AcademicPres

Available online: www.notulaebiologicae.ro

Print ISSN 2067-3205; Electronic 2067-3264

Not Sci Biol, 2015, 7(1):66-71. DOI: 10.15835/nsb.7.1.9477

Notulae Scientia Biologicae

Androgenesis Induced in *Nicotiana alata* and the Effect of Gamma Irradiation

Ayman EL-FIKI[1]*, Gamal EL-METABTEB[1], Abdel-Hadi SAYED[2], Mohamed ADLY[1]

[1]*Egyptian Atomic Energy Authority, National Centre for Radiation Research and Technology, Nasr City, Cairo, Egypt; aymana.elfiki@eaea.org.eg (*corresponding author); gamalelmetabteb@yahoo.com; adly4000@yahoo.com*

[2]*Faculty of Agriculture, Al-Azhar University, Cairo, Egypt; a_sayed@fastmail.fm*

Abstract

Nicotiana alata anthers cultured on different modified media based on MS, MT and N were used to obtain haploid plants through direct and indirect ways. The haploid plants resulting on MS medium ranged from 52-80%, on MT medium ranged from 32-52% and on N medium ranged from 28-44%. Accordingly, the best medium used for haploid induction was MS supplemented with 0.2 mgl^{-1} NAA + 0.5 mgl^{-1} KIN. On the other hand, MS medium supplemented with 0.4 mgl^{-1} NAA + 0.5 mgl^{-1} KIN or 1.0 mgl^{-1} BAP + 0.5 mgl^{-1} NAA were the best mediums for callus induction and plant regeneration, respectively. Morphologically, the leaf size, stem highest and diameter, flower size and diameter, anther length and number were about 67% of the diploid plants growth. Irradiated anthers with doses of 0, 2.5, 5, 7.5, 10, 15, 20 and 25 Gy caused reducing the number of haploid plants with increasing gamma radiation dose. For the haploid plants irradiated with same doses, the mortality percentage of bud survival was increasing with increasing gamma radiation dose. The irradiated callus with doses of 0, 5, 10, 15 and 20 Gy was affected negatively on growth rate and morphology. Proline content in irradiated plantlets increased with increasing gamma radiation dose. As well, total soluble protein content was increased with gamma irradiation up to 10 Gy. However, the higher doses caused a severe decrease of total soluble proteins. The production of proline and total soluble proteins in haploid plants were 48.6% and 69.5%, respectively comparing with diploid plants.

Keywords: androgenesis, anther culture, haploid, radiation, tobacco

***Abbreviations*:** Murashige and Skoog medium (MS), Murashige and Tuker medium (MT), Nitsch medium (N)

Introduction

Nicotiana alata is an ornamental plant, member from Solanaceae family and the diploid cells contain 18 chromosomes. Tobacco is an ideal plant for obtaining haploid cultures in direct way. Tobacco cultures produce an explosion of haploids, which are now used in hybridization processes. Some authors stimulated the production of female gametes (gynogenesis) or male gametes (androgenesis) in haploid individuals produced directly. In androgenesis, which is carried out only *in vitro*, vegetative or generative nuclei from pollen grains are stimulated to develop haploid plants without fertilization. The literature on *in vitro* androgenesis (Bhojwani and Razdan, 1986; Pierik, 1979) clearly shows that species from the Solanaceae family are capable for regeneration of haploids from isolated anthers. Haploid plants can be obtained by isolation of anthers *in vitro* in two ways: directly, with formation of embryo from the pollen grains (microspore), and indirectly, with callus development and formation of haploid embryoids or adventive buds (Pierik, 1979). Gamma rays are often used on plants in developing varieties that are agriculturally an economically important and have high productivity potential (Jain *et al*., 1998). They are useful for mutations in breeding programs and *in vitro* mutagenesis in order to develop required features of plants and increase the genetic variability. Many mutant varieties, which are resistant to biotic and abiotic stress and with high quality, have been developed (Jain *et al*., 1998). Several attempts of mutagenic treatment on cultured anthers have been reported in higher plants (Ling *et al*., 1991; MacDonald *et al*., 1988; Sangwan and Sangwan, 1986). However, the protocol of mutation induction at haploid level has not been established.

