Effect of Gamma Irradiation on Production of Phytoplasma-Free Hibiscus Plantlets

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ABSTRACT:

Controlling phytoplasma-causing disease through combination of tissue culture technique and radiation treatment represented a short method for high level management. To investigate the effect of gamma rays to produce healthy plantlets, the hibiscus witches' broom phytoplasma was transmitted by grafting into healthy hibiscus plants, the *in vitro*-infected hibiscus explants were exposed to gamma radiation at different doses - 5 gray (Gy), 10Gy, 15Gy, 20Gy and 25 Gy- emitted from cobalt 60 (^{60}Co) for 30 minute. All applied doses resulted in phytoplasma-free hibiscus plantlets with different survival activity. The presence of phytoplasma 16Sr DNA was examined using PCR detection before and after treatments. The different treatments of radiation resulted in different survival rates and phytotoxic effects including leaf yellowing and lack of growth. The 5Gy dose was proved to be best effective dose for controlling the phytoplasma without affecting the *in vitro* growth and survival rate, while the higher doses leaded to strongly reducing the survived hibiscus explants with increasing the inability to re-shoot.

Key words: Hibiscus, phytoplasma, gamma irradiation, *in vitro*, PCR, graft transmission, tissue culture.

INTRODUCTION

Hibiscus *rosa-sinensis* L. (*Malvaceae*) is one of perennial ornamental shrubs widely planted in Egypt and has high commercial value as a landscaping plant with amazing flowers increase the beauty of surrounding areas and provide calmness to the eyes. Flowers of hibiscus *rosa-sinensis*, have some potential in cosmetic skin care as an anti-solar agent by absorbing ultraviolet radiation (Nevade *et al*., 2011) and the whole plant with high medical and therapeutic potential, principally for bronchial catarrh, demulcent in cough, anodyne, laxative, regulation of menstrual cycle, curing hypoglycemia, potentiate hair growth and anti-hypertensive (Anil and Ashatha, 2012).

Phytoplasmas are pleomorphic but nonhelical bacteria lacking a cell wall; inhabiting the phloem of plants and the hemolymph of insect vectors (Nicoletta, *et al*., 2012). Phytoplasmas can also be spread mechanically by grafting infected plant parts onto healthy plants (Al-Zadjali, *et al*. 2007). Molecular DNA-based techniques such as, polymerase chain reaction (PCR) is a specific and rapid technique in their detection using universal and specific primers (Lee *et al*., 2000, Heinrich *et al*., 2001), and exceptionally, nested PCR used to overcome the low concentration problem of phytoplasma in infected plants through amplifying the direct PCR product (Bertaccini and Duduk, 2009)

Phytoplasma transmission through the hibiscus-trees scattered widely in Egypt (Mokbel *et al*., 2013) lead to serious threat as a alternative source of natural host and provides an ideal starting point for the establishment of a disease in important field crops furthermore, each infected seedling provides a phytoplasma reservoir for subsequent secondary spread by vectors having the ability to migrate in presence of wind currents, causing reduced yields, decreasing of

fruits quality and death of plants are probably the final result of this disease (Chaturvedi *et al*., 2010).

During recent years, increasing attention has been devoted to the control of phytoplasma diseases by controlling the vector using substances with antibiotic activity like tetracycline, eliminating the pathogen from the infected plants through tissue culture technique alone or combined with thermal treatment (Bertaccini 2007, Wongkaew and Fletcher 2004, Tassart-Subirats *et al*., 2003). After these treatments, phytoplasma associated with diseases-plant have been still found in fields causing growth retardation and very low propagation percentages of these plants (Chung 2008, Singh *et al*., 2007).

Research topic was come from successful inactivation of some plant viruses by gamma irradiation like *Citrus tristeza virus* (Ieki and Yamaguchi, 1984), *Necrotic ring spot virus* and *Prune dwarf virus* (Megahed and Moore, 1969). Gamma irradiation has been also used to sterilize agricultural products in order to increase their conservation time or to reduce pathogen when being traded from a country to another (Melki and Salami, 2008). Gamma radiation is high-energy radiation emitted from certain radioactive isotopes as ^{60}Co, these isotopes are potential sources of gamma radiation (Rosenthal, 1992). Therefore, this research was conducted to find out the inactivation possibility of HibWB-phytoplasma using gamma irradiation through tissue culture technique with clarify their effect on *in vitro* growth and survival rate.

