gıda, Tarım ve Hayvancılık Müdürlüğü Çerezlik Kabak Çalıştayı, p.26-27.
- Tatum TC, Nunez L, Kushad MM, Rayburn AL (2006) Genome Size Variation in Pumpkin (*Cucurbita sp.*). Annals of Applied Biology, 149 (2):145-151.
- Tiwari S, Arnold R, Saxena A, Mishra RM, Tiwarii AS, Rajak A, Singh P (2013) Studies on Rapid Micropropagation of *Stevia Rebaudiana* Bertoni: A Natural Sweetener. Int J of Pharm & Life Sci (IJPLS). 4(5):2667-2671.
- Turkish Statistical Institute, (2018) <http://www.tuik.gov.tr>.
- Whitaker TW (1930) Chromosome Numbers in Cultivated Cucurbits. American Journal of Botany, 17(10):1033-1040.
- Whitaker TW, Davis GN (1962) Cucurbits. Cucurbits: Botany, Cultivation and Utilization. New York: Interscience, p. 250.