Gamma rays belong to ionizing radiation and interact to atoms or molecules to produce free radicals in cells. These radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism, e. g. dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds (Kim *et al*., 2004; Kovacs and Keresztes, 2002; Wi *et al*., 2005).

The aim of this work was to investigate some composition of culture media to induce haploid plants through direct and

Received: 23.11.2014. Accepted: 20.01.2015.

indirect ways and the effect of gamma rays at different stages of microspore development in *Nicotiana alata* anther culture.

Materials and methods

The anthers of *Nicotiana alata* were excised from flower buds and surface sterilized by dipping in Clorox (30%) for ten minutes followed by three times rinses in sterile distilled water. Anthers were aseptically removed and placed on nutrient medium and cultured for 8 weeks at 25± 2 ° C. They were then exposed to 16/8 photoperiod (3,000 lux).

Over 43 modified culture media based on those of Murashige and Skoog (1962)- MS, Murashige and Tuker (1969)- MT and (Nitsch 1969)- N were tested (Table 1) with *Nicotiana alata* anthers with some growth regulators combination (IAA, NAA, IBA, KIN, BAP and/ or 2,4- D). A further 25 anthers were tested for each culture medium.

Determination of the ploidy level

Cytologically, immature floral buds and root meristems were collected from test plants, fixed in Carnoy's fluid and stored at 4 °C. Slides were prepared using the squash method and pollen mother cells were stained using acetocarmine according to Ramana and Parkken (1967).

Morphologically, plantlets derived from anther culture, cultivated in sterile jars containing peat moss and sand with ratio 1:1. Plastic caps were removed and covered with plastic boring sheets. After one week the plastic cover sheets were removed and the anthers were left in growth chamber one week before transfer the green house (Fig. 1).

Irradiation was carried out with ^{60}Co source at the dose rate 1 Krad/ min at National Centre for Radiation Research and Technology, Cairo, Egypt.

Flower buds were irradiated with different gamma radiation doses (0, 2.5, 5, 7.5, 10, 15, 20 and 25 Gy). The irradiated anthers were cultured on MS medium

Fig. 1. Adaptation stages of haploid plants

Table 1. Composition of culture media

No	Basic medium	Growth regulators (mgl^{-1})						Induced plantlets	Callus formation	Plant regeneration
		IAA	NAA	IBA	KIN	BAP	2,4-D			
1	Murashige and Skoog	2.0			0.02			16		
2		0.2			0.2			16		
3		0.2			0.5			20		
4		0.2			1.0			13		
5				0.1		0.5		16		
6				1.0		0.2		14		
7				0.3		1.5		15		
8			0.05	0.2				17		
9			0.05	0.5				14		
10			0.1	0.1				16		
11			1.0	0.5				15		
12						0.1	0.1		Small	
13						0.2	0.2		Small	
14						0.5	0.3		Large	
15						1.0	0.5		Medium	
16			0.1		0.2				Medium	
17			0.3		0.4				Medium	
18			0.4		0.5				Large	
19			0.5		0.5				Medium	
20			0.1			0.5				10
21			0.2			1.0				16
22			0.5			1.0				33
23			0.5			1.5				12
24			1.0			1.5				0
25						2.0				0
26	Murashige and Tuker			0.1		0.5		9		
27				0.2		1.0		12		
28				0.3		1.5		11		
29	1/8X			0.1		0.5		13		
30	1/8X			0.2		1.0		10		
31	1/8X			0.3		1.5		12		
32	1/4X			0.1		0.5		11		
33	1/4X			0.2		1.0		11		
34	1/4X			0.3		1.5		8		
35	1/2X			0.1		0.5		10		
36	1/2X			0.2		1.0		9		
37	1/2X			0.3		1.5		9		
38	Nitsch	0.2		0.5				11		
39		0.5		0.5				9		
40		1.0		0.5				7		
41		1.0		1.0				11		
42		1.0						11		
43		0.1						9		

supplemented with 0.2 mgl^{-1} NAA + 0.5 mgl^{-1} KIN. One hundred anthers were cultured to each dose.