MATERIALS AND METHODS

Phytoplasma source and grafting inoculation:

T-bud grafting (Rasha, 2012) was carried out with 30 scions taken from identified HibWB-phytoplasma (Mokbel *et al*., 2013) and 30 healthy hibiscus plants from the same variety as root stocks those obtained from commercial nurseries in Giza governorate. The graft unions were placed in an insect-free greenhouse for 4 weeks. Phytoplasma detection in hibiscus plants has been carried out through visual monitoring of symptoms as well as by molecular analysis using PCR.

Surface sterilization and plant materials:

One hundred and fifty stem segments were gathered from one month old infected hibiscus plants (young shoots) that being maintained in the greenhouse, washed under running tap water for 30 min to remove dirt on the stem, surface were sterilized with 20% commercial Clorox solution containing one drop of Tween 20 (polyoxy ethylene sorbiton monolaurate) for 20 min then rinsed 3 times with sterile distilled water for 5 min each.

Nodal segments of approximately 2.0 cm in length were cut from sterilized stems, each three nodal segments were cultured in one sterilized culture jar containing 25ml solid half strength MS medium (Murashige and Skoog, 1962) supplemented with 20 gL^{-1} sucrose and 8 gL^{-1} agar without any additions of growth regulators, pH 5.7 and then were incubated under artificial conditions at 27ºC, 16h photoperiod, 2000 Lux for 6 weeks (Chew *et al*. 2012).

Irradiation source and treatments using ^{60}Co:

In order to investigate the effects of various doses of gamma irradiation on controlling HibWB-phytoplasma as well as its effect on the survival rate and development of hibiscus shoots. After incubation period, five jars with three explants each were irradiated individually for 30 min with five doses- 5, 10, 15, 20 or 25-

Gy of gamma irradiation. Five non-irradiated culture jars were served as negative control. The source of gamma irradiation was ^{60}Co gamma cell 3500, from the Middle Eastern Regional Radioisotope Center for the Arab countries, Giza, Egypt.

Three days after irradiation, developed plantlets were sub-cultured on solid half strength MS medium supplemented with 20 gL^{-1} sucrose and 8 gL^{-1} agar under the same incubation conditions for 4-6 weeks and then were detected using PCR assay.

Molecular screening for phytoplasma:

DNA extraction:

To evaluate the effect of the gamma irradiation on HibWB-phytoplasma DNA, newly shoots from tissue cultured plantlets which produced from hibiscus plants those previously irradiated with five different doses were tested for the infection of phytoplasma using nested PCR. Non-irradiated infected hibiscus plantlets (*in vitro*) and healthy plants from greenhouse were used as a control treatment.

Total DNA was extracted from true leaves using column based DNA extraction protocol utilizing Plant DNA Mini Kit (Geneaid-Taiwan) according to the manufactures' instructions.

Nested-PCR:

The Universal phytoplasma-specific primers, P1/P7 and R16F2n/R16R2 (Lee *et al*., 2004) were used for the PCR screening of the irradiated and none irradiated hibiscus plant leaves. The detection of phytoplasma was performed in nested PCR (Bhat *et al*., 2006). The P1/P7 primer was used in the first step while, the R16F2n/R16R2 primer pair was used to amplify a 1.2 kbp fragment of 16Sr RNA gene in the second step nested-PCR as described by Wang and Hiruki (2001). The extracted total DNA was used as template for PCR. The PCR mixture of 25µl total volume contained 1 µl DNA; 25 pmol of each primer; 200 µM of each dNTP; 1x polymerase reaction buffer; 2.5 mM $MgCl_2$; 1.25 U of dream-Taq polymerase (Fermentas) and sterile water to a final volume of 25 µl. The first round PCR of the DNA amplification was started with a denaturation step at 94°C for 2 min followed by 35 cycles of three steps started by denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min. A final extension step was added for 10 min 72°C. To run the second round PCR; one µl of the first round PCR product (with primer pair P1/P7) was used - at 1:10 dilution- as template for the second round PCR. The second round PCR was started with a denaturation step at 94°C for 2 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing for 2 min at 50°C, and primer extension at 72°C for 3 min. A final extension step was added for 10 min 72°C.

The electrophoresis analyses:

The electrophoresis analyses were performed for all PCR products. The gel star (Lonza, USA) was used to stain all PCR products then separated on 1% agarose gel with 1x TBE buffer and analyzed using (Gel Doc 2000 Bio.RAD). The PCR products were compared with 1 kbp DNA ladder to determine the molecular weight of the amplified fragments.

