Pak. J. Bot., 44: 311-314, Special Issue March 2012.

IN VITRO INDUCTION OF MUTATION IN TOMATO (*LYCOPERSICON ESCULENTUM* L.) CV. ROMA BY USING CHEMICAL MUTAGENS

MUHAMMAD ISHFAQ[1], IDREES AHMAD NASIR[2], NASIR MAHMOOD[3] AND MUHAMMAD SALEEM[1]

[1]*Department of Botany, University of the Punjab, Lahore*-54590.
[2]*National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore.*
[3]*Institute of Mycology and Plant Pathology, University of the Punjab, Lahore.*
Corresponding author Email: molec.biologist@gmail.com; *Tel: 0333-4246280,Fax: 92-42-9231862*

Abstract

The aim of present study was to optimize tissue culture conditions for tomato (*Lycopersicon esculentum.* L) *cv*. Roma and to induce mutagenesis with different chemical mutagens (Cholchicine, Sodium azide, and Methyl-N-Nitro-N-Nitrosoguanidine abbreviated as MNNG). Tomato seeds were surface sterilized with 0.05 % $HgCl_2$ and placed on the Petri plates padded with wet cotton, obtained plantlets in 6-7 days. Meristem was isolated from the germinated plants to get pathogen free plants. Media with different hormone compositions were used for callus induction and regeneration. Media having BAP (2.22 µM) proved to be the best callus induction media, while media having IAA (0.75 µM) and zeatin (9.12 µM) was found to be best for regeneration. The calli were treated with different concentrations (0.1 mM- 0.5mM) of the mutagens colchicine, sodium azide and MNNG for ten days. After mutagenic treatment, calli were transferred in regeneration medium with said concentration of hormones and growth parameters of the mutated plants were recorded in comparison to control plants.

Introduction

Tomato (*Lycopersicon esculentum* L.) belongs to the family *Solanacae* and is an important vegetable all over the world as described by Abu-El-Heba *et al*., (2008). According to Garvey & Hewitt (1991) tomato is highly prized for its monitory gain and nutritional value especially for its richness in vitamins and minerals (Vitamin A 1500-1200 IU, Vitamin B-1 26 IU, Vitamin B-2 26 (mg/100 gm of fruit) and Vitamin C-25 (mg/100 gm of fruit). Leonardi *et al.,* (2000) found that tomato also contains 0.3% ascorbic acid, 4.8% K, 0.24% Ca, 0.55% P and 0.32% S. According to Bal & Abak, (2007), tomato behaves like a basic diploid, mutations of many types can be clearly identified in its phenotype. Most abundant are modifications of form, size, color and venation of the leaf as well as plant height and number of fruits per plant. The cultivar Marglobe serves as the standard or normal wild type against which mutants are usually compared.

Improvent of plant varieties either through conventional or non-conventional breeding is the objective of the plant breeders (Sajjad *et al*., 2011) but parental varieties with unusual features are usually produced by mutagenesis at first stage and later subjected to breeding experiments to produce more stable and modified plant lines (Stubee, 1975; Evans & Sharp, 1983; Evan, 1989). Tissue culture is the back bone of methodologies leading to mutated plants, so types of mutagens and optimized tissue culture conditions lead to successful mutated plants (Srivastave *et al*., 1995). Shoot apical meristems isolated from 7 days old seedlings of some genotypes (cultivated and F1 hybrids) were cultured *in vitro* on MS medium supplemented with varying concentrations and combinations of cytokinin and auxins under defined environmental conditions. Benzyladeninepurine (BAP) 0.0225- 2.25 mg/liter, IAA at 0.175-1.75 mg/liter and NAA at 0.0186-0.930 mg/litre were used individually or in combination to obtain 10 phytohormonal balanced media on which 24 genotypes were investigated. The *in vitro* response of the genotypes differed significantly and were dependent on the culture medium condition for callus formation which occurred in all BAP-NAA combinations, with a profuse growth rate at low BAP and high NAA levels. Regenerated plants were grown to maturity in soil and were fertile and morphologically true-to-type (Mirghis *et al*., 1995).

Ali & Li, (1994) used leaf explants from terminal leaflets of *Lycopersicon esculentum* cultivars Beijing, Early, Floredal and Trapies and *L. pimpinellifolium* and cultured on MS medium in the presence of various concentrations and combinations of growth regulators. They found that the calli initiated at a greater rate on MS media supplemented with 3.0 mg/litre zeatin or 0.6 mg IAA+2.0 mg BAP /liter in all the genotypes. Callus differentiated at the highest rate on MS medium containing 0.6 mg IAA +2.0 BAP mg/ liter.

