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RESEARCH

Induction of *Tomato ring spot virus* resistant plants using the chemical mutagens (NaN_3 and EMS)

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ABSTRACT

Background: Tomato (*Solanum lycopersicon*) is affected by many biotic and abiotic stresses which became major limiting factors for growth, fruit yield quality and quantity. *Tomato ringspot virus* (ToRSV) is considered one of the most devastating members of the genus Nepovirus and distributed in most parts of the world and can infect herbaceous, ornamental and woody species including tomato, tobacco, grape, apple, peach, cherry, apricot and raspberry. Both of sodium azide (NaN3) and ethyl methanesulfonate (EMS) are known as chemical mutagens.
Objective: Study the effect of chemical mutagens (NaN3and EMS) on the resistance of (ToRSV) on two different tomato cultivars and characterization induction mutagens by ISSR molecular method.
Methods: ToRSV was isolated from infected tomato, grapevine and pelargonium plants and detected using double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). Host range study was carried out using mechanical inoculation into fourteen different diagnostic host plant species and cultivars belonging to 6 families. Tomato seeds were treated with four different concentration of $NaN_3$and EMS (1.0, 2.0, 3.0 and 4.0 mM) and (0.3%, 0.4%, 0.5%and 0.6%) respectively, then planted into a tissue culture media, induced plants were checked for resistance to ToRSV by ELISA. ISSR reaction was used to confirm the DNA variations.
Results: ToRSV was transmitted mechanically into11 plant species and cvs., belonging to 6 families while 3 plant species and cvs. from 2 families gave negative results with DAS-ELISA and were symptomless. Treated plant with different concentrations of $NaN_3$and EMS showed that high concentrations of both chemicals led to death of plantlets while, concentrations 3.0 and 4.0 mM of NaN_3 and EMS 0.4%, 0.5% showed no symptoms of ToRSV in spite of concentrations 1mM and 2mM of NaN_3 and 0.3% of EMS were showing symptoms and gave positive results with ELISA. EMS (0.40%) treated tomato cv. CR and 3mM NaN_3 treated tomato cv. SSB revealed 3 unique fragments with 98-A ISSR primer, 3 unique fragments appeared in SSB cv. treated with 3mM NaN_3 with 98-B ISSR primer, and E-9 ISSR primer revealed 6 fragments in EMS (0.40%) treated CR cv. and 5 fragments in 3mM NaN_3 treated SSB cv. Indicting changes in the genetic make-up of the treated tomato plants. No similar fragments were observed in the control samples.
Conclusion: NaN_3 at 3mM concentration and EMS at 0.4% concentration gave the highest effect in resistance to ToRSV on tomato and revealed in different unique fragments in ISSR PCR reaction, without harmful effect on the produced plants.

Keywords: Tomato; ToRSV; DAS-ELISA; Chemical mutagenesis; NaN3; EMS; ISSR.

BACKGROUND

Tomato (*Solanum lycopersicum* L.) is a fleshy vegetable used all over the world. It belongs to the family *Solanaceae*, and is the world's most widely cultivated vegetable (Villarael, 1980) with an estimated global production of 162 million tons in 2014 (FAO, 2017). However in Egypt, tomato is grown over an area of 214016 ha. annually, which produces 8288043 tons/ha. with 38.37 tons/ha. (FAO, 2014). The tomato crop production value was estimated at $1.7 billion in 2014 (FAO, 2017).

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Tomato have a nutritional and medicinal value for its content from vitamin A and C, besides carotenoids pigments which conceders as an antioxidant which protects humans body from free radicals damages and reduce the risk of getting cancer. Tomato fruits are also cholesterol free **(**Bhowmik *et al.,* 2012)

Tomato ring spot virus (ToRSV) is one of the most destructive viruses and is a member of the Nepovirus group which includes about 46 viruses, is widespread in the temperate regions of North and South America, Europe, and Asia (Stace-Smith 1996; Card *et al*., 2007). The virus is distributed in most parts of the world and can infect herbaceous (Anonymous, 2005), ornamental and woody species including grape, apple, peach, cherry, apricot and raspberry. It also infects many herbaceous plants as tomato and tobacco (Moini, 2010).

ToRSV was isolated from grapevines in Niagara (Dias, 1977). Darwish (2005) isolated ToRSV from grapevines for the first time in Egypt. The infected vines have small and poor berries. The vines died within few years. In severely affected vines they have many winter-killed buds and weak, stunted shoot growth. By about 9 weeks after the start of vine growth, shoot and foliage symptoms are conspicuous on one or more shoots (Yang *et al*., 1986). Fruit clusters are reduced in size with many berries aborting (Sutic *et al.,* 1999).

On field-grown tomatoes there is a conspicuous curling and necrosis of the terminals of one or more actively growing shoots. The basal portion of younger leaves develop brown, clearly defined, necrotic rings and sinuous lines. The petioles of the necrotic leaves and adjacent stem tissue are often marked with necrotic streaks and rings. If fruits are infected early, they develop faint to conspicuous, grey to brown, corky, superficial and frequently concentric rings or portions of rings (Brunt *et.al.,* 1996 and Ben Moussa *et al.,* 2000).

The host range of the virus includes herbaceous ornamental and woody species including grape, apple, peach, cherry, apricot and raspberry. It also infects many herbaceous plants as tomato and tobacco (Moini, 2010).

Mutation is the ultimate source of all genetic variation. Spontaneous mutations occur at very low frequency, meanwhile induced mutations facilitate the development of improved varieties (Zaky Zayed *et al.,* 2014). Many studies used chemical mutagens such as sodium azide (NaN_3) and ethyl methane sulfonate (EMS) to produce plants resistant to biotic stress such as bacterial canker disease in tomato, *Fusarium oxysporium* in banana, and to induce resistant banana plants of BBTV and BMV (Ozer *et al.,*2012; Bhagwat and Duncan, 1998; Afifi, 2011) and abiotic stress such as salinity stress (El Kaaby *et al.,* 2015). Several DNA markers have been used to identify mutants like Inter-Simple Sequence Repeat (ISSR). ISSR exploits primers that are complementary to the simple sequence repeats that anneal to and amplify DNA sequences between the adjacent inversely oriented repeats. ISSRs are randomly distributed throughout the genome, allowing the detection of multiple loci simultaneously, and requiring no prior sequence information (Tomlekova *et al.,* 2012; Wannajindaporn *et al.,* 2014; Aswandy *et al.,* 2015).

In the present study, sodium azide (NaN_3) and ethyl methane sulfonate (EMS) were used as seed treatment to produce tomato plants resistant to *Tomato ring spot virus.* Tomato plantlets were produced from treated seeds using 3 successive types of media used in tissue culture. The aim of this work is to check the effect of two different chemical mutagens in three different concentrations on resistance to *Tomato ring spot virus* (ToRSV) infection and molecular characterization of mutagens by ISSR analysis.