RESULTS

Infectivity of phytoplasma

The infectivity of phytoplasma was confirmed through grafting transmission from infected hibiscus plants onto healthy ones. Symptoms of HibWB-phytoplasma include yellowing and deformations of the newly formed leaves were observed on 27 grafted plants out of 30 (Fig. 1). The PCR

results (Fig. 2) using the DNA extracted from the inoculated plants showed a clear band at the expected size (~ 1200bp). These results, both PCR and Hib-WB symptoms confirmed the successful transmission of the phytoplasma by grafting from infected to a healthy Hibiscus plant with percentage reached 90% in hibiscus graft unions (Al-Zadjali, *et al*. 2007).

Fig. (1): Grafting transmission for the HibWB-phytoplasma. **A:** Healthy hibiscus plant. B: Affected hibiscus plant after grafting transmission; showing the typical witch's broom symptoms.

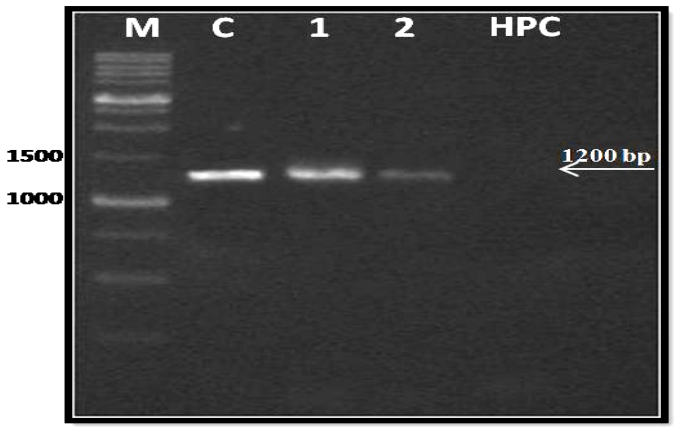

Fig. (2): Electrophoresis analysis for the PCR products amplified from hibiscus plants inoculated by grafting. M: 1 Kbp DNA ladder. C: is a positive control for phytoplasma infected Hibiscus plant that used as a source for inoculation. 1 and 2: are inoculated plants using grafting transmission. HPC: Healthy Hibiscus plant control.

Effect of gamma irradiation on phytoplasma-infected plantlets:

118 out from 150 (78.6%) of nodal segments of hibiscus infected with WB-phytoplasma were successfully developed shoots after six weeks on MS medium. Five groups, fifteen explants each, were irradiated for 30 min at five different doses of gamma radiation; 5, 10, 15, 20 or 25 Gy (Fig. 3). Non-irradiated explants served as control treatment (Fig. 4).

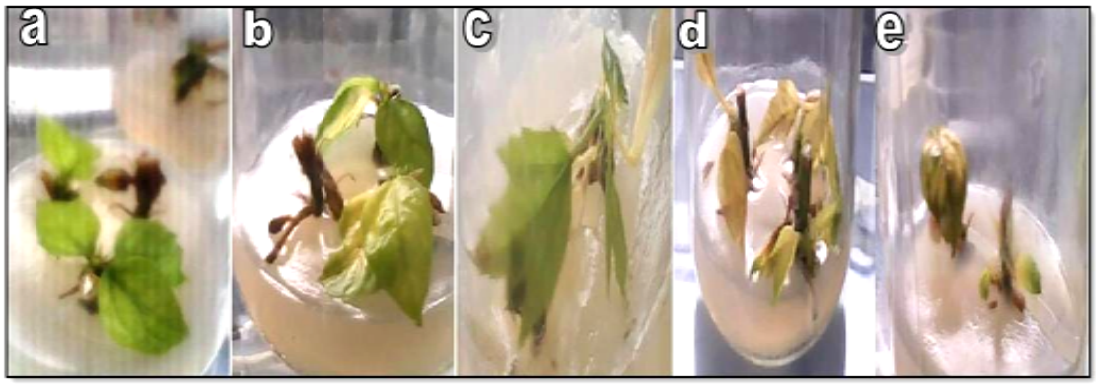

Fig. (3): Effect of different doses of gamma irradiation 5 Gy **(a)**, 10 Gy **(b)**, 15 Gy **(c)**, 20 Gy **(d)** and 25 Gy **(e)** on *in vitro* hibiscus explants with different degrees of yellowing and phytotoxic symptoms.