Mass cultures of *in vitro* grown haploid plantlets derived from single nodes were treated with doses of gamma rays (0, 2.5, 5, 7.5, 10, 15, 20 and 25 Gy). A further 100 haploid plantlets were irradiated with gamma rays at each dose. However, the calli grown on MS + 0.4 mgl^{-1} NAA + 0.5 mgl^{-1} KIN were irradiated with doses: 5, 10, 15 and 20 Gy.

The proline content was estimated according to Batels *et al*. (1973) in irradiated and non- irradiated plantlets.

The total soluble protein in irradiated and non-irradiated plantlets was estimated according to Bradford (1976).

Results and discussions

Over 43 modified culture media based on those of Murashige and Skoog (1962)- MS, Murashige and Tuker (1969)- MT and (Nitsch, 1969)- N were used to obtain haploid plants through direct way with formation of embryoid by isolation of anthers *in vitro* (microspore) and through indirect way with callus development and formation of haploid embryoid or adventive buds.

Data in Table 1 illustrated that all the composition media used for haploid induction in *Nicotiana alata* were positive, but the percentage of haploid plants was variable, dependent on the type of medium and hormone combinations (direct way). Twenty five anthers were tested for each culture medium. The results showed that higher haploid plant numbers were on MS medium, whereas the haploid plants produced a range from 13 to 20 anther cultures (52% - 80%), consequently MT medium with a range of 8 to 13 anther cultures (32% - 52%) and the last performant was N medium with a of range 7-11 anther culture

Fig. 2. The effect of MS medium with different BAP and NAA concentrations on plant regeneration

(28% - 44%). Accordingly, the best results for haploid plants induction were observed with MS medium, especially with NAA and KIN addition. Therefore, MS medium supplemented with 0.2 mgl^{-1} NAA + 0.5 mgl^{-1} KIN were used for further experiments.

On the other hand, the induction of haploid plants through callus culture (indirect way) was also promising. MS medium was used for callus induction and plant regeneration

Fig. 3. The difference between diploid (D) and haploid (H) plants

with different hormone combinations (2,4-D, BAP, NAA and/or KIN). The MS medium supplemented with 0.4 mgl^{-1} NAA + 0.5 mgl^{-1} KIN gave the best results for callus induction. The obtained calli were transferred to regeneration medium, using MS medium containing different hormones combination (BAP and/or NAA). The regeneration of plants was observed after 40 days and ranged between 10 to 33 plantlets/callus (Fig. 2). The best combination of hormones used with MS medium for plant regeneration was 1.0 mgl^{-1}

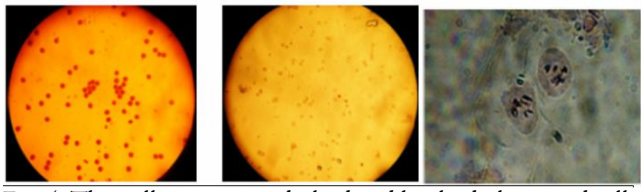

Fig. 4. The pollen grains in diploid and haploid plants and cells from root meristem of a haploid regeneration

BAP + 0.5 mgl^{-1} NAA. The ability to form callus mainly depends on the physiological state of the donor plants and the season during which the anthers are explanted. It has been reported that flower buds which have developed after plant regeneration give rise to pollen grains of flower androgenetic ability (Maheshvari *et al*., 1980; Shtereva *et al*., 1998; Zhou, 1996). Microspore developmental stage at the time of anther excision is one factor determining the induction of androgenesis. Some researchers indicate early meiosis as the

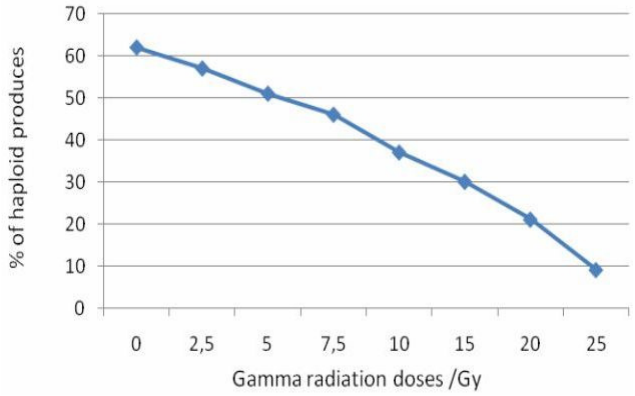

Fig. 5a. Effect of gamma radiation doses on anthers (to produce haploid plants)