MATERIALS AND METHODS

Virus isolation

One hundred samples of (grapevine, tomato, and geranium) representing symptoms suspected to be caused by *Tomato Ring Spot Virus* (ToRSV) were collected from three governorates (El- Behera, El- Qaliobeia and El- Fayom).

Collected samples were checked for the presence of ToRSV by Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) as described by Boscia *et al*., (1995) for grapevine samples and (Clark and Adams, 1977) for samples of other plant species. ELISA kit completely ready to use was supplied by SANOFI (Sante Animols, Paris, France) was used.

The samples which gave positive reaction in ELISA were used for mechanical inoculation of *Chenopodium amaranticolor* which was used as local lesion host of ToRSV. Single local lesion assay (Kuhn, 1964) was used for biological purification of ToRSV. Back inoculation into tomato seedlings (cv. Castle Rock) was carried out. The tomato isolate of the virus was used in the present investigation.

Host range

Seventeen different diagnostic host plant species and cultivar belonging to 7 families (*Geraniaceae, Cucurbitaceae, Chenopodiaceae, Vitaceae, Solanaceae, Fabaseae,* and *Amranthaceae*) were tested. Ten seedlings of each plant species were mechanically inoculated and observed for symptoms development. Infectious sap was prepared from tomato leaves using 1:3 (w/v) 0.1 M phosphate buffer, and from midribs of young grape leaves and shoots using the same buffer + 2.5% nicotine 98% (Martelli,1993). The same numbers of healthy seedlings were inoculated with the same buffer used in inoculum preparation to serve as control.

The inoculated seedlings were kept under greenhouse conditions at $25^{o}C\pm2$ until symptoms development. Data were confirmed by DAS-ELISA using the polyclonal antibodies against ToRSV.

Effect of different concentration of the mutagenic NaN_3 and EMS on resistance of tomato to ToRSV

This experiment was carried out in the following different steps:

Tomato Seed treatments

About 250 seeds for each of two tomato cultivars (Castel Rock and Super Strain "B") were soaked for four hrs. with sodium azide (NaN_3) at concentration (1.0, 2.0, 3.0 and 4.0 mM). and for 12 hrs in (EMS) at concentration (0.3%, 0.4%, 0.5% and 0.6%). Group of seeds about 50 seeds for each cultivar were left without treatment (soaked in water) as check treatment. The percentage of germination of tomato seeds were calculated in all treatments (El Kaaby *et al*., 2015; Aswandy *et al.,* 2015).

Seed Sterilization

The treated seeds were washed three times to remove excess NaN_3 with sterilized distilled water. Subsequently, treated and untreated seeds were surface disinfected with ethanol at concentration 70% for 2 min, followed by sodium hypochlorite at concentrations (6%) for 20 min and rinsed for 15 min with sterilized distilled water three times (5 min each time) according to Ozer Calis *et al.,* (2012); El Kaaby *et al.,* (2015).

Media and sterilization

The treated and untreated seeds for both cultivars were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) prepared and adjusted to pH 5.8, filled into culture bottles and sterilized by autoclaving at 121°C at 15 psi for 15 min. About 50 ml of the medium was dispensed into each previously sterilized culture jar while still hot under the laminar flow hood and allowed to cool. Jars were kept at incubation room until used. The seeds were inoculated onto sterile, cooled and solidified germination medium (GM). The various media used in this investigation are as showed in table (1) and as follows:

Germination medium (GM): The treated and non-treated seeds were inoculated onto (GM) at dark for 5 days and with light at 10 days at 25±2 °C (Cortina *et al.,* 2004).

Shooting medium (SM): modified of the media of Cortina *et al.,* (2004); Tingting *et al.,* (2005). The immerging shoots were cut and cultured on regeneration medium and incubated for 21 days at 25±2 °C.

Rooting medium (RM): The regenerated shoots were sub cultured into the (RM) for 21 days 25±2 °C (Davis *et al.,* 1994).

Table 1: The contents of the three different media used in this investigation.

Media	Germination	Shooting	Rooting
MS	4.4g	4.4g	4.4g
Sucrose	30g/ L	30g/L	15g/L
Thiamine	-	0.1 mg/L	-
Zeatin	-	1mg/L	-
IAA	-	0.1 mg/L	-
NAA	-	-	0.1ml/L
Agar	7g/L	7g/L	6 g/L

Ms= Murashige and Skoog, IAA= Indol acetic acid, NAA=Naphthaline acetic acid

Seedling establishment

According to methods described by Raj *et al.,* (2005), rooted plantlets were washed three times to remove the residual of the rooting medium, then the plantlets were transferred individually in pots (5 cm in diameter) in a hardening medium consisting of peat and vermiculite (1:1) to make the medium sturdy enough to keep the plants upright. Pots were kept in humidity chamber for 2 weeks before planting in soil, still under humidity chamber and gradually acclimatized in the screen house environment.

Mechanical Inoculation of the immerging plantlets treated with different concentration of NaN_3 and EMS

After 45 days from acclimations, the produced tomato plants were mechanically inoculated by ToRSV. Inoculated plants were maintained in insect- proof greenhouse at 25±2 °C until symptoms development. Results were confirmed by DAS-ELISA.

Evaluation of genetic variability in *in vitro* NaN_3 and EMS- induced tomato mutants

This experimental was carried in Biotechnology Lab, Horticultural Research institute, AGR, Giza, Egypt.

DNA extraction

Total genomic DNA was isolated from young and fresh tomato leaves. The samples were collected separately from cv. CR treated with 0.40% EMS and cv. CR control. Another samples

were collected from cv. SSB treated with 3mM NaN_3 and cv. SSB control. Samples were ground using liquid nitrogen to a fine powder. The bulked DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN).

Inter Simple Sequence Repeats (ISSRs) Polymerase Chain Reaction (PCR) analysis

ISSR-PCR reactions were conducted using twelve primers. PCR was performed in 30 µL volume tubes that contained the following: dNTPs (2.5 mM) 3.00 µL, $MgCl_2$ (25 mM) 3.00 µL, Buffer (10 x) 3.00 µL, Primer (10 pmol) 2.00 µL, Taq DNA polymerse 0.20 µL, Template DNA (25 ng) 2.00 µL, and H_2O (d.w.) 16.80 µL. The PCRs were programmed for one cycle at 94º C for 4 min followed by 45 cycles of 1 min at 94 ºC, 1 min at 57 ºC, and 2 min at 72 ºC. The reaction was finally elongation step at 72 ºC for 10 min (Williams *et al.* 1990).