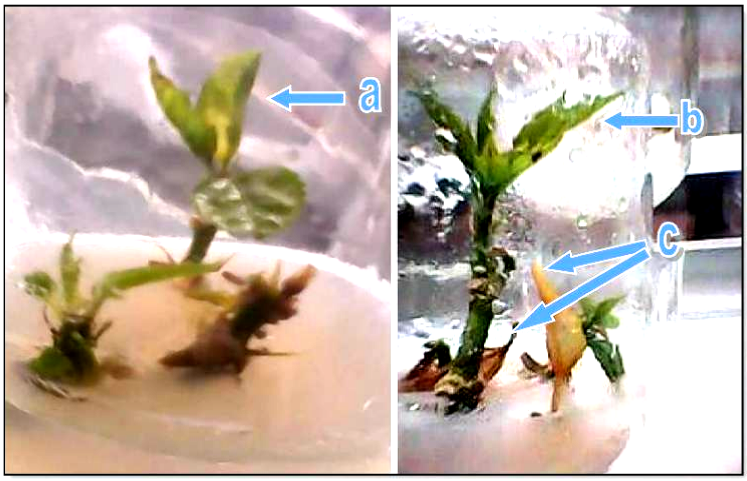

Fig. (4): Non-irradiated *in vitro* hibiscus explants with different symptoms caused by phytoplasma disease, yellowing and browning **(a)** and deformation of leaves **(b)** or shortened and increased in the size of internodes **(c)**.

PCR detection of phytoplasma in irradiated plants:

After undergoing the treatments, the nested-PCR was used for the detection of WB-phytoplasma in hibiscus plantlets compared with the non-irradiated plants. The PCR was performed utilizing the universal-phytoplasma specific primer pairs; P1/P7 and R16F2n/R16R2. Phytoplasma characteristic 16Sr DNA fragments of 1.8 kbp fragment that expected from the first round PCR could not be visualized by electrophoresis. While the characteristic fragment of approximately 1.2 kbp that specific for the phytoplasma infection was amplified by the second round PCR, using R16F2n/R16R2 primer pair, only from non-irradiated hibiscus plants, on the contrary, the irradiated hibiscus plants didn't show any fragment specific for phytoplasma infection (Fig. 5). Electrophoresis analysis for the PCR products showed clear bands at the expected size 1.2 kbp, only when the template DNA was extracted from the non-irradiated hibiscus samples.

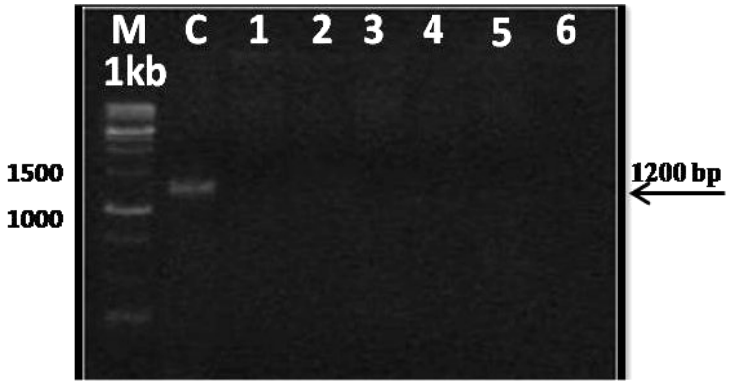

Fig. (5): Electrophoresis analysis for the PCR products amplified from irradiated and none irradiated hibiscus plants. **(C)**: None irradiated infected hibiscus plant. **(1)**: sample from the 1st irradiation treatment (5Gy). **(2)**: 2nd irradiation treatment (10Gy). **(3)**: 3rd irradiation treatment (15Gy). **(4)**: 4th irradiation treatment (20Gy). **(5)**: 5th irradiation treatment (25Gy). **(6)**: Healthy hibiscus plant from greenhouse. M: 1 Kb DNA Laddar.

Vitality of the irradiated hibiscus plants:

The changes on the plant growth after irradiation treatments and sub-culture were clearly observed in Fig.(6); and the percentage of plant survival were recorded and shown in Fig. (7); by changing the amount of radioactive cobalt in the radiation source. A low dose 5Gy gamma irradiation stimulated the plant growth which just took 4 weeks for development with new shoots (Fig. 6-a) and survival rate 93.3% (14/15) as compared to non-irradiated explants which took 6 weeks with survival rate 86.66% (13/15). While, dose of 10 or 15Gy gamma irradiation caused same pattern (11/15) of surviving plants 73.3% with growth retardation (Fig. 6-b). On the contrary, 20 and 25 doses of gamma irradiation proved to be the most lethal doses in which phytotoxic symptoms were observed vigorously and reduced the percentage of survival plants to 20% (3/15) and 13.3% (2/15) respectively with explants not able to re-shoot (Fig. 6-c).