Therefore in the current research article, different chemical mutagens were screened and tomato callus induction and regeneration conditions were optimized with variable amount of growth hormones in order to establish an optimized process for genetic manipulation in this important vegetable crop.

Materials and Methods

Imported seeds of tomato. *cv.* Roma was purchased from the Sunny Seed Company, egertan road, Lahore. Seeds were soaked in water for 3-5 hours. Subsequently, seeds were soaked in 0.05% $HgCl_2$ solution for 10 minutes and rinsed with autoclaved distilled water five times. For germination, seeds were placed in petriplates padded with moist cotton and kept in dark at 24^{o}C for 4 days (Table 1). After germination, plantlets were transferred in the test tubes supplemented with MS basal medium (4.43gm/lit), sucrose (30gm/lit) and phytagel (2.23gm/lit). After plantlet growth, the apical meristem of the tomato *cv*. Roma was isolated using sterilized blade under the microscope in aseptic conditions and sterilized with 5% household bleach coupled with thorough rinsing with autoclaved distilled water five times. Meristems were transferred into the test tubes containing MS

medium (De-Calciumpantothenate 8.39µM, GA_3 1.44µM, NAA .054µM, sucrose 30 mg/l and phytagel 2.23 mg/l). pH was adjusted to 5.7. Root, leaf node and internode pieces of regenerated plantlet derived from metristem, were used as an explant for the callus induction.

Table 1. Seed germination on different media versus days.

No. of days	Seeds germination (%)		
	Cotton	Filter paper	Ms media
2	-	-	-
4	80%	50%	-
6	90%	50%	-
8	90%	70%	20%
10	100%	80%	40%

No. of treatments = 25 each treatment has 10 seeds / Petri plates.

Tomato callus induction and regeneration media comprised of MS media (Murashige & Skoog, 1962) with variable amounts of plant growth hormones. The pH was adjusted to 5.6-5.8. The phytohormone, vitamins and antibiotic stocks were filter sterilized using disposable micro filter (0.2µm) size and added to media after autoclaving. All the glassware was heat sterilized at 140°C for 3 hours. All tissue culture work was carried out under aseptic conditions in a laminar airflow cabinet. On the callus induction media 200 explants of each root, leaf node and internodes were placed to observe the callus induction response. Six different media, CM-0 (Control-without hormones), CM-1 (90 µM 2.4-D), CM-2 (2.22 µM BAP), CM-3 (50 µM NAA), CM-4 (9.05 µM 2.4-D + 1.162 µM kinetin) and CM-5 (40 µM NAA + 0.57 µM IAA), each containing 4.43gm/lit MS-medium, 30gm/lit sucrose, 2.23gm/lit phytagel and pH-5.7 were tested for callus induction. The petri plates containing MS callus induction media amended with phytohormones were kept at 24 ± 2°C for 2-3 weeks. Fragile, light green calli were obtained to be used for mutagens treatment.

The mutagens used to induce mutation in tomato were colchicine, sodium azide and MNNG (Kucharska, & Maluszynski, 1998). The concentrations 0.0mM, 0.1mM, 0.2mM, 0.3mM, 0.4mM, and 0.5mM of the each mutagen were used for the treatment of callus and were added to the callus induction media after the filter sterilization of their stock solutions. 1mM stock solution of each mutagen was used. Best callus inducing media (Table 2) which showed better callus induction response and for each type of mutagen was divided into 5 treatments with 40 Petri plates containing 5 calli pieces of $1cm^3$ in each treatment. Each treatment refers to one specific concentration of each chemical mutagen. After sealing with parafilm, these petri dishes were kept for 16 hours under 3000 lux white florescent light at 24 ± 2°C for 10-days. After 10 day treatment of callus with the each mutagen (200 calli for each mutagen concentration) the calli were shifted to the regeneration media. Six regeneration media, Reg-0 (Control-without hormones), Reg-1 (9.12 µM zeatin + 0.75 µM IAA, After 3 weeks same medium but without IAA), Reg-II (9.29 µM Kinetin), Reg-III (8.88 µM BAP), Reg-IV (5.37µM NAA + 2.22µM BAP), Reg-V (0.5µM NAA + 2.5µM GA + 0.2µM BAP) each containing 4.43gm/lit MS-medium, 20gm/lit sucrose, 2.23gm/lit phytagel and pH-5.6 were tested for the regeneration from the callus, where Reg-0 medium was control without growth harmones. 200 calli pieces of $1cm^3$ were used for each media. After 10 days, Reg-I medium proved best regeneration media (Table 3).