The PCR products were separated on a 1.5% agarose gels and fragments sizes were estimated with the 100 bp ladder marker. The gels were stained with ethydium bromide solution and photographed with gel documentation system. Twelve primers were used to generate reproducible polymorphic DNA products. Table (2) lists the base sequences of these DNA primers that produced informative polymorphic fragments (El-Abhar, 2018; Wannajindaporn *et al.*, 2014; Aswandy *et al.*, 2015; Tomlekova *et al.*, 2012).

Table 2: ISSR base sequences of the DNA primers.

No.	Primers	5' Sequences 3'	No.	Primers	5' Sequences 3'
1	844-A	CTCTCTCTCTCTC	2	844-B	CTCTCTCTCTCTCTCTGC
3	17898-A	CACACACACACAAC	4	17898-B	CACACACACACAGT
5	HB-14	CTCCTCCTCGC	6	44A	CTCTCTCTCTCTCTCTAC
7	49A	CAC ACA CAC ACA AG	8	98A	CAC ACA CAC ACA CA
9	E-9	AGAGAGAGAGAGAGGG	10	HB-15	GTG GTG GTG GC
11	HB-10	GAG AGA GAG AGA CC	12	E-8	CACACACACACACACAA

RESULTS

Virus isolation

Twenty five of 100 samples gave positive results by DAS-ELISA. After about 15-18 days post inoculation. The inoculated *Ch. amaranticolor* represented local lesions, and the tomato seedlings representing vain clearing and chlorotic spots 18-21 days after inoculation with single local lesion *Ch. amaranticolor*.

Host range reaction

Eleven plant species and cvs. belonging to 5 families were infected by ToRSV while 6 others belonging to 4 families gave negative results with DAS-ELISA and didn't show symptoms. Symptoms were developed after 15-16 days on *Nicotiana glutinosa* and *Datura mettel L.* after 15to 22 days on *Necotiana Tabacum* L, 18 to 21 days on *Cucumis sativus* L ,*Ch. Amaranticolor* L and *Ch. quinoa* L. and after about 21 days on *Lycopersicon esculentum* and *Pelargonium sidoides. Phaseolus vulgaris* L showed symptoms after 21 to 28 days. As shown in table (3) and figs. (1, 2 and 3).

Table 3: Host rang reaction to ToRSV expressed by symptoms and ELISA readings:

Tested plant species and cvs.	Symptoms Observed	Days after inoculation	ELISA reading
Geraniaceae *Pelargonium sidoides*	M	21 days	0.562
Chenopodiaceae *Ch. amaranticolor L* *Ch. quinoa L*	Ch.L.L.→N.L.L Ch.L.L.	18 →21 18 →21	0.492 0.489
Cucurbitaceae *Cucumis sativus L*	L.L→ M	18 →21	0.511
Cucurbita pepo L	-		0.186
Solanaceae *Datura mettel L* *Necotiana tabacum L* *Nicotiana glutinosa* *Capsicum annuum* *Lycopersicon esculentum.* Castel Rock, Super strain B	N.S,V.C, B,Ml Ch.l.l, mottle, N Mottle, V.C Ch.L.L Ch.R.S.→ NL	15 days 15→22 16 days 21days 21-28 days	0.479 0.571 0.562 0.451 0.582
Fabaceae *Phaseolus vulgaris L* *Pisum sativum* L	N.L.L -	21-28 days	0.481 0.201
Amranthaceae *Gomphrena globosa L.*	-		0.211
Vitaceae *Vitis venifera* Flame, Global Red, Early Superior cvs.	-		0.199

M=mosaic, Ch.L.L= chlorotic local lesion, L.L,= local lesion, N.S= necrotic spots, V.C= vein clearing, B= blisters, ML= malformation, N= necrosis, N.L.L= necrotic local lesion, ChL= chlorosis.

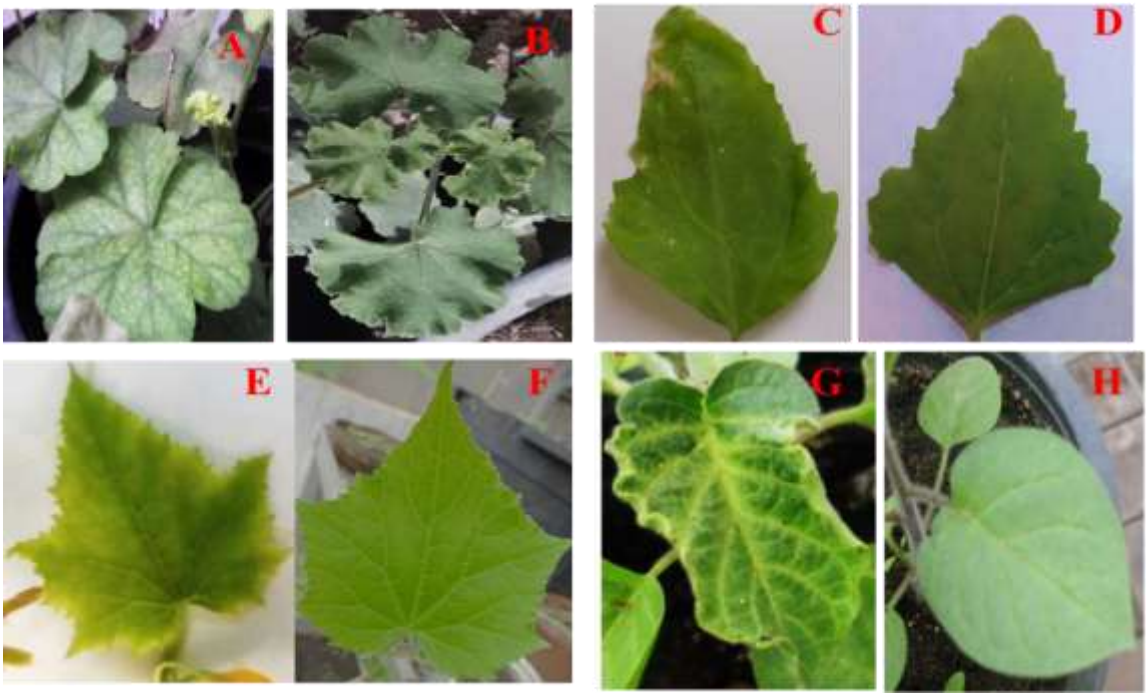

Fig. 1: *Pelargonium sidoides* showing interveinal mosaic (A), *Pelargonium sidoides* healthy control (B), *Ch. amaranticolor* L showing chlorotic local lesions (ChLL) turned to necrotic local lesion (NLL) (C), *Ch. amaranticolor* L healthy control (D), *Cucumis sativus* L showing mosaic (M) on the new leaves (E), *Cucumis sativus* L healthy control (F), young leaves of *Datura mettel* L showing vein clearing and malformation (G), and *Datura mettel* L healthy control (H).