Fig. (6): The growth development stages, observed after subculture of irradiated explants with 5 Gy **(a)**, with 10 Gy or 15 Gy **(b)** and with 20 Gy and 25 Gy **(c)**.

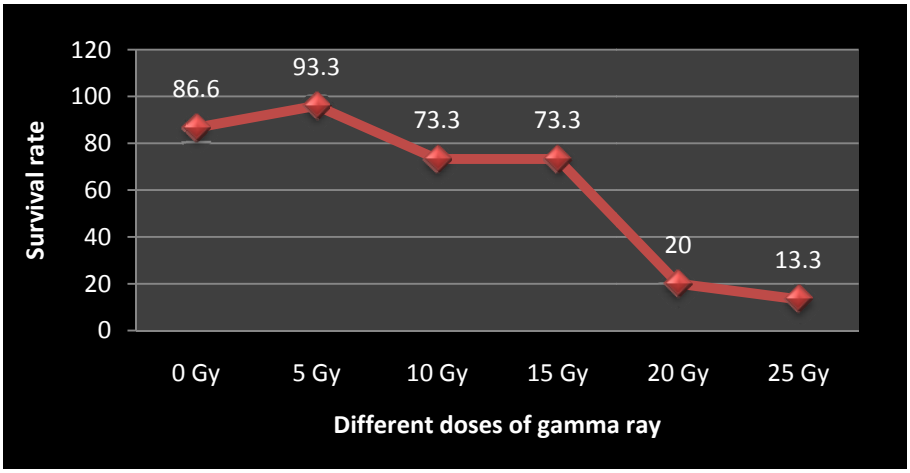

Fig. 7: Effect of gamma irradiation doses on *in vitro* survival rate of hibiscus explants.

DISCUSSION

A management strategy for phytoplasma disease control is critical. In this regard, so far no work has been reported on control of phytoplasma, through gamma irradiation in a tissue-culture line of diseased hibiscus.

The present study was undertaken to get rid of the phytoplasma pathogen, strain Witches' Broom, which affected hibiscus plants recently in Egypt (Mokbel *et al*., 2013) and one of the most promising means to achieve this goal is by the use of radiotherapy tools based on gamma irradiation in tissue culture line for disease control; that may help to propose effective strategies for obtaining healthy planting material. However, our results showed that the treatment with gamma irradiation had a positive effect on controlling HibWB-phytoplasma and consistent with many studies in previous years, have shown that irradiation as a mean of controlling both the fungal pathogens (*Rhizoctonia solani Kühn* and *Sclerotium rolfsii Sacc*) that attacked sugar beet plant (Tarek *et al*., 2003) as well as human pathogens such as *E. coli* (Thayer and Boyd, 1993) or food-borne pathogens (Sumner and Peters, 1997).

To get our infected plant materials, HibWB-phytoplasma was successfully transferred by grafting into healthy hibiscus *rosa sinensis* plants with percentage reached 90% in hibiscus graft unions, causing symptoms composed of yellowing and deformation of the newly formed leaves after 4 weeks.

The detection method applied in the present study is widely used to identify and detect phytoplasma thus, the interpretation of absence of the phytoplasma DNA in plants exposed to radiation unlike the infected plant may be a quite similar to mechanism of gamma ray during sterilization processes for the control of food contaminating microorganisms like bacteria, however it would not be a sterilizing step as it is for bacteria that has a cell wall and are ten times larger than phytoplasma, and the presence of cell wall is a step for irradiation resistance (Gherbawy, 1998) taking in account, the phytoplasmas are very small prokaryotes which are related to bacteria, but in contrast to bacteria they do not have a cell wall and thus collapsed with the effect of high-energy gamma photons (1.33 MeV) given off by Cobalt-60. This energy can penetrate plant cell and hit and break down the double helix of the pathogen-DNA or split the water molecules and generate free hydrogen (H^+), hydroxyl (OH^-) and oxygen (O^{-2}) radicals that capable of

killing the pathogen by deactivating and damage pathogen-DNA (Smith and Pillai, 2004), which in turn causing defects in the genetic instructions and disrupting its function and is therefore inhibiting pathogen reproduction (Kátia, 2012) through a safe and nontoxic treatment, without any radioactivity even if the high doses are used up to 10 kGy (WHO, 1981).