Table 2. Callus induction on different media and explants.

Media	Explants			
	Root	Leaf	Node	Internode
CM-0	-	-	-	-
CM-1	+	++	+	++
CM-2	++	+++	++	++
CM-3	+	++	++	++
CM-4	+	++	+	++
CM-5	+	++	+	++

+ Poor, ++ Good, +++ Excellant

Table 3. Callus regeneration versus days.

Number of Weeks	Regeneration percentage					
	REG-0 control	REG-I	REG-II	REG-III	REG-IV	REG-V
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	50%	2%	5%	10%	-
4	-	60%	35%	25%	30%	10%
5	-	60%	40%	30%	35%	20%

Regeneration percentage, recorded from 100 calli for each medium.

After 3 weeks, the calli were subcultured on the same media but without IAA. Plantlets were produced from the mutagen treated calli on these media without IAA. These mutant plantlets were subcultured on the MS basal media. When the mutant plantlets developed upto 6-8cm in height and had appropriate number of vigorous roots, they were transferred to pots containing sandy loam soil and well decomposed leaf matter in 3:1 ratio. Before transplanting the roots were washed with luke warm water to remove the medium and dipped in 0.5% solution of fungicide (Dithane M-45) to avoid any fungal attack. Pots were covered with transparent polythene bags to conserve moisture and placed in growth room at 25°C until these were established. Potted plants were gradually exposed to environmental conditions on daily basis and different growth parameters (inter-node distance, plant height, leaf venation and leaf color) were recorded in mutant plants with reference to control.

Results and Discussion

Tomato seeds sterilized by using three sterilents which were 0.05% mercuric chloride. 8% bleach and 70% ethanol but mercuric chloride proved best sterilent as compared to others. Mercuric chloride is an exceedingly

toxic substance for plants as well as animals (Pierik, 1997). From the germinated plants apical meristems were isolated under aseptic conditions and were transferred to the meristem culture media and disease free plantlets were obtained. Apical meristems in the infected plants are generally free or carry a very low concentration of the viruses. Meristem-tip culture has also enabled plants to be free from other pathogens including viriods, mycoplasma, bacteria and fungi (Grout, 1999). Five different media were observed for callus induction response from tomato leaf. The media having 2.22µM BAP produced best callus (Table 2), according to Davis *et al*., 1994 and Soniya *et al.,* 2001, the highest percentage of callus induction was obtained in the presence of 2.75µM BAP. Different media were used to select the best media for the tomato callus regeneration. Media with 9.12µM zeatin and 0.75µM IAA gave excellent regeneration of the callus (Table 3). Shtereva & Atamassorva, (2001) have developed a media for the regeneration from callus. It was consisted of MS salts. 1% agarose (type VII). 4% sucrose 2.8µM zeatin and 0.06-0.1µM GA_3. Approximately 95% of excised elongated shoots produced roots when subcultured on media supplemented with 5µM of IBA solution. Regenerated platelets were initially transferred to plastic cups and subsequently to soil, where they grow into phenotypically normal plants which flowered and set seeds (Geetha, *et al*., 1998).

Calli obtained from the tomato *cv*. Roma were treated with different concentrations of the chemical mutagens, which were colchicine, sodium azide and MNNG (Table 4). The concentrations were 0.1mM 0.2mM 0.3mM 0.4mm and 0.5mM. Calli treated with 0.2mM and 0.3mM of colchicine which produce mutants after 3-weeks while at remaining concentration no plants were obtained (Table 4). The Mutants produced from two different concentrations had broader leaves, long branches and small petiole as compared to the control. Colchicine ($C_{22}H_{25}O_6$ Mw= 339.43) is a poisonous drug, used to arrest mitosis at metaphase and induce chromosome duplication without separation resulting in diploidization of haploid cells (homozygous diploidization) and polyploidization of diploid cells (Edward and Hartung, 1954, Kalyani *et al*., 1989, Wan & Widholm, 1995, Zhou, 1995). Youssef *et al*., (1998) reported that subjecting *in vitro* shootlets of *Melalenca armillaris* to various concentrations of colchicine caused a significant influence on their growth behaviour and their production of pigments and volatile oils. Low concentration of cholchicine give the heaviest mass of fresh shootlets, at 100 or 200ppm resulted in heaviest mass of dry shootlets.