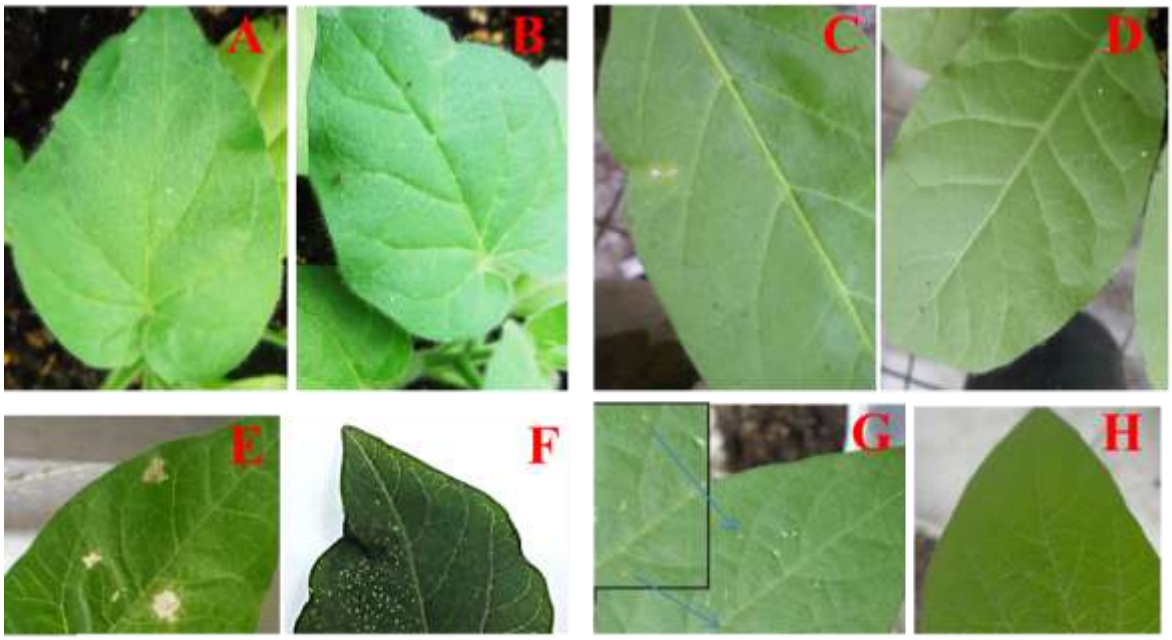

Fig. 2: *Nicotiana glutinosa* leaves exhibiting mottle and vein clearing (A), *Nicotiana glutinosa* healthy control leaves (B), *Necotiana Tabacum* L infected leaves showing chlorotic local lesion turned to necrosis (C), *Necotiana Tabacum* L healthy control (D), The inoculated cotyledons of *Phaseolus vulgaris* L showing necrosis (E), healthy control of *Phaseolus vulgaris* cotyledons (F), and the new leaves showing necrotic local lesion in (G), healthy control of *Phaseolus vulgaris* (H).

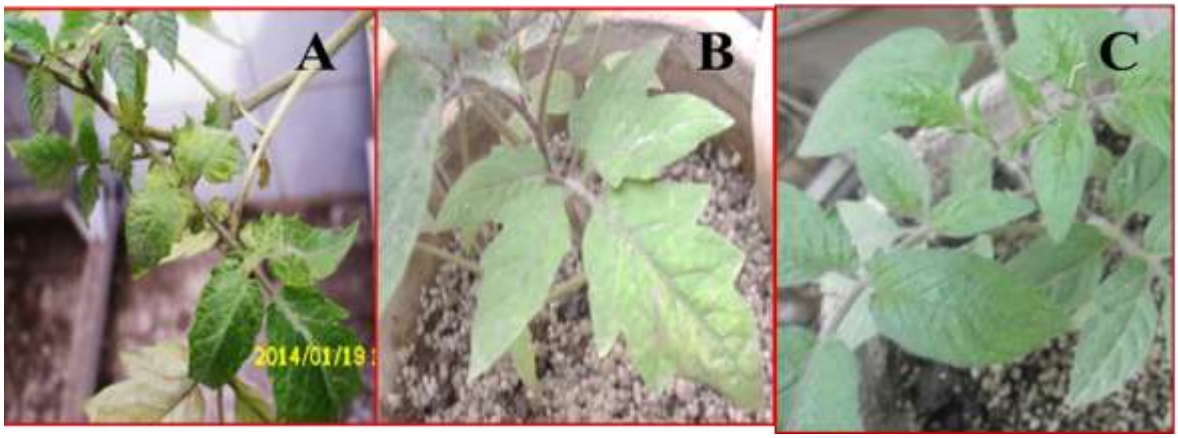

Fig. 3: Chlorotic ring spots on *Solanum lycopersicum* L (A), Necrotic lesions and vein necrosis. (B) and (C) *Solanum lycopersicum* L healthy control.

Effect of different concentration of the mutagenic NaN3 and EMS on resistance of tomato plants to ToRSV

Seeds Germination percentage

The results showed that the germination percentage in the two tomato cultivars was decreased with the increasing of the concentration of NaN_3 and EMS (Figs. 4 and 5). NaN_3 at 1mM concentration showed 86% seed germination in the two cultivars. The germination rate was 74% and 80% for cultivars CR and SSB respectively treated with 2mM concentration. At concentration 3mM and 4mM the rate of germination was reduced in the two cultivars (Fig. 4).

At 0.3% concentrations of EMS the seed germination ranged from 76% and 86% to 86% and 92% in the control samples for cultivars CR and SSB respectively (Fig. 5). The lowest germination percentage in the two cultivars CR and SSB (46% and 42%) respectively was at 0.6%. concentration the germination rate was decreased within the increased of EMS concentration.

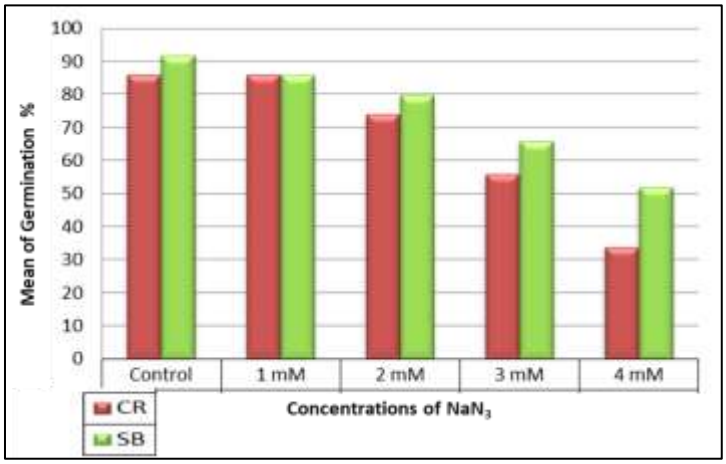

Fig. 4: Effect of treatment with NaN_3 in four different concentrations on seed germination % of two tomato cultivars Castel Rock and Supper Strain B.