The results obtained from figures 6 and 7 showed that, the influences of irradiation on plant growth and development depend mainly on the dose of the gamma irradiation. Many studies carried out on the stimulation and inhibition of plant growth by applying gamma irradiation, mentioned that, irradiation at 1-2 Gy lead to slightly increasing the growth of Arabidopsis seedlings (Wi *et al*. 2006), the growth stimulation of red pepper seeds occurred between 2 to 8 Gy and was affected at 16 Gy (Kim *et al*. 2004), the growth stimulation of *in vitro* key lime explants occurred at 5 Gy after 4 weeks of regeneration (Fatemeh *et al*. 2011), the stimulation of citrus occurred at 10 Gy and inhibited above 10 Gy (Ling *et al*. 2008), and finally, the inhibition of more than 50% of the *in vitro* regenerating *gerbera jamesonii* explants occurred at 25 Gy (Nor *et al.* 2012). So, the results of our study are completely consistent with the results obtained by Kim *et al*. (2004) and Fatemeh *et al*. (2011) or the suggestion by El Sherif *et al*. (2011) and Minisi *et al*. (2013) that increasing the doses of gamma irradiation caused severe effects on the plant development and the surviving plants percentage, which decreased linearly in our study with increasing doses of gamma irradiation, as evident in figure 6 that may be attributed to the inhibition of DNA synthesis, destruction of the membrane system of mitochondria and chloroplasts (Ladanova, 1993), or other physiological damages and complications after irradiation treatments, like disruption of protein synthesis, hormone balance, enzyme activity (Kovacs and Keresztes, 2002; Nor *et al*., 2012). Or may be due to enhance the production of antioxidative compounds such as phenolics and flavonoids during the radiation treatments (Harrison and Were, 2007) in related with the presence of browning in culture media that mainly to reduce the effects of irradiation stress and avoid the oxidative damage (El-Beltagi *et al*., 2011) taking in account, the browning dramatically reduced after sub-culture of irradiated explants (Fig. 6) unlike non-irradiated explants (Fig. 4) which still found to the defense against stresses of phytoplasma (Dixon and Paiva, 1995) as evident by symptoms of phytoplasma, such as yellowing of some leaves (Fig. 4a) due to breakdowns of chlorophyll (II) that could be the result of an enhanced chlorophyllase activity in phytoplasma-infected leaves (Bertamini *et al*. 2002 and Bertamini *et al*., 2004), leaves deformed (Fig. 4b) and shorted or increase in size of the internodes (Fig.2c) due to profound disturbances in the normal balance of plant hormone or growth regulators (Lee *et al*., 2000).

Finally**;** the results obtained in this research for controlling witches' broom phytoplasma through combination of tissue culture technique and radiation can help future studies to use this environmentally safe and inexpensive mechanism to apply as starting point to overcome phytoplasma diseases. The low dose (5Gy) has been seemed as strategy solution for controlling phytoplasma disease and avoiding the degradation of *in vitro* plants but it is recommended to fully explore doses below of 5Gy with different irradiation intervals.

REFERENCES

Al-Zadjali, A.D., Natsuaki, T. and Okunda, S. (2007). Detection, identification and molecular characterization of a phytoplasma

associated with Arabian jasmine (*Jasminum sambac* L.). J. Phytopathology 155: 211-219.

Anil, K. and Ashatha, S. (2012). Review on *Hibiscus rosa sinensis*. International Journal of Research in Pharmaceutical and Biomedical Sciences 3 (2): 534-538.

Bertaccini, A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. Frontiers in Bioscience 12: 673-689.

Bertaccini, A. and Duduk, B. (2009). Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathol. Mediterr. 48:355-378.

Bertamini, M., Grando, M.S. and Nedunchezhian, N. (2004). "Effects of Phytoplasma Infection on Pigments, Chlorophyll-Protein Complex and Photosynthetic Activities in Field Grown Apple Leaves". Biol. Plant. 47 (2): 237-242.

Bertamini, M., Nedunchezhian, N., Tomasi, F. and Grando, S. (2002). Phytoplasma Stolbur subgroup (Bois Noir-BN) infection inhibits photosynthetic pigments, ribulose-1, 5-biphosphate car- boxylase and photosynthetic activities in field grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. Physiol. Mol. Plant Pathol. 61:357-366.