Calli treated with sodium azide produced mutants only at 0.2mM (Table 4) after 3-4 weeks. Phenotypically, these mutant plants were stunted in height and leaves were yellowish-green color as compared to control plants. Calli at the remaining concentrations failed to produce plants even after 10-weeks. Sodium azide is a white crystalline powder consisting of positive sodium ion and negative azide ions. It is a metabolic poison; this inhibits certain key enzymes and is a potent mutant in plants, causing reciprocal translocation, chromosome breakage and genetic sterility in the absence of high frequencies of visible chromosome aberrations. In the last decade, the mutagenic effect of sodium azide along with its effect on general growth of plants has been extensively investigated by Sander & Muchlbaver. 1977. In plant growth, the effects include delay in germination and retardation in growth, which consequently damages to bio-chemical, physiological, cytological and general processes of the plant. Sodium azide as an efficient mutagen produces significant frequencies of chlorophyll deficient mutation. A comparative study of SCV versus induced mutagenesis indicated that two sources of variability are different in their effect changing the pattern of segregation in *Zycopersicon esculenium* (Gavazzi *et al*., 1987) and *Brassica napus* (Jain & Newton, 1989). Sodium azide and ethyl methane sulphonate mutagens showed that mutagen-induced gain in terms of reduction in plant height, fertility and spike length was not outstanding in regenerated plants compared with the untreated control (Rackovska & Dimova, 2000).

Table 4. Percentage of mutants, regeneration from treated calli.

Con. mM	Mutagens		
	Colchicine	NaN3	MNNG
0.1	-	-	-
0.2	50%	40%	-
0.3	30%	-	-
0.4	-	-	-
0.5	-	-	-

No. of treatment 5 and each treatment has 40 Petri plates containing callus pieces of 1 cm3 for each mutagen.

As regards MNNG, no mutant plants were obtained at all concentrations even after 10-weeks (Table 4). MNNG is a potent mutant and has ability to induced severe chromosome damage (Francis *et al*., 1989). MNNG is used as a tool to isolate morphological mutants or mutants with variations in calli components-pigments or proteins. Levy & Ashri (1974) reported the differential physiological sensitivity of peanut varieties to seed treatment with sodium azide, ethidium bromide and MNNG, they found significant interaction between two varieties.

References

Ali, Y. and S.J. Li. 1994. Effect of various growth regulators on the initiation, Size, color and differentiation of callus in different genotypes of tomato. *Sarhard J. of Agri.,* 10: 399-405.

Abu-El-Heba, G.A, G.M. Hussein and N.A. Abdalla. 2008. A rapid and efficient tomato regeneration and transformation system. *Agric. For. Res*., 58: 103-110.

Bal, U. and K. Abak. 2007. Haploidy in tomato (*Lycopersicon esculentum* Mill.): a critical review. *Euphytica,* 158: 1-9.

Davis, D.G., K.A. Breiland, D.S. Frear and G.A. Secor. 1994. Callus initiation and regeneration of tomato (*Lycopersicon esculentum.* L) cultivars with different sensitivities to metribuzin. *Plant Growth Regulator society of America Quarterly*, 22: 65-73.

Edward, F. and H. Hartung. 1954. Study of Cholchicine effects in cell division. *Ann. J. of P. Sci.,* 13: 190-200.

Evans, D.A. and W.R. Sharp. 1983. Single gene mutation in tomato plants regenerated from tissue culture. *Science,* 221: 949-951.

Evans, D.A. 1989. Somaclonal variation gene: bases and breeding applications. *Trends Genet.,* 5: 46-50.

Francis, A.R., T.K. Shetty and R.K. Bhattacharya. 1989. Modulating effect of plant flavonoids on the mutagenicity of N-methyl-N′ -nitro-N-nitrosoguanidine. *Carcinogenesis,* 10(10): 1953-1955.

Garvey, T. and J. Hewitt. 1991. Starch, vitamins and minerals accumulation in two accessions of *cheesmanni*. *J. of the Am. S. for Hort. Sci.,* 46: 381-396.

Gavaazi, G.C., G. Tonelli, E. Todesco, F. Arreghini, F. Raffaldi, G. Vecchio and M.G. Barbuzzi. 1987. Somaclonal variation versus chemically induced mutagenesis in tomato (*Lycopersicon esculentum* L). *Theo. Appl. Genet.,* 74: 733-738.