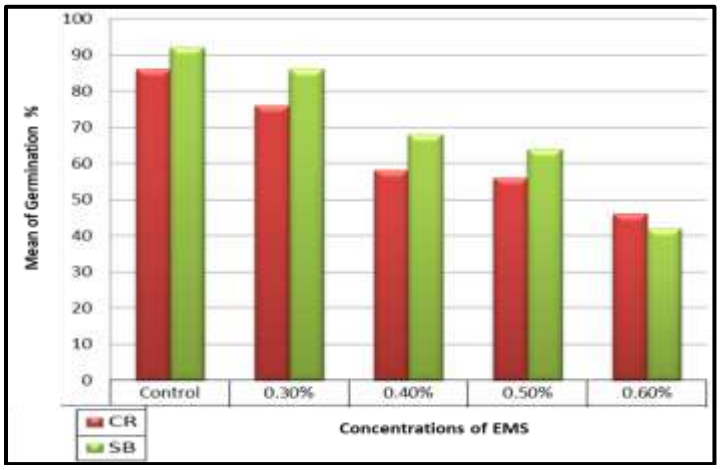

Fig. 5: Effect of treatment with EMS in four different concentrations on seed germination % of two tomato cultivars Castel Rock and Supper Strain B.

Effect of treatment with NaN_3 and EMS on some growth parameter of two tomato plantlets cvs.

The plantlets resulting from seeds treated with the two mutagenic substances (NaN_3 and EMS) were observed for growth morphology. In all treatments plantlets didn't give branches compared with control except one plantlet treated with 1mM concentration of NaN_3 in cultivar SSB and one plantlet at 2mM concentration of NaN3 in cultivar CR. At concentration 0.3% of EMS there were two plantlets from cultivar CR gave branches from 512 plantlets.

The mean length of shoots in the two tomato cultivars was almost good if compared with control. At 1, 2mM concentrations of NaN3 than at 3mM, and 4mM concentration, significant decrease in mean shoot length was remarkable (Fig. 7) at 2mM concentration of NaN3 many of plantlets showed whiteness (Fig. 6).

Fig. 6: Whiteness of tomato leaflets at 2mM concentration of NaN3.

At 0.3% concentration of EMS the mean shoot length was (9.7% and 9.4%) for cultivars CR and SSB respectively. The mean shoot length showed significant decrease at 0.3% and 0.4% concentrations of EMS for the two tomato cvs. At 0.6% concentration of EMS, the most of plantlets of the two cultivars died. Mean shoot length showed significant decrease by the increasing of EMS concentrations, while lethality increased with increasing EMS concentration. At 0.6% concentration of EMS the plantlets exhibited filiform shape of leaflets.

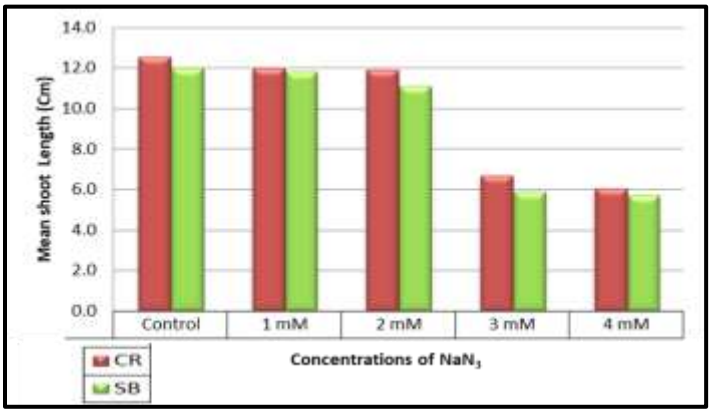

Fig. 7: Effect of treatment with NaN_3 in four different concentrations on shoot length (cm) of two tomato cultivars Castel Rock and Supper Strain B.

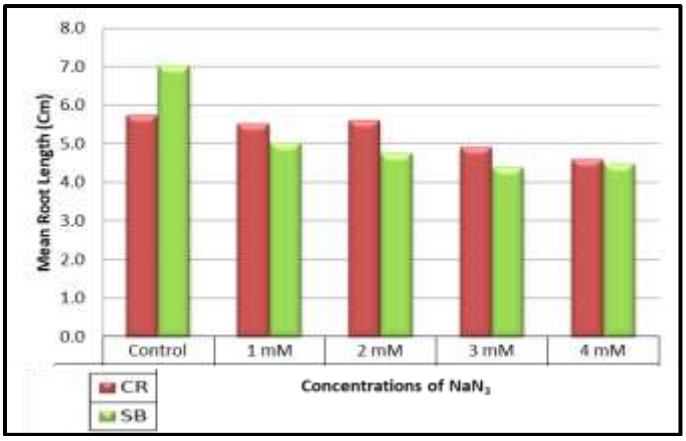

Fig. 8: Effect of treatment with NaN_3 in four different concentrations on root length of two tomato cultivars Castel Rock and Supper Strain B.

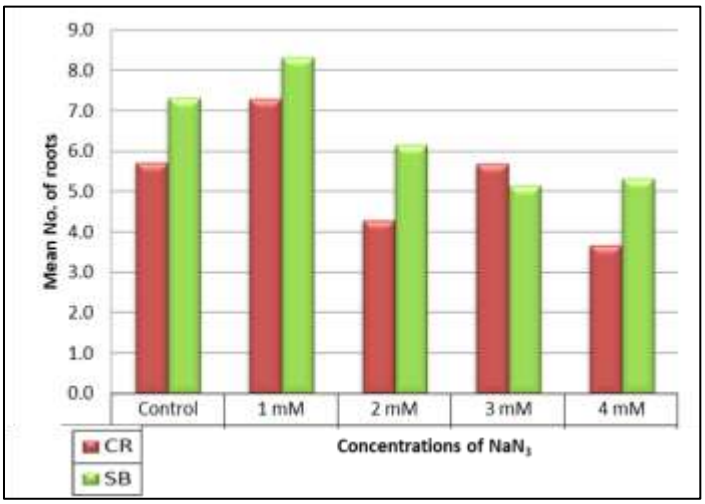

Fig. 9: Effect of treatment with NaN_3 in four different concentrations on roots number of two tomato cultivars Castel Rock and Supper Strain B.

