Full Length Research Paper

Effect of physical and chemical mutagens on morphological behavior of tomato (*Lycopersicon esculentum* L.,) CV. “Rio Grande” under heat stress conditions

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Given the importance to tomato production under heat stress conditions in hot climates of Pakistan, the objective of this research work was to study the influence of temperature and application of physical and chemical mutagens on tomato (*Lycopersicon esculentum* L.,) CV. “Rio grande” Seeds were treated with chemical mutagen (Ethyl Methane Sulphonate) and physical mutagen (Gamma radiation). Plants were grown in open field conditions under day/night temperatures regimes at the experimental area of Department of Horticulture, faculty of crop and food sciences, PMAS-AAUR. The experiment was set nine nineteen treatments and four replications in Complete Randomize Block Design. It is observed that by increasing dose of mutagens shows lethal effect and morphological parameters reduced significantly. But lower doses of EMS and Gamma radiation improve therotolerance capacity significantly. Plants were investigated for different morphological parameters i.e Plant height, no. of leaves, leaf area, relative water contents of leaves, chlorophyll contents, cell viability (TCC Assay) and pollen germination. Among gamma rays 5Kr were the only dose which survived and bear fruits. Among EMS 4mM, 8 mM, 16mM performed better under heat stress conditions than other treatments.

Key words: Mutagens, Gamma Rays, EMS, Tomato, heat stress.

INTRODUCTION

Tomato (*Lycopersicon esculentum* L.,) is one of the most economically important and widely grown plants in Solanaceae family. (Mourvaki et al., 2005).It is grown throughout the world, either outdoors or indoors, because of its wide adaptability and versatility. The estimated world production of tomato is about 16179834 tones. (FAOSTAT, Database 2012) and total yield is 33681.2 kg/ha. The leading producers are China (with 25.3% of the total production), USA, India, Turkey and Egypt. (FAOSTAT, 2012). The popularity of tomato as fresh and processed food has made it an important source of vitamin A and C in diets. Tomatoes are being consumed throughout the world on a large scale and serve many benefits for heart and other organs. They contain the carotene lycopene, one of the most powerful natural antioxidants (Mourvaki et al., 2005). Its fruits are used in salads or cooked as a vegetable, processed into tomato paste, sauce and puree. In Pakistan, tomato is cultivated in all the provinces but its production is mainly concentrated in Sindh and Balochistan. Total tomato production in year 2012 was 560,000 tones while total yield recorded was 101818.9 (kg/ha) and area harvested 4803680 hectors. (FAOSTAT, 2012). The average yield of tomato in Pakistan is very low as compared to other countries of the world for which many factors are responsible, some of them are failure to ripen, uneven or blotchy ripening, Tomato yellow shoulder disorder but the major problem in different regions of Pakistan is the failure to set fruit or poor fruit set due to day temperatures above 45°C-50°C combined with low humidity and/or drought night temperatures above 21°C or below 10°C. Hot drying winds can add to the problem. Dry soil can cause blossoms to dry up and drop. Cold soils at planting time can stunt growth and...
Treatments with EMS and Gamma Radiation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. of EMS</th>
<th>Gamma radiation Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T_1</td>
<td>Control (0)</td>
<td>Control (0)</td>
</tr>
<tr>
<td>T_2</td>
<td>4mM</td>
<td>5Kr</td>
</tr>
<tr>
<td>T_3</td>
<td>8mM</td>
<td>10Kr</td>
</tr>
<tr>
<td>T_4</td>
<td>16mM</td>
<td>15Kr</td>
</tr>
<tr>
<td>T_5</td>
<td>24mM</td>
<td>20Kr</td>
</tr>
<tr>
<td>T_6</td>
<td>32mM</td>
<td>25Kr</td>
</tr>
<tr>
<td>T_7</td>
<td>40mM</td>
<td>30Kr</td>
</tr>
<tr>
<td>T_8</td>
<td>48mM</td>
<td>35Kr</td>
</tr>
<tr>
<td>T_9</td>
<td>56mM</td>
<td>40Kr</td>
</tr>
<tr>
<td>T_{10}</td>
<td>64mM</td>
<td>45Kr</td>
</tr>
</tbody>
</table>

delay or eliminate flowering. Lack of air circulation can inhibit the movement of pollen to the flower pistils. The tomato flower happens in the three patterns that are organizational and flowers that are simple appear as well as branched and simple cymes. Flowers number that appears in inflorescence is based on environmental elements including temperature (David et al., 1996). Heat stress is identified as the enhancement in temperature below a threshold level for some time is enough to prompt irreversible harm to crop growth and improvement. As a whole, a temporary increase in temperature 10°–15°C above normal, can lead to heat stress or shock (Wahid et al., 2007). Shortage of tomatoes occurs during summer season due to high temperatures, thus resulting in poor growth, reproductive development and yield (Hall, 1992; Hussain et al., 2006 Singh et al., 2007). When temperature is up, injury of cellules and death may happen within minutes that could be related to a disturbance of cellular structure (Schoffl et al., 1999).