Bhat, A.I., Madubala, R. Hareesh, P.S. and Anandaraj, M. (2006). Detection and characterization of the phytoplasma associated with a phyllody disease of black pepper (*Pepper nigrum* L.) in India. Scientia Hortic. 107: 200-204.

Chaturvedi, H.Y., Rao, G.P., Tiwari, A.K., Duduk, B. and Bertaccini, A. (2010). Phytoplasma on Ornamentals: Detection, Diversity and Management. Acta Phytopathologica et Entomologica Hungarica 45 (1): 31-69.

Chew, T.D., Janna, O., Parameswari, N. and Siti, H.R. (2012). Sterilization of Hibiscus *rosa-sinensis* L. Vegetative Explants Sourced from Plants Grown in Open Environment and Influences of Organic Ingredients on *In Vitro* Direct Regeneration. American J. of Plant Sciences 3: 791-798.

Chung, B.N. (2008). Phytoplasma detection in Chrysanthemum and lily. In: N. A. Harrison, G. P. Rao and C. Marcone (eds): Characterization, Diagnosis and Management of Phytoplasma. Studium Press LLC, Texas, USA. pp. 175-194.

Dixon, R.A. and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097.

El Sherif, F., Khattab, S., Ghoname, E., Salem, N. and Radwan, K. (2011). Effect of Gamma Irradiation on Enhancement of Some Economic Traits and Molecular Changes in Hibiscus *Sabdariffa* L. Life Science Journal 8 (3): 220-229.

El-Beltagi, H.S., Ahmed, O.K. and El-Desouky, W. (2011). Effect of low doses γ -irradiation on oxidative stress and secondary metabolites production of rosemary (*Rosmarinus officinalis* L.) callus culture. Radiat Phys. Chem. 80: 968-976.

Fatemeh, Sh., Akbar, H.P., Farkhondeh, R., Amin, Sh. and Mohammad, M. (2011). The preservation of lime witches' broom phytoplasma in key lime by tissue culture. Bulletin of Insectology Journal. Italy 64: S201-S202.

Gherbawy, A.M. (1998). Effect of gamma irradiation on the production of cell wall degrading enzymes by *Aspergillus niger*. Int. J. Food Microbiol. 40:127-131.

Harrison, K. and Were, L.M. (2007). Effect of gamma irradiation on total phenolic content yield and antioxidant capacity of almond skin extracts. Food Chem.102: 932-937.

Heinrich, M., Botti, S., Caprara, L., Arthrofer,W., Strommer, S. and Hanzer,V. (2001). Improved detection method for fruit tree phytoplasmas. Plant Mol. Biol. Rep.

19: 169-179.

Ieki, H. and Yamaguchi, A. (1984). Inactivation of citrus tristeza virus by gamma ray irradiation. Ann. Phytopathol. Soc. Jap. 50:656-658.

Kátia, A. (2012). Sterilization by Gamma Irradiation, Gamma Radiation, Prof. Feriz Adrovic (Ed.), ISBN: 978-953-51-0316-5, InTech, DOI: 10.5772/34901. Available from: http://www.intechopen.com/books/gamma-radiation/sterilization-by-gamma-irradiation.

Kim, J.H., Baek, M.H., Chung, B.Y., Wi, S.G. and Kim, J.S. (2004). Alterations in the photosynthetic pigments and antioxidant machineries of red pepper (*Capsicum annuum* L.) seedlings from gamma-irradiated seeds. J. Plant Biol., 47: 314-321.

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

Ladanova, N. (1993). The ultrastructural organization of pine needles after radiation exposure. Radiobiologia 33 (1): 25-30.

Lee, I.M., Bertaccini, A., Vibio, M. and Gundersen, D.E. (2000). Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85: 728-735.

Lee, I.M., Davis, R.E. and Gundersen-Rindal, D.E. (2000). "Phytoplasma: Phytopathogenic Mollicutes". Ann. Rev. of Microbiol. 54: 221-255.

Lee I. M., Martini M., Marcone C., Zhu S. F., (2004). Classification of phytoplasma strains in the elm yellows group (16SrV) and proposition of '*Candidatus* Phytoplasma ulmi' for the Phytoplasma. Int. J. Syst. Evol. Microbiol. Mar 54(Pt 2):337-47.

Ling, A.P.K., Chia, J.Y., Hussein, S. and Harun, A.R. (2008). Physiological responses of *Citrus sinensis* to Gamma Irradiation. World Applied Sciences Journal 5 (1): 12-19, 2008.