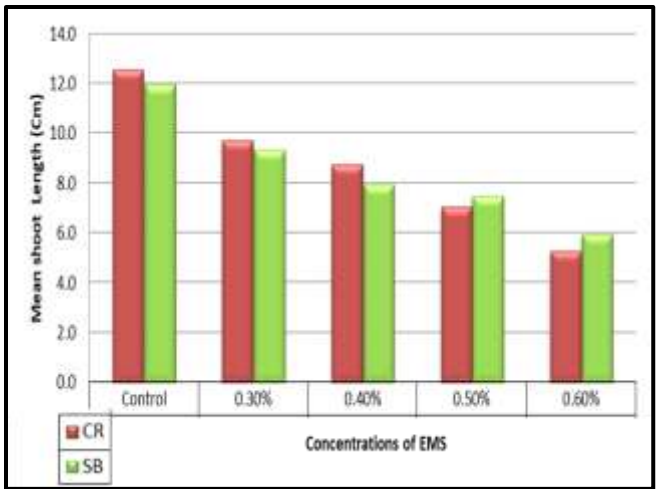

Fig. 10: Effect of treatment with EMS in four different concentrations on shoot length of two tomato cultivars CR and SSB.

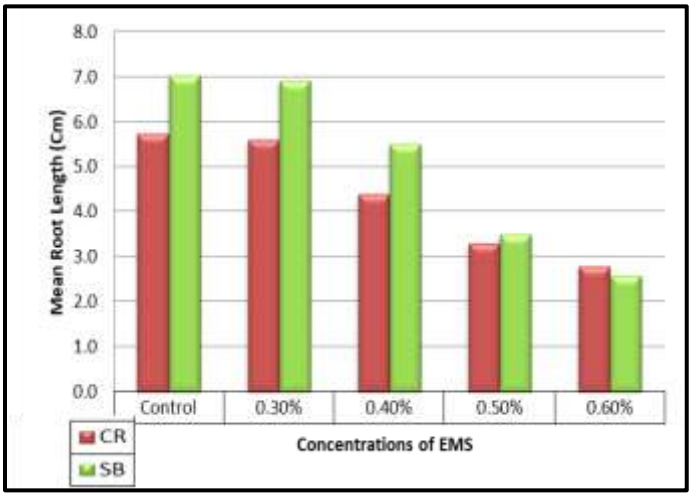

Fig. 11: Effect of treatment with EMS in four different concentrations on root length of two tomato cultivars CR and SSB.

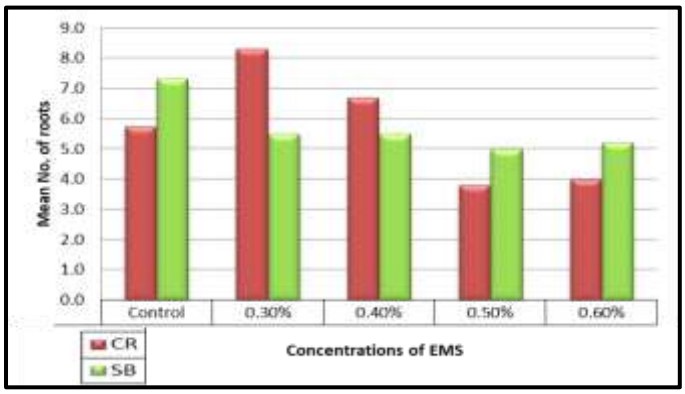

Fig. 12: Effect of treatment with EMS in four different concentrations on roots number of two tomato cultivars CR and SSB.

Effect of different concentration of NaN_3 and EMS on percentage of infection with *Tomato ring spot virus* on tomato cv. Castle Rock (CR) and Super Strain B(SSB) produced by tissue culture

Results in table (4) show that, at 3 and 4mM concentration of NaN3 for both tomato cv. plants didn't show any external symptoms, while results of ELISA readings showed that they were infected, but the concentration of the virus expressed by ELISA reading was low compared with positive control and the other treatments. While concentration of 1mM showed symptoms of chlorotic spots and necrotic lesions in both cvs. At concentration 2mM the plants showed chlorotic spots only. Percentage of infection with ToRSV was reduced from 86.04% to 26.92% by increasing NaN_3 concentrations in cv. SSB. In Castel Rock cv. percentage of infection was reduced from 81.39% to 29.41% by increase of NaN_3 concentrations. For EMS at, 0.3% concentration plants showed chlorotic spots and necrotic lesions and recorded high ELISA reading. While concentrations 0.4%, 0.5% and 0.6% no symptoms were observed on plants although concentration 0.4% and 0.5% gave positive ELISA reading and 0.6% gave negative ELISA reading. Percentage of infected with ToRSV was reduced from 76.74% to 23.80% by increasing EMS concentrations in cv. SSB. In Castel Rock cv. percentage of infection was reduced from 73.68%to 26.08% by increase of EMS concentrations.

Table 4: Effect of four different concentrations of NaN3 and EMS on percentage of infection with *Tomato Ring Spot virus* on tomato cv. Castle Rock(CR) and Super Strain B(SSB) produced by tissue culture:

Treatment		Cultivars					
Concentration		CR	SSB		SSB		
		ELISA Reading	Infection%□	Sympt.	ELISA Reading	Infection%□	Sympt.
NaN3	1mM	0.507	35/43=81.39%	ChS,NL	0.511	37/43=86.O4%	ChS,NL
	2mM	0.394	25/37=67.57%	ChS	0.327	30/40=75%	ChS
	3mM	0.283	10/28=35.71%	NS	0.272	11/33=33.33%	NS
	4mM	0.261	5/17=29.41%	NS	0.248	7/26=26.92%	NS
EMS	0.30%	0.542	28/38=73.68%	ChS,NL	0.662	33/43=76.74%	ChS,NL
	0.40%	0.273	15/29=51.72%	NS	0.347	20/34=58.82%	NS
	0.50%	0.264	12/28=42.85%	NS	0.282	14/32=43.75%	NS
	0.60%	0.25	6/23=26.08%	NS	0.251	5/21=23.80%	NS
Positive Control		0.762		ChS, N, NR	0.757		ChS, N, NR
Negative control		0.254		NS	0.254		NS
Blank		0.286			0.286		

NS=no symptoms, ChS= Chlorotic Spots, N=necrosis of terminal growing shoots, NR= necrotic rings, NL= necrotic lesions
□ = No. of infected plants/ No. of inoculated plants x 100

Detection of DNA fragment polymorphism by the obtained ISSR patterns

Analysis of ISSR pattern was carried out. A total of 12 random Inter Simple Sequence Repeats (ISSRs) primers were tested as a preliminary tool of mutation screening. The plantlets imerged from seeds treated with EMS 0.40% cv. Castel Rock and with NaN_3 3mM cv. Super Strain B gave results with the three primers 98-A, 98-B, and E-9. Fig. (13) show that using primer 98-A resulted in detection of 6 DNA fragments in control samples of cv. CR and 8 fragments in the treated one. Three DNA fragments were observed in cv.CR tomato plants treated with EMS (0.40%) with molecular weight 421, 362 and 211. While there is a unique band found in cv. CR control. There were two monomorphic fragments common with the CR cv. and SSB cv. control and the two treatments. Data showing in fig. (14), using 98-B primer with the same samples show ١٠fragments in the control of CR cv. and 9 fragments in the treated CR cv. With 0.40% EMS, the hidden band was at molecular weight 508 bp.