Fig. 5b. The effect of gamma irradiation on anther

Table 2. Effect of gamma irradiation on *in vivo Nicotiana alata* haploid plants

Radiation dose/Gy	Shoot length cm / days			
	40	60	80	%
0.0	54.0	86.6	104	100
2.5	42.9	71.5	93.0	89
5	38.6	63.0	79.7	76
7.5	31.5	56.0	71.6	69
10	26.6	48.0	63.8	61
15	18.6	41.0	51.6	49
20	13.3	33.4	42.6	41
25	11.4	27.8	37.0	35

optimal stage, while according to others it is the uninucleate stage (Sasmita *et al*., 2001; Summers *et al*., 1992; Zagoraska *et al*., 1982). The growth regulator specific action depended both on genotype and physiological state of the donor plant. A similar tendency of phytohormone influence on organogenesis was also observed by Dewi *et al*. (2004), Miceska (2011), Shtereva *et al*. (1998).

The regenerated plants can be obtained by isolation of anther *in vitro*, whether by direct or indirect way, therefore it was investigated morphologically and cytologically to confirm the haploidy.

Morphologically, it has been proved by observation and measurements that the highest haploid plant was of 104 cm comparing with diploid plant (155 cm); measurements were also made for steam diameter, leaf size, flower size and diameter,

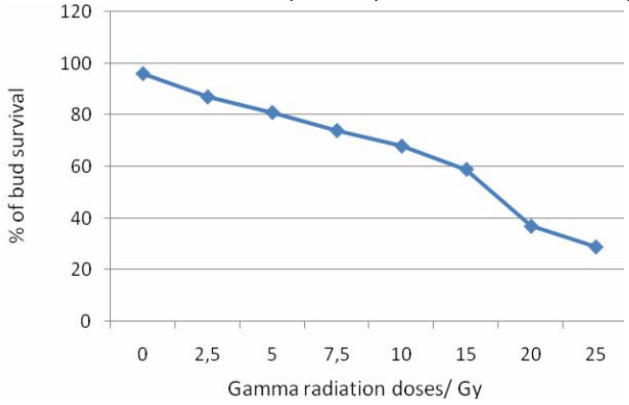

Fig. 6a. Effect of gamma radiation doses on % of bud survival in haploid plants

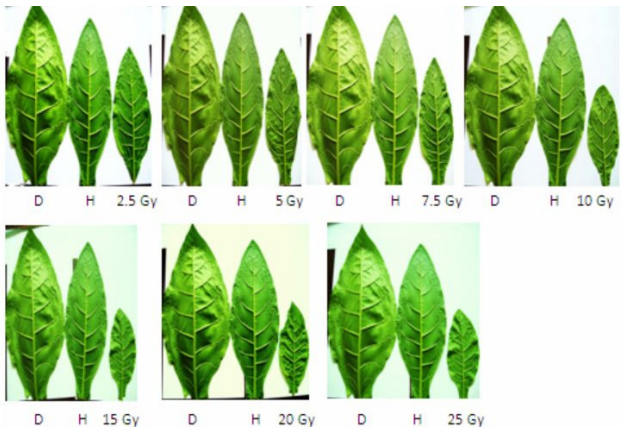

Fig. 6b. The difference between diploid (D), haploid (H) and irradiated haploid leaves

anther length and number. The haploid plants presented about 67% growth of the diploid plants (Figs. 3 and 6b).

Cytologically, with used squash method for each pollen grains and root meristem examination, the pollen grains in haploid plants were invalid. However, the root meristem investigation confirmed the haploid level got half of