When temperature is optimum, damages or death may happen after long term exposure. Direct damages can be happened in high temperatures such as denaturation and aggregation of protein, and enhanced membrane lipids liquidity. Indirect or slower heat damages can be occurred in terms of enzymes inactivation in chloroplast and mitochondria, limitation in protein production, degradation of protein and loss of integrity of membrane (Howarth, 2005). In general, it is obvious that high temperature influences markedly plants anatomy at the tissue, cellular, and sub-cellular levels. The additional impacts of all these alterations in high temperature stress can lead to crop low growth and yield (Wahid et al., 2007). Plant responses to stress and adaptive mechanism form the basis for developing tools/protocols to improve stress tolerance. Based on the temperature stress response several techniques have been developed to assess the stress effects. Mutation breeding is one of the major technique to develop stress resistant plants, its advantages is that mutants with multiple traits can be identified. The chances of survival of mutant varieties are much higher under rapid fluctuating climatic conditions.

The use of nuclear techniques for developing new varieties under the changing climatic conditions would be the most ideal approach before any new cost effective techniques are developed which are freely available without too many regulations. The purpose of induced mutations is to enhance the mutation frequency rate in order to select appropriate variants for plant breeding. The mutation frequency rate of spontaneous mutations is rather very low and difficult to exploit by the plant breeders. Mutations are induced by physical (e.g. gamma radiation) and chemical (e.g. ethylmethane sulfonate) mutagen treatment of both seed and vegetatively propagated crops. The mutagen treatment breaks the nuclear DNA and during the process of DNA repairs mechanism; new mutations are induced randomly which are heritable. The changes can occur in cytoplasmic organelles and results in chromosomal or genomic mutations and that enable plant breeders to select useful mutants such as flower color, flower shape, disease resistance and early flowering types. A specific advantage of mutation induction is the possibility of obtaining unselected genetic variation, improvement of vegetative propagated plants when one or few characters of an outstanding cultivar are to be modified.

MATERIALS AND METHODS

The present study was conducted during March-July 2013 in Horticultural research area of PMAS-Arid Agriculture University Rawalpindi. Seeds of tomato variety Rio grande was treated with physical (Gamma irradiation) and chemical mutagen (EMS). Nine different doses of radiation and EMS were used each dose were considered as a single treatment. Details of doses are shown in table 1.

Seeds were subjected to EMS (Ethylmethane sulphonate) mutagenesis. Set of 400 seeds per treatment were imbibed in 200ml distilled water for 24 hours at room temperature. The seeds were then incubated in 100ml solution of EMS (Sigma-Aldrich, St. Louis) at different concentrations as shown in table 1.with gentle shaking for 12hr. After the EMS treatment, the seeds were washed twice in 200ml of 3% sodium thiosulfate...
buffer for 20 min at room temperature, with gentle shaking, followed by three washes in 200 ml distilled water. The used EMS and wash solutions and all equipment were treated in sodium thiostoslate. Another set of 400 seeds per treatment undergoes the treatment with gamma irradiation in the laboratory of NIFA (Nuclear Institute of Food and Agriculture, Peshawar). The seeds of both treatments were sown in germination trays in Soil: FYM: Sand in the ratio of 2:1:1 and covered with coconut husk. Nursery was ready after one month and transplanted into open field conditions at P x P distance 2.6 feet, all application of required irrigation and management practices were done to evaluate crop for its vegetative and reproductive responses in heat stress conditions.

Among them plant height and internodal distance were measured by using measuring tape, no. of leaves and branches were counted manually. Leaf area was measured with the help of digital leaf area meter ADC model AM 100 meter (Analytical Development Company Ltd., Herts, U.K.) at full maturity stage. Leaf chlorophyll concentration will be recorded by Spad–502 chlorophyll meter (Minolta Camera Co. Ltd., Osaka, Japan). Pollen germination was observed by using method of by Abdul-Baki, (1992) by growing pollen in a growth medium containing 0.29 M sucrose, 1.27 mM Ca(NO3)2, 0.16 mM H3BO3, and 1 mM KNO3 (pH 5.2) to which 0.001% fluorescein diacetate (FDA) was added. Pollen viability can be evaluated within 30 min by determining percent fluorescing pollen in a sample. The RWC of leaves was determined in the fully expanded leaf stage by using the method of (Pirzad et al., 2011). Cell viability test was performed by using TCC analysis, leaf punches were made from the top third leaf at the full leaf expended stage. After exposing the leaf punches to induction temperature of 38 °C 2h followed by high temperature of 45°C for 2h,they were incubated in 0.2% TTC solution for 4h and cell viability was measured by taking the absorbance at 485nm. (Senthil-kumar et al., 2002c; 2002d).