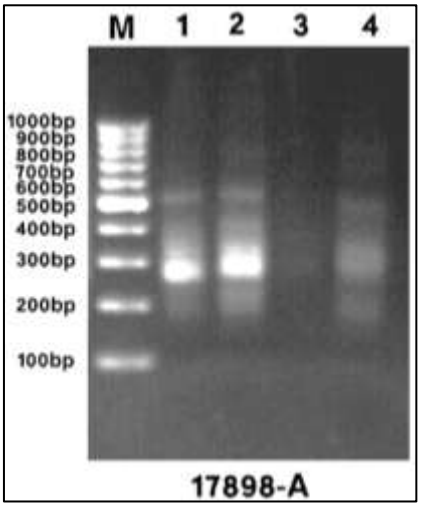

Fig. 13: Agarose gel electrophoresis analysis of ISSR using the primer 17898-A, M: marker (100 bp), lane 1: cv.CR control, lane 2: cv. CR treated with (0.40%) EMS, lane 3: cv. SSB control and lane 4: cv. SSB treated with 3mM NaN_3.

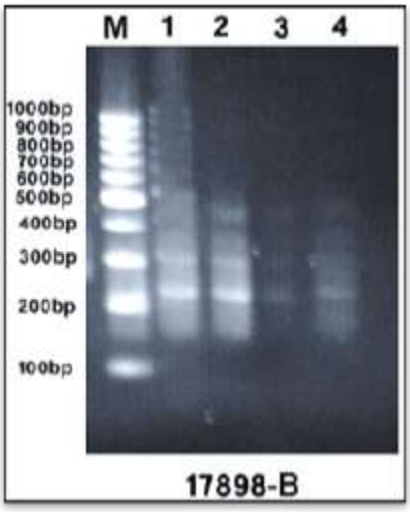

Fig. 14: Agarose gel electrophoresis analysis of ISSR using the primer 17898-B, M: marker (100 bp), lane 1: cv.CR control, lane 2: cv. CR treated with (0.40%) EMS, lane 3: cv. SSB control and lane 4: cv. SSB treated with 3mM NaN_3.

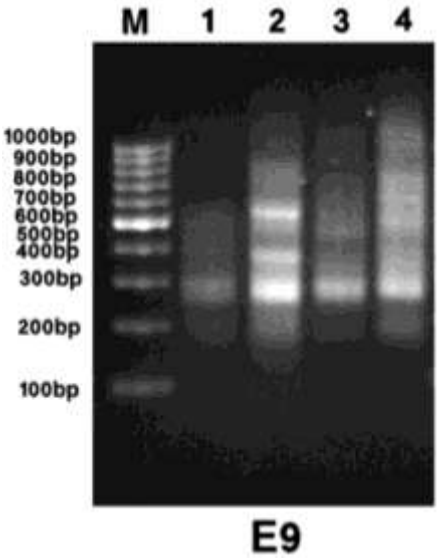

Fig. 15: Agarose gel electrophoresis analysis of ISSR using the primer E9, M: marker (100 bp), lane 1: cv.CR control, lane 2: cv. CR treated with (0.40%) EMS, lane 3: cv. SSB control and lane 4: cv. SSB treated with 3mM NaN_3.

Fig. (13) show that using primer 98-A resulted in detection of 2 DNA fragments in control treatment of cv. SSB and 4 DNA fragments in NaN_3 3mM treated. SSB cv. treated with NaN_3, three DNA fragments were observed with molecular weight of 331, 319, and 188 but not found in SSB control. Using 98-B primer, SSB cv. gave 4 DNA fragments with control and 7 DNA fragments with the SSB cv. treated with 3mM NaN_3.

The observed fragments at 3mM concentration of NaN_3 were at molecular weight 226, 177, and 155 bp. These fragments were not found in control. When 98-B primer was used there were two monomorphic fragments were common with the CR cv. and SSB cv. control and the two treatments (Fig.14)

In fig. (15), the E-9 primer gave three monomorphic DNA fragments in control treatment of the two tomato cvs. and the two treated cvs. with molecular weight at 286, 264, and 194 bp. In CR cv. Control treatment, 6 fragments appeared with E-9 primer and 11 fragments appeared in CR cv. treated with 0.40% EMS. There was one unique band in CR cv. control at molecular weight 473 bp which disappeared in treatment. At 0.40% EMS treated CR cv. showed 6 fragments with molecular weight at 1162, 1050, 857, 799, 709, and 549 not found in the control. SSB control gave 10 fragments with E-9 primer and 11 fragments appeared in the SSB cv. treated with 3mM NaN_3. There were 4 unique fragments appeared in SSB cv. control with molecular weight at 725, 932, 658, and 459 bp and were not detected in the treated. Five fragments appeared in SSB cv. treated with 3mM NaN_3 with molecular weight at 799, 709.3, 624.2, 511.9, and 369.4bp. and not found in the control treatments (Fig.15).

DISCUSSION

Tomato ring spot virus (ToRSV) is one of the most destructive viruses and is a member of the Nepovirus group which includes about 46 viruses. The virus is distributed in most parts of the world and can infect herbaceous (Anonymous, 2005), ornamental and woody species (Moini. 2010). Tomato artificially inoculated of tomato plants with ToRSV showed symptoms typical to those describe by Stace-Smith (1984); Darwish (2005). The host range of the virus was tested by

mechanical inoculation into 11plant species and cvs. geranium, *Chenopodium amaranticolor* , *C. quinoa, Cucumis sativus* , *Nicotiana tabacum* , and *Phaseolus vulgaris* exhibited symptoms similar to these mentioned by Stace-Smith(1984); Brunt *et al* ., (1996); Pourrahim *et al.,* (2004).

Mutation is the ultimate source of all genetic variation (ZakyZayed *et al.,* 2014). The tissue culture technique is an important way of mutant plant production with mutagen and thus speeds up the breeding program (Al-Qurainy and Khan, 2009). Aswandy *et al.,* (2015) mentioned that, tomato is an ideal model plant of assessing the effects of mutation on specific genome due to the availability of its complete genome.