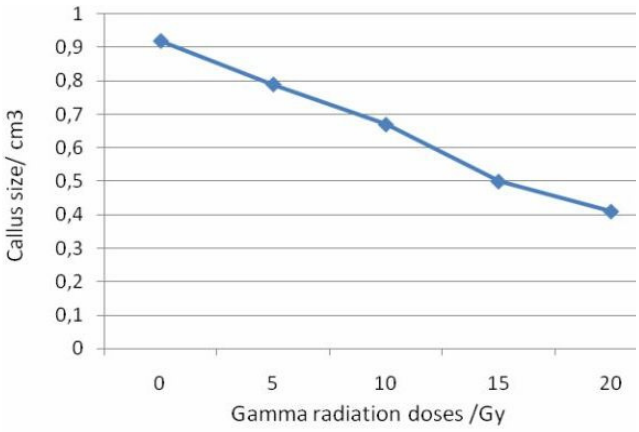

Fig. 7. Effect of gamma radiation doses on proline content in haploid plants

chromosomes number set (Fig. 4). These results are in agreement with Zagorska *et al*. (1998) and Miceska (2011). The various techniques for anther culture and induction of androgenesis are summarized by Chlyah *et al*. (1990) and Summers (1997) for tomato and Vagera (1990) for pepper.

Anther radio-sensitivity in Nicotiana alata

The investigation of produced haploid plants from anther culture by direct way with sensitivity gamma radiation doses (0.0, 2.5, 5, 7.5, 10, 15, 20 and 25 Gy) proved that irradiated anthers decreased the number of haploid plants and also shoot length with increasing gamma radiation dose (Figs. 5a and b). As well as when haploid segments were irradiated with same doses it was observed the same results (Table 2, Figs. 6a and b). On the other hand, the effect of gamma radiation doses (5, 10, 15 and 20 Gy) on callus morphology and growth rate was investigated. The callus morphology changed from friable to massive greenish and the callus growth rate decreased with increasing gamma radiation dose (Fig. 7). However, the irradiated callus failed to regenerate

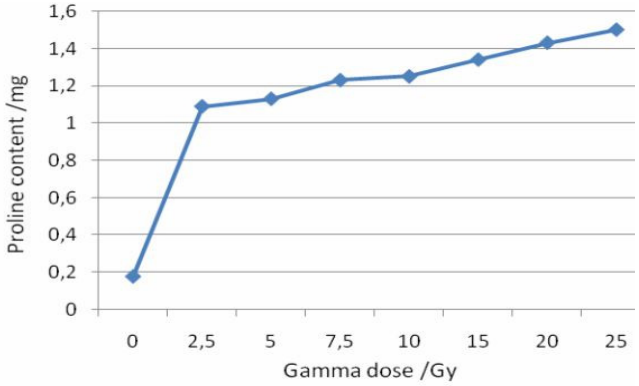

Fig. 8. Effect of gamma radiation doses on total soluble protein in haploid plants

plants. The results showed by the radiation sensitivity test based on survival percentage of irradiated and non-irradiated plantlets that a significant reduction in survival percentage was observed with increasing gamma dosage. These results were in accordance with radiation sensitivity test done by Hasegawa *et al*. (1995) for tobacco, El-Fiki (1997) for potato, El-Fiki *et al*. (2005a and b) for alfalfa, Norfadzrin *et al*. (2007) for tomato and okra and Kiong *et al*. (2008) for *Orthosiphon stamineus*.

Proline content estimation

Proline content in irradiated plants with doses of 2.5, 5, 7.5, 10, 15, 20 and 25 Gy and non-irradiated plants was estimated. The results showed that the proline content was increased with increasing gamma radiation dose (Fig. 8). The haploid plants contained 0.18 mg/ 100 g fresh weight proline, while diploid plants had 0.37 mg/ 100 g fresh weight (48.6%).

Total soluble protein

Total soluble protein was estimated in irradiated plants with different doses (2.5, 5, 7.5, 10, 15, 20 and 25 Gy) and non-irradiated plants. The results demonstrated that the total soluble protein was increased with increasing gamma radiation dose up to 10 Gy. However, the higher doses have had a negative impact on total soluble protein, as they began to decline until it reached the lowest for the dose of 25 Gy (Fig. 9). The amount of total soluble proteins in haploid plants was equivalent to 69.5% compared to the amount found in diploid plants. The total soluble proteins in diploid plants was 1.41 mg/ 100 g fresh weight, while the haploid plants were containing 0.98 mg/ 100 g fresh weight total soluble proteins.