There are no reports in this regard according to our knowledge specifically in Pakistan so the study aims to:

1. To screen best doses of EMS and Gamma radiation that will be helpful to make stress tolerant tomato mutant plants for future.
2. Efficiency evaluation of various mutagenesis techniques.

RESULTS AND DISCUSSION

All treatments of Gamma radiation with doses higher then 10kr proved to be lethal and complete mortality was observed in seedlings in 3rd week after sowing. While treatments with Chemical mutagen EMS doses higher then 32mM were also proved to be lethal. All reaming treatments with control were transplanted into open field condition and evaluate for various parameters and daily mean temperatures were recorded that exceeds up to 45°C and following results were obtained.

Plant height (cm)

Through data analysis different doses of gamma radiations and EMS plants were found to have high variability among them. A look on the data revealed that among treatments there was maximum average plant height (60.75 cm) in (EMS 4mM) whereas minimum average plant height (28 cm) was found in gamma radiation (10Kr). While EMS doses 8mM (58.25cm) and 24mM (57.25cm) shows non-significant results with each other. Chromosomes were damaged through radiations and the connection of damaged chromosomes and non-damaged chromosomes have an important correlation with the growth decline (Sparrow and Gunckel, 1955). Considerable phenotypic variations have been shown in studies of induced mutations. The mutation frequency, however, might be controlled by several factors, for instance the mutagen mechanism of action (Griffiths et al., 1993), the size of target gene and composition of nucleotide (Bichara et al., 1995), position of gene in genome (Swoboda et al., 1993), structure of chromatin and DNA repair efficiency. Higher doses level of mutagens shows lower plant height because these mutagens restricted the somatic cells division and was also observed reduced viability, growth abnormalities and decreased fertility. Thus, there is one most effective dose for each mutagen that brings about the maximum degree of mutagenesis and it depends upon the plant genotype, duration and dose level.

Number of Branches

Data analysis showed both mutagenic treatments had significant differences for number of branches. Minimum number of branches (4 and 2) was recorded in EMS 24mM and 32mM respectively and gamma radiation 10Kr was non-significant with them (4) The number of branches was found to be higher in EMS 16Mm (12) that is highly variable among the other doses and control. With the increase in doses of gamma radiation and EMS a gradually decreasing pattern was observed in case of average number of branches. These results revealed that higher doses level of gamma rays give less secondary branches appeared on plants.

Number of leaves

The interaction of gamma radiations and EMS in case of number of leaves was observed significantly different
among all treatments with control. The highest numbers of leaves were counted in EMS 8mM (70) and 16mM (61). As the dose level of EMS increased growth parameters decreased and lowest leaves number calculated 29 and 20 in 24mM and 32mM respectively. On the other hand gamma radiation 10kr also shows lower no. of leaves 18. Irfaq and Khalid (2001) found that higher doses of gamma radiations had adverse effects on different plant growth characters. A decreasing trend was observed in treatment by increasing doses of gamma radiations and EMS. It is clear from the Table that 20 were minimum number of leaves in EMS 32mM and 18 in 10kr Gamma irradiation caused different types of changes in plant structure and function. It depends upon the dose rate and time period of irradiation (Piri et al., 2011). Higher doses might have stopped the enzymes that are necessary for leaves initiation. Another reason found that higher doses blocked cell division by decreasing the rate of physiological processes and genetic material was also damaged (Neary et al., 1957).

Leaf Area

Interaction of gamma radiation and EMS doses gave significant results relating to leaf area. Control had a minimum area of leaves (94.95 cm²) while EMS had maximum (158.5 cm²) leaf area at the dose level of 4mM. Generally, the leaf area was reduced by increasing the doses of gamma radiations and EMS. Among the different levels of gamma radiations there was a lowest value of leaf area (133.4 cm²) following by (103.7 cm²) at 5Kr and 10kr respectively. Shahin et al., (2011) reported that a dose range of 5-10 gray produced highest leaf area in grapes cultures and at 30 Gy gave lowest leaf area. Extension of cell wall is decreased by stress and high pH which results in the form of structural change in the plant cell wall. Rate of growth for these stressed plants was very low than unstressed plants. Various factors such as biochemical factors were involved in the expansion of leaves and cells. In addition to this, there were many other evidences which supported the slow growth rate of plants according to stress like protein synthesis, processes within the cell wall, cell division and biosynthesis of membranes (Bursssens et al., 2000). The results of leaf area were in agreement with the results of Pakorn et al. (2009) who demonstrated that leaf area in Anubias congensis decreased with the increasing intensity of irradiation due to destruction of genetic material and reduction of cell division and ultimately growth retarded.