Megahed, E.S. and Moore, J.D. (1969). Inactivation of *necrotic rings pot* and *prune dwarf* viruses in seeds of some *Prunus* spp. Phytopathology 59:1758-60.

Melki, M. and Salami, D. (2008). Studies of the effects of low dose gamma rays on the behavior of chickpea under various conditions. Pakistan J. of Biological Sci. 11: 2326-2330.

Minisi, F.A., El-mahrouk, M.E., El-Din, M., Rida, F. and Nasr, N. (2013). Effects of Gamma Radiation on Germination, Growth Characteristics and Morphological Variations of Moluccella *laevis* L. American-Eurasian J. Agric. and Environ. Sci. 13 (5): 696-704.

Mokbel, A.S., El Attar, A.K. and Farag, A.G. (2013). Detection and molecular characterization of phytoplasma associated with Hibiscus Witches'-Broom in Egypt. Egyptian J. of Virology. 10:162-171.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Bilogia Plantarum. 15: 473-497.

Nevade, S.A., Sachin, G. L. and Kalyane, N.V. (2011). Study on anti-solar activity of ethanolic extract of flower of Hibiscus rosa-sinensis Linn. Research Journal of Pharmacy and Technology 4 (3) :472-473.

Nicoletta, C., Assunta, B., Samanta, P., Helena, M.W. and David, W.G. (2012). Axenic culture of plant pathogenic phytoplasmas. Phytopathologia Mediterranea 51 (3): 607-617.

Nor, A., Rosna, M., Azani, S. and Noraini, M. (2012). Irradiation effect on *in vitro* organogenesis, callus growth and plantlet development of Gerbera jamesonii. http://dx.doi.org/10.1590/S0102-05362012000200012. Hortic. Bras. J.

Rasha, M.M.I. (2012). Studies on

Controlling Phytoplasma Infecting Some Economic Crops Under Oraganic Agriculture Conditions. Ph.D Thesis Ain Shams University.

Rosenthal, I. (1992). Electromagnetic radiations in food science. Springer-Verlag, pp: 244.

Singh, S.K., Aminuddin, S.P., Singh, B.R. and Khan, J. A. (2007). Production of phytoplasma - free plants from yellow leaf diseased *Catharanthus roseus* (L.) G. Don. *J Plant Dis Prot*. 114: 2-5.

Smith, J. S. and Pillai, S. (2004). Irradiation and Food Safety. An IFT Scientific Status Summary, Food Technology. 58 (11): 48-55.

Sumner, S. and Peters, D.L. (1997). Microbiology of vegetables. In: Smith, D.S. (Ed.), Processing Vegetable Science and Technology. Technomic Publishing Co. Inc., Lancaster, PA, USA, pp. 87-106.

Tarek A. Moussa, A. and Rizk, M.A. (2003). Impact of Gamma Irradiation Stresses and Control of Sugarbeet Pathogens *Rhizoctonia solani Kühn* and *Sclerotium rolfsii Sacc*. Pakistan J. of Plant Pathology 2 (1):10-20.

Tassart-Subirats, V., Clair, D., Grenan, S., Boudon-Paradieu, E. and Larrue, J. (2003). Hot water treatment: Curing efficiency for phytoplasma infection and effect on plant multiplication material. In Proceedings of the 14th Meeting of the International Council for the Study of Viruses and Virus-like Diseases of the Grapevine Conference, Locorotondo, Italy pp. 69-70.

Thayer, D.W. and Boyd, G. (1993). Elimination of *Escherichia coli* O157:H7 in meats by gamma irradiation, Appl. Environ. Microbiol. 59:1030-1034.

Wang, K. and Hiruki, C. (2001). Use of heteroduplex mobility assay for identification and differentiation of phytoplasmas in the aster yellows group and the clover proliferation group. Phytopathology 91 (6): 546-52.

Who (1981): Wholesomeness of irradiated food. Report a joint FAO/IAEA/WHO expert Committee. Technical Report Series 659. World Health Organization, Geneva: 7-34.

Wi, S.G., Chung, B.Y., Kim, J.S., Kim, J.H., Baek, M.H., Lee, J.W., Kim, Y.S. (2006). Effects of gamma irradiation on morphological changes and biological responses in plants. Micron 38: 553-564.

Wongkaew, P. and Fletcher, J. (2004). Sugarcane white leaf phytoplasma in tissue culture: long-term maintenance, transmission, and oxytetracycline remission. *Plant Cell Rep*. 23: 426-434.