The most crucial function of plant cell is to respond to gamma stress by developing defense mechanisms. This defense may be affected by alteration in the pattern of gene expression (Corthals *et al*., 2000), which may led to modulation of certain metabolic and defensive pathways (Zolla *et al*., 2003). Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein contents were obvious in the study of Corthals *et al*. (2000). These proteins play an important role in signal transduction, antioxidative defense, antifreezing, heat shock, metal binding, antipathogenesis or osmolyte synthesis, which are essential to a plant's function and growth (El-Fiki *et al*., 2003, 2004; Gygi *et al*., 1999).

Conclusions

Nicotiana alata was considered a model plant for production of haploid plants. Through direct and indirect ways, haploid plants were obtained. Gamma radiation doses have had a negative impact of haploid production, while the irradiated callus did not have positive effect on plant regeneration. The haploid plants irradiated with gamma rays and cultured on MS medium had a negative effect on vitality and growth rate.

Acknowledgements

This research was carried out in National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority.

References

Batles LS, Waldern RP, Teare ID (1973). Rapid determination of free praline for water stress studies. Plant and Soil 39:205.

Bhojwani SS, Razdan MK (1986). Plant tissue, a classified bibliography, Elsevier, Amsterdam 1-179.

Bradford M (1976). A rapid and sensitive for the quantitative of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem (27):248-254.

Chlyah A, Taarji H, Chlyah H (1990). Anther culture and induction of androgenesis, p. 442-455. In: Bajaj, Y. (Ed) Biotechnology in agriculture and forestry, Vol. 12. Haploids in crop improvement. Springer, Berlin Heidelberg New York.

Corthals G, Gygi S, Aebersold R, Patterson SD (2000). Identification of proteins by mass spectrometry. Proteome research 21(1):197-231.

Dewi IS, Purwoko BS, Aswidinnoor H, Somantri IH (2004). Kultur antera padi pada beberapa formulasi media yang mengandung poliamin. Journal Bioteknologi Pertanian 9 (1):14-19.

El-Fiki AAM (1997). Induction of genetic variability by using gamma radiation and selection for salt tolerance *in vitro* in potato *(Solanum tuberosum).* J Genetics & Breeding 51:309-312.

El-Fiki AA, El-Khalal SM, Aliwa NE (2003). *In vitro* induction of mutation in banana (*Musa* sp.) by using gamma irradiation. Egypt J Biotechnol 13:37-49.

El-Fiki AA, Sayed AIH, Moustafa MAM (2004). Gamma radiation effects on growth and patterns of protein in organ and tissue culture of sweet potato (*Ipomea batatas* L. Lam). Arab J of Nuclear Sci and Appl 37(3):253-263.

El-Fiki A, Sayed AIH, Abdel-Hameed AAM (2005a). The combined effect of gamma radiation and mannitol on callus formation and regeneration in alfalfa (*Medicago sativa* L.). Int J Agric & Bio 7(6):966-972.

El-Fiki A, Sayed AIH, Abdel-Hameed AAM (2005b). Genotype, explant, medium, light and radiation effects on the *in vitro* plant regeneration in alfalfa (*Medicago sativa* L.). Isotope and Radiation Research 37(2):437-453.

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999). Correlation between protein and mRNA abundance in yeast. Molecular Cell Biology 19(1):1720-1730.

Hasegawa H, Takashima S, Nakamura A (1995). Effect of gamma ray irradiation on cultured anthers of tobacco (*Nicotiana tabacum* L.) radiosensitivity and morphological variants appearing in the haploid plants. Plant Tissue Culture Letters 12(3):281-287.

Jain SM, Ahloowalia BS, Veilleux RE (1998). Somaclonal variation and induced mutation in crop improvement. Kluwer Academic Publishers 12(3):203-218.

Kim JH, Baek MH, Chung BY, Wi SG, Kim JS (2004). Alterations in the photosynthic pigments and antioxidant machineries of

red pepper (*Capsicum annum* L.) seedlings from gamma-irradiated seeds. Journal of plant Biotechnology 47(2):314-321.

Kiong ALP, Lai AG, Hussein S, Harun A (2008). Physiological responses of *Orthosiphon stamineus* plantlets to gamma irradiation. American- Eurasian journal of sustainable agriculture 2(2):135-149.

Kovacs E, Keresztes A (2002). Effect of gamma and UV-B/C radiation on plant cell. Micron 33(4):199-210.