Chlorophyll Contents of Leaves

Treated plants showed better growth than the control plants for 5Kr, 10Kr and EMS 8mM and 16mM as the photosynthetic pigments, that is, Chlorophyll contents were increased by 60.37, 59.8, and 57.1, 58.7 respectively, over the control plants (39.1)., with increasing dose of EMS treatment, the leaf Chlorophyll contents decreased continuously and that decrease was recorded up to 44.2 for 32mM EMS. These results were similar in findings with Jitendra et al., 2012 by using gamma radiation and EMS in Safflower shows higher number of chlorophyll a and b contents as the intensity of mutagens decreases. Moreover Exposure to high temperature induced chlorophyll loss, led to marked decline in the photochemical efficiency of PSII and caused considerable degradation of Rubisco in plant leaves. Plants with lower doses of mutagens shows protective response to heat stress, which involves structural alterations in the photosynthetic apparatus as compared to higher doses. Prolonged irradiations damage to reaction centers is usually followed by chlorophyll loss (Krause, 1998).

Exposure of leaves to higher temperatures causes inhibition of both photosynthesis and respiration hence during the vegetative stage chlorophyll content degradation ultimately lowers carbohydrate synthesis. The heat stress directly affects the heat sensitive sites, mainly Oxygen Evolving Complex of Photosystem II and Rubisco activase. It leads to subsequent indirect effects, such as changes of the redox status of individual components on thylakoid membrane in chloroplast and increase in production of Reactive Oxygen Species (ROS). Hence, the redox signaling plays the crucial role in enhancement of alternative electron pathways such as cyclic electron flow as well as triggering the signal transduction pathways resulting to heat stress response. The redox signaling in chloroplast is closely associated with ROS signaling, which interferes with regulation also out of chloroplast. Marian Brestic, (2013).

Inter-nodal length

Data regarding inter-nodal length depicted significant interaction between the gamma radiations and EMS applications. It was found that inter-nodal length was minimum in gamma radiation treatment 10kr (3.63) and EMS treatment 32mM (4.75). Among different treatments, inter-nodal length was maximum 6.65 cm in 5Kr of gamma radiation and 6.08 cm EMS 16mM. With the increasing level of gamma radiations and EMS there was a decrease in the internodal length and ultimately plant height. The findings of Motilal et al. (2012) are matched with the findings of the current study. He explained that there was a decrease in inter node length with the increase dose level of EMS in asteracantha longifolia L. Major impact of high temperatures on shoot growth is a severe reduction in the first internode length resulting in premature death of plants (Hall, 1992). For example, sugarcane plants grown under high temperatures
### Table 2: Results of morphological traits in response of mutagenic treatments in heat stress conditions