Sodium azide: a chemical mutagen has become important tool to enhance agronomic traits of crop plants. It is being used to produce resistance in various susceptible crops to improve their yield and quality traits against harmful pathogens (Khan *et al.,* 2009). Sodium azide is a chemical mutagen that creates point mutation in the genome of plants by producing metabolite and thus produced protein in mutant plant has different function from the normal plant (Al-Qurainy and Khan, 2009), Sodium azide creates point mutation, A.T-->G.C base pair transition and transversion, and hence all DNA based markers cannot apply for point mutation detection.

Ethyl Methane sulfonate (EMS) is effectively proven in inducing random point mutation (Till *et al.,* 2004). EMS has been used to obtain high frequency of gene mutation and chromosomal alteration in different crops which led to evolve a number of mutant varieties (Wei-Seng Ho *et al,* 2014). Ethyl methane sulfonate (EMS), a compound of the alkaline sulfonate series is widely known to induce a higher frequency of mutations in higher plants. EMS usually causes high frequency of gene mutations and low frequency of chromosome aberrations, but loss of a chromosome segment or deletion can also be occurred in plants. EMS alkylates guanine bases due to mispairing-alkylated G pairs with T instead of C, resulting in primarily G/C to A/T transitions. EMS produces a large genome number of non-lethal point mutations approximately 10,000 to saturate the genome with mutations. For instance, in Arabidopsis, EMS can produce more than four non-lethal point mutations per Mb. Thus, EMS has the potential of altering loci of particular interest without inducing a great number of closely linked mutations (ZakyZayed *et al.,* (2014).

In the present work NaN_3 and EMS at 4 different concentrations were checked as mutagenic as seed treatment of two tomato cvs. The higher concentration of NaN_3 affected some biological activities such as specific enzymes which are involved in seed germination processes and reduced germination percentages and other growth parameters (El Kaaby *et al*., 2015). Azide ion plays an important role in causing of mutation by interacting with enzymes and DNA in the cell. This is due to that NaN_3 is strong mutagen and growth of plant parts are strongly inhibited with increasing its concentration and treatment duration. Its impact has been observed on tomato and it was very effective in inducing mutations with respect to germination percentage, root length, seedling height, seedling survival, number of branches per plant and yield per plant (Al-Qurainy and Khan, 2009).

The survival percentage decreased progressively as the dosage increased (Mensah and Obadoni 2007). Mensah and Akomeah (1992) have reported that the higher the mutagenic dose, the lower the survival percentage, and Adegoke (1984) reported that, sodium azide induces chromosomal damages leading to bridge formation during mitotic division and hence increased phenoltypic aberration. An overall decrease in seed germination was observed with increasing concentration and exposure of EMS (Saba and Mirza, 2002). Mahla *et al.,* (2010) gradual reduction in germination and subsequent survival of the treated population with EMS was observed with the increase of mutagen dose while higher concentration was detrimental for treated plants. The obtained results showed that the 3mM NaN_3 concentration and the (0.40%)

EMS concentration was the better based on for growth parameters and for inactivity with ToRSV inoculation.

These results could be attributed to the effect of mutagens on the meristematic tissues of the seeds. These may be due to physiological and acute chromosomal damage, delay in the onset of mitosis, chromosomal aberrations induced enzyme activity such as catalase and lipase and hormonal activity resulted in reduced germination and survivability. Disturbance in the formation of enzymes involved in the germination process may be one of the physiological effects caused by EMS and SA leading to decrease in germination. Reduced growth due to higher doses was also explained differently by different workers. It may be attributed to one or more of the following reasons (i) the increase in growth promoters, (ii) the sudden increase in metabolic status of seeds at certain levels of dose, (iii) the increase in destruction of growth inhibitors, (iv) drop in the auxin level or inhibition of auxin synthesis and (v) decline of assimilation mechanism. Taking these as the preliminary consideration (Roychowdhury and Tah, 2011).

In the present investigation 12 ISSR primers were used in ISSR-PCR for preliminary screen for mutants resulting from treatment of seeds of two tomato cvs. with NaN_3 and EMS. Polymorphic fragments were produced by 3 of 12 ISSR primers. Polymorphic and monomorphic DNA fragments were detected when primers 98-A, 98-B, and E-9 were used. ISSR (Inter simple sequence repeat) has been found to be the most economical among PCR based markers recently. It has many advantages like low quantities of template DNA, no need of sequence data for primer construction, random distribution throughout the genome, high level of polymorphic bands, even using only a few primers, less investment in time and labour than other markers, and exhibit Mendelian inheritance. Therefore, ISSR markers have been widely used for DNA fingerprinting studies, genetic diversity studies, phylogenetic studies and identification of closely related cultivars (ZakyZayed *et al.,* 2014). Putative mutants were evaluated for genetic variability compared to untreated control plants using inter-simple sequence repeat (ISSR) analysis (Wannajindaporn *et al.,* 2014).

The absence of ISSR fragments or the presence of additional ISSR fragments in 15 mutants may result either from the loss/gain of primer binding sites as a result of changes in the nucleotide sequences (e.g., point mutations) or changes that alter the size or prevent the successful amplification of a target DNA (e.g., deletions, duplications, inversions, or translocations). These mutants will be transferred to a greenhouse for further evaluation of desirable traits and multiplication. It should be noted that changes in ISSR profiles may not reflect changes in morphological traits. This research could benefit in agriculture sector particularly in the study of chemical mutagenesis and research for genes of resistance to ToRSV (Wei-Seng *et al.,* 2014).

CONCLUSION

The virus was isolated from infected grapevine, from tomato and from geranium which exhibiting necrotic rings and sinuous lines symptoms. The virus was checked in the suspected samples by ELISA using the specific antiserum. ToRSV was transmitted mechanically into11 plant species and cvs., belonging to 5 families.

Treated plant with different concentrations of $NaN_3$and EMS showed that high concentrations of both chemicals led to death of plantlets. Concentrations 3.0 mM of NaN_3 and EMS 0.4%, showed no symptoms of ToRSV.

EMS (0.40%) treated tomato cv. CR and 3mM NaN_3 treated tomato cv. SSB revealed 3 unique bands with 98-A ISSR primer, 3 unique bands appeared in SSB cv. treated with 3mM NaN_3 with 98-B ISSR primer, and E-9 ISSR primer revealed 6 bands in EMS (0.40%) treated CR

cv. and 5 bands in 3mM NaN_3 treated SSB cv. Indicting changes in the genetic make-up of the treated tomato plants. No similar bands were observed in the control samples.

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