Ling DX, Luckett DJ, Darvey NL (1991). Low-dose gamma irradiation promotes wheat anther culture response, Aust J Bot 39:467-474.

MacDonald MV, Hadwieger MA, Aslam FN, Ingram DS (1988). The enhancement of anther culture efficiency in *Brassica napus* ssp. oleifera Metzg. (Sinsk.) using low doses of gamma irradiation. New Phytol 110:101-107.

Maheshvari SC, Tyagi AK, Malhotra K (1980). Induction of haploidy from pollen grains in angiosperms: the current status. Theor Appl Genet 58:193-206.

Miceska G (2011). Determination of the level of androgenesis in tobacco (*Nicotiana tabacum* L.). Journal of Central European Agriculture 12(3):515-518.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assay with tobacco tissue cultures. Physiol Plant I 5:473-497.

Murashige T, Tucker DPH (1969). Growth factors requirements of citrus tissue culture. Proc. 1st intl. Citrus Symp (3):1155-1161. J Amer Soc Hort Sci (1995), 120(6):902-905.

Nitsch JP (1969). Experimental androgenesis in *Nicotiana*. Phytomorphology (19)389-404.

Norfadzrin F, Ahmed OH, Shaharudin S, Abdul Rahman D (2007). A preliminary study on gamma radiosensitivity of tomato and okra. International Journal of Agricultural and Research 2(7):620-625.

Pierik RLM (1979). *In vitro* culture of higher plants, Department of Horticulture, Wageningen Agriculture University, The Netherland.

Ramana MS, Parkken R (1967). Structure of homology between pachytene and somatic metaphase chromosomes of the tomato. Genetica 38:115-133.

Sangwan RS, Sangwan BS (1986). In: Nuclear techniques and *in vitro* cultured plant tissues, p. 181-185. IAEA, Vienna.

Sasmita P, Dewi IS, Purwoko BS (2001). Pengaruh generasi kalus terhadap regenerasi tanaman pada kultur antera padi (*Oryza sativa* L.) kultivar Gajah Mungkur. Sain Teks (Edisi Khusus):179-188.

Shtereva LA, Zagorska NA, Dimitrov BD, Kruleva MM, Oanh HK (1998). Induced androgenesis in tomato (*Lycopersicon esculentum* Mill). II. Factors affecting induction of androgenesis. Plant Cell Report 18:312-317.

Summers WL (1997). Haploid plantlet production in tomato, p. 219-231. In: Jain SM, Spory SK, Veilleux RE (eds). *In vitro* haploid production in higher plants. Kluwer, Dordrecht.

Summers WL, Jaramillo J, Bailey T (1992). Microspore developmental stage and anther length influence the induction of tomato anther callus. HortScience 27:838-840.

Vagera J (1990). Pepper (*Capsicum* spp): *In vitro* induction of haploids, p. 374-391. In: Bajaj YBS (Ed) Biotchnology in agriculture and forestry, Vol. 12. Haploids in crop improvement I. Springer, Berlin Heidlberg New York.

Wi SG, Chung BY, Kim JH, Baek MH, Yang DH, Lee JW, Kim JS (2005). Ultrastructural changes of cell organelles in Arabdopsis stem after gamma irradiation. J Plant Biol 48(2):195-200.

Zagoraska N, Abadjieva M, Georgiev G (1982). Morphogenesis and plant differentiation in anther culture of the genus *Lycopersicon* Mill. Proc 5th Int Cong Plant Tissue cult Tokyo. p. 539-540.

Zagorska NA, Shtereva A, Dimitrov BD, Kruleva MM (1998). Induced androgenesis in tomato (*Lycopersicon esculentum* Mill). I. Influence of genetype on androgenesis ability. Plant Cell Report 17:968-973.

Zhou H (1996). Genetics of green plantlet regeneration from anther culture of cereals, p. 169-187. In Jain SM, Sopory SK, Veilleux RE(Eds.). *In Vitro* Haploid Production in Higher Plants. Vol. 2. Applications. Kluwer Acad Publ, Netherlands.

Zolla L, Timperio AM, Walcher W, Huber CG (2003). Proteomics of light harvesting proteins in different plant species. Plant Physiol 131(2):198-214.