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant Height</th>
<th>No. of Branches</th>
<th>No. of Leaves</th>
<th>Leaf Area</th>
<th>Chlorophyll content of Leaves</th>
<th>Inter-nodal length</th>
<th>RWC</th>
<th>Pollen germination(%)</th>
<th>TCC Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56 d</td>
<td>8.25cd</td>
<td>37e</td>
<td>95.94g</td>
<td>62.25a</td>
<td>5.75bc</td>
<td>0.25f</td>
<td>12f</td>
<td>42.6f</td>
</tr>
<tr>
<td>GI 5Kr</td>
<td>59.75ab</td>
<td>7.75d</td>
<td>48d</td>
<td>133.4d</td>
<td>60.38ab</td>
<td>6.65 a</td>
<td>0.42cd</td>
<td>68b</td>
<td>72.2b</td>
</tr>
<tr>
<td>GI 10Krr</td>
<td>28e</td>
<td>4.25e</td>
<td>18fg</td>
<td>103.7f</td>
<td>59.80ab</td>
<td>3.63 e</td>
<td>0.35e</td>
<td>0g</td>
<td>34.4g</td>
</tr>
<tr>
<td>EMS 4Mm</td>
<td>60.75a</td>
<td>9.5bc</td>
<td>58c</td>
<td>158.5a</td>
<td>51.13c</td>
<td>5.56bcd</td>
<td>0.48cd</td>
<td>75a</td>
<td>75.5a</td>
</tr>
<tr>
<td>EMS 8Mm</td>
<td>58.25bc</td>
<td>9.75b</td>
<td>70a</td>
<td>149.7b</td>
<td>57.13b</td>
<td>5.62bcd</td>
<td>0.49cd</td>
<td>68b</td>
<td>65.7c</td>
</tr>
<tr>
<td>EMS 16Mm</td>
<td>59.25ab</td>
<td>12a</td>
<td>61b</td>
<td>144.0c</td>
<td>58.73ab</td>
<td>6.08ab</td>
<td>0.59 a</td>
<td>66c</td>
<td>66.8c</td>
</tr>
<tr>
<td>EMS 24Mm</td>
<td>57.25bc</td>
<td>4e</td>
<td>29f</td>
<td>126.7e</td>
<td>50.88c</td>
<td>4.88cd</td>
<td>0.6 a</td>
<td>52d</td>
<td>59.3d</td>
</tr>
<tr>
<td>EMS 32Mm</td>
<td>56.25d</td>
<td>2f</td>
<td>20g</td>
<td>132.8d</td>
<td>44.20d</td>
<td>4.75d</td>
<td>0.53bc</td>
<td>34e</td>
<td>45.7e</td>
</tr>
</tbody>
</table>

Exhibited smaller internodes, increased tillering, early senescence, and reduced total biomass (Ebrahim et al., 1998).

### Relative Water Contents

By observing results after statistical analysis for relative water contents of leaves reviles that higher RWC was observed in treatments EMS 16mM and 24mM 0.6 and 0.59% while non-significant results were obtained in treatments with 4mM and 8Mm. Among gamma radiation treatments 5Kr (0.35) shows higher RWC then 10kr(0.25) shows least value for RWC. Its shows the plants which are treated with mutagens have more ability to absorb water when transpiration rate were higher in heat stress conditions as compared to plants which are in control. High temperature causes loss of cell water content for which the cell size and ultimately the growth is reduced. Reduction in net assimilation rate (NAR) is also another reason for reduced relative growth rate (RGR) under high temperature but mutagen treatments improves the ability of more intakes of water and nutrients from soil to maintain turgidity and overall plant vigor in stress conditions. As the heat stress hampered the leaf water content, plants exposed to heat stress indicated an accumulation of several heat stable proteins which appeared to be associated with heat tolerance. Results are similar in correspondence with the results of Morales et al., (2003) who stated that preconditioned tomato plants with heat indicated a better osmotic adjustment and maintained leaf water potential than non-preconditioned ones.

### Pollen Viability test

Data presented in table 2 shows that treatment with lower doses of EMS provides more viable pollen growth as compared to control under heat stress conditions. While 5kr dose of gamma radiation also increases the number of viable pollen grains. EMS 4Mm (75%) shows higher percentage of viable pollen at temperature above 40°C and EMS 8Mm and gamma radiation dose 5kr shows non-significant result (68%) with each other but significantly different from control (12%). In tomato, a dramatic decrease in fruit set in response to heat stress has mainly been attributed to the impairment of pollen development occurring in anthers 8–13 days before anthesis, corresponding to the shift from pollen mother cells to uni-cellular microspores. This heat stress sensitivity of the male reproductive organs has been observed after short episodes of high temperatures at 40 °C and after chronic exposure to 32 °C/28 °C (day/night), which is just few degrees above the optimal physiological growth range of 26 °C/22 °C (day/night) observed by Filomena et al., (2013). But mutagen treatment dramatically increases the ability of plants to produce viable pollen at higher temperature stress conditions.

### Cell Viability (TCC Assay)

TCC assay is useful technique to assess cell viability at higher temperature stress conditions. By showing results it's confirmed that applications of EMS and Gamma radiation significantly increases the viability of cells under heat stress. Application of EMS 8mM has significantly higher number of viable cells 75.5 while 16mM and 24mM show non-significant results with each other. Gamma radiation dose 5kr also increases thermostability of cells at higher temperatures as compared to control conditions. The TTC reduction reflects overall cell viability, which is often related to extent of generation of cytotoxic compounds, integrity of macromolecules and membrane-bound organelle integrity (Wu and Wallner, 1983). Further, this technique is
amenable for large-scale screening.

CONCLUSION

From all the tests and procedures, our results indicated that significant genetic variation could be created by physical and chemical mutagens. Further, the TTC test, pollen viability test, and chlorophyll contents could be used to assess genetic variability in this mutant population under heat stress conditions.

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