Geetha, N., P. Venkatachalam, P.S. Reddy and G. Rajaseger. 1998. *In vitro* plant regeneration from leaf callus cultures of tomato (*Lycopersicon esculentum* Mill.). *Adv. Plant Sci*., 11: 253-257.

Grout, G.W.W. 1999. Meristem-tip culture for propagation and virus elimination. In: *Methods in Molecular Biology*, (Ed.): R.D.Hall. Vol III. Plant Cell Culture protocols. Humana press Inc., Totowa, N.J., 115-125.

Jain, S.M. and R.J. Newton. 1989. Evaluation of protoclonal variation versus chemically induced mutagenesis in *Brassica napus*. *Curt. Sci*., 58: 176-180.

Kalyani, D., S.K. Datta and K. Datta. 1989. Palynological interpretation of gamma ray and colchicines induced mutation in chrysanthemum cultivars. *Israel j. of P. Sci.,* 199-207.

Kucharska, M. and M. Maluszynski. 1998. Estimation of induced Mutation rate of four esterase genes in barley (*Hordeum vulgare.* L). *Gen. of App. Sci.,* pp. 141-145.

Leonardi, C., P. Leonardi, F. Ambrosino, T. Esposito and V. Fogliano. 2000. Antioxidative activity and carotenoid and tomatine contents in different typologies of fresh consumption tomatoes. *J. of Agri. and F. Chem.,* pp. 4723-4727.

Levy, A. and A. Ashri. 1974. Differential physiological sensitivity of peanut varicties to seed treatment with the mutagens ethidium bromide. MNNG and sodium azide. *Rad. Bot.,* 13: 369-373.

Mirghis, E. 1995. Analysis of tomato cultivars and hybrids for *In vitro* callus formation and regeneration. *Acta Horticulturae*., 112: 111-116.

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

Pierik, R.L.M. 1997. Sterilization of plant material, chap. 9. *In vitro* tissue culture of higher plants. Kluwer Academic Publisher, Dordrecht/Boston/London. pp. 91.

Rackovska, G. and D. Dimova. 2000. Effect of Sodium azide and Gamma rays on MI quantitative characteristics of the productivity and their connection with M2 mutation changes in winter common wheat. *Rasteniev dni Nauki.,* 37: 413-419.

Sajjad, M., M. Ashfaq, A. Suhail and S. Akhtar. 2011. Screening of tomato genotypes for resistance to tomato fruit borer (*Helicoverpa armiger*, Hubner) in Pakistan. *Pak. J. Agri. Sci*., 48: 59-62.

Sander, C. and F.J. Muchlbaver. 1977. Mutagenic effect of sodium azide and gamma irradiation in Pisum. *Environ. Exp. Bot.,* 17:43-47.

Scholthof, K-B.G. 2000. Tobacco mosaic virus. The Plant Health Instructor. DOI: 10.1094/PHI-I-2000-1010-0.

Sirvastava, D.k., M.M. Kruleva and H.K. Oanh. 1995. *In vitro* selection and characterizationof water stress tolerant culture of tomato *(Lycopersicon esculentum.L)*. *Indian J. of P. Phy*., 38: 99-104.

Shtereva, L. and B. Atamassorva. 2001. Callus induction and plant regeneration via anther culture in Mutant tomato (*Lycopersicon esculentum.* Mill). *Israel J. of p. S.,* 49: 203-208.

Soniya, E.V., N.S. Banerjee and M.R. Das. 2001. Genetic analysis of somaclonal variation among callus derived plants of tomato. *Current Science*., 80: 1213-1215.

Stubee, H. 1972b. Mutation in wild totato *(Lycopersicon esculentum.* L). *Mill iv. Kulturpflanze.* 19: 231-263.

Youssef, E.M.A., E.L. Alfy and A.K. Hassan. 1998. Effect of various gamma irradiation and colchicines treatments on the growth behavior and pigments and volatile oil products of *Melalenea amillaris* smith explants *in vitro* Egypt. *J. Hort*., 25: 337-395.

Wan, Y. and J.M. Widholm. 1995. Effect of chromosome doubling agents of somaclonal variation in the progeny of doubled haploids of maize. *Plants Breed.,* 114: 253-255.

Zhou, L., G. Heping and Y. Chengwan. 1995. Preliminary study on the use of colchicines in induced mutation breading of pepper. *China Vegetables*, 5: 22-24.

Received for publication 12 February 2012)