MUTAGENIC EFFECT OF ETHYL METHANESULFONATE AND SODIUM AZIDE ON *IN VITRO* CULTURE OF *PHILODENDRON BIPINNATIFIDUM*

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Abstract

The mutagenic effects of different concentrations of Ethyl Methanesulfonate (EMS) and Sodium Azide (SA) on *in vitro* culture of *Philodendron bipinnatifidum* were examined. Results obtained generally revealed that most of treatments gave high percentages of survival, except the explants immersed in SA at 80 mg/l for 8 min which decreased the survival to 55.55%. Most of treatments gave good results for shootlet length, shootlet number and leaves number except explants immersed in 80 mg/l SA for 8min which recorded the lowest values of shootlet length, number and leaves number. The genetic fidelity of the mutagenic plants was tested by 5 RAPD primers. It was found that all DNA fingerprints with these primers displayed polymorphic band profiles indicating between the different mutagenic treatments and mother plant.

Keywords: *Philodendron bipinnatifidum*, Mutagenic, EMS, SA and RAPD

Abbreviations: EMS: Ethyl Methanesulfonate, **SA**: Sodium Azide, **RAPD**: random amplified polymorphic DNA

INTRODUCTION

Uses of tissue culture techniques are important tools for varietal improvements of plants *via* genetic engineering, *in vitro* mutagenesis and somaclonal variation induction (Ganesan *et al.* 2005). The genus Philodendron contains more than 700 species making it the second largest genus in the family Araceae (Croat, 1997). It is propagated from cuttings and also propagated from seed in native regions. Philodendrons have many uses, including houseplants as tabletop plants or floor specimens. For interior landscape plantings in offices, lobbies or shopping malls, philodendrons are used as upright plants or highly suitable as groundcovers, depending on the variety selected (Xiong, 2009).

In vitro mutagenesis is an important tool to induce phenotypic and genotypic variations. Spontaneous variation found in regenerated plants has been termed as "somaclonal variation" and this is a common phenomenon in callus-derived plantlets. Both somaclonal variation and induced mutations result in the production of new genotypes with limited change in their original genome. Compared with spontaneous

mutation, mutagen induced mutations provide tools for the rapid generation of variability in crops. Chemical mutagens Ethyl Methanesulfonate (EMS) and Sodium Azide (SA) have been widely used to induce a large number of functional variations in ornamental plants. Chemicals induce mainly point mutations and are thus ideal for producing missense and nonsense mutations, which would provide a series of change of function mutations. On the other hand, ionizing radiations normally induce chromosomal rearrangements and deletions (Bhat *et al.*, 2007 and Talebi *et al.*, 2012).

Genetic markers have become indispensable tools for understanding, managing, and improving natural and planted populations (Pijut *et al.*, 2007). The many marker systems and their uses, as well as, the choice of optimal system for various research goals are well reviewed (Gillet, 1999; Mohler and Schwarz, 2004 and Ziegenhagen and Fladung, 2004). The RAPD technique provides genetic markers which have been used extensively in many different applications and in different plant species because of its simplicity (Ulanovsky *et al.*, 2002; Samaee *et al.*, 2003 and Gabr and El-Bahr, 2013).

Therefore, the aims of this study to investigate the effect of EMS and SA on shooting behaviour and genetic on *P. bipinnatifidum*.

MATERALS AND METHODS

Plant materials

This study was carried out in the laboratory of Tissue Culture, H. R. Inst., A. R. C. and Dep. of Plant Biot. Genetic Eng. N. R. C. The experiments were carried out throughout 2013 – 2014. The aims of this study to investigate the effect of EMS and SA on shooting behaviour and genetic on *P. bipinnatifidum*.

Shoot tip, as explants of *P. bipinnatifidum* (syn. *P.* selloum) were sterilized by mercuric chloride ($HgCl_2$) at 1.5 g/l for 30 min and sodium hypochlorite (NaOCl) at 3.0% for 30 min. The explants were rinsed three times with sterile distilled water and then cultured on a basal MS medium (Murashige and Skoog, 1962) enriched with sucrose 30 g/l and agar 7 g/l. The media were adjusted to pH 5.7 ±.0.1, then poured at 25 ml in 200 ml capacity glass jars before autoclaving at 121°c and 1.2 kg/cm^2 for 15 min. Cultures were incubated for 4 weeks in a growth chamber at 24±2° C. The photoperiod was 16 hours light/8 hours darkness, and Illumination intensity was 3000 lux from cool fluorescent lamps.

Shoot cultures

The survival explants was cultured on MS medium supplemented with 3.0 mg/l Benzyl adenine (BA) as described by Chen *et al.* (2012). This step aimed to provide a stock of *P. bipinnatifidum* shoot cultures which will be subjected in the mutagenic treatments. Then explants were cultured on MS medium supplemented with 3.0 mg/l Indolbutric acid (IBA) for rooting stage.

Mutagen Treatments

Explants were aseptically soaked in 125 ml-capacity Erlenmeyer flasks containing distilled solutions of Ethyl Methanesulfonate (EMS) and/or Sodium Azide (SA) at the concentrations of 0.00, 20, 40 or 80 mg/l. Besides, untreated water solution was used as a control, After 2, 4 and 8 mins of EMS and SA exposure the explants were rinsed and planted into solidified MS-medium.

Therefore, twenty-one treatments [control and three concentrations from each of EMS and SA were used for three duration treatments] were done and in each treatment twenty-five explants in five replicates were used.

Micropropagability of explants *in vitro* study

The parameters of the micropropagability of explants were recorded:

- Survival capacity of explants was estimated as percentage
- Number of the formed shootlets per explants
- Length of the developed shootlets in (cm)
- Number of the initiated leaves per shootlet.

Extraction and Purification of Genomic DNA

Leaves from *in vitro* mutagenic cultures were harvested after explants were containing roots. DNA was isolated in order to perform RAPD analysis, to detect the variation a modified, CTAB (hexadecyltrimethyl ammonium bromide) procedure based on the protocol of Doyle and Doyle (1990).

RAPD analysis

A set of five random 10-mer primers was used in the detection of polymorphism among 22 samples, (21 mutagenic treatments and the mother plant). These primers were synthesized on Metabion international AG (D-82152 Martinsried/ Deutschland). RAPD-PCR was carried out according to the procedure given by Gabr and El-Bahr (2013). The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 2 mM dNTPs, 1 µM primer, 1 U *Taq* DNA polymerase and 25 ng templates DNA.

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (*PE* Applied Bio-systems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at

94ºC for 1 min, an annealing step at 36ºC for 1 min, and an elongation step at 72ºC for 1 min.

The primer extension segment was extended to 7 min at 72ºC in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts.

Table (A). Sequence of the five decamer arbitrary primers assayed in RAPD-analysis to detect polymorphism among the 21 mutagenic treatments and the mother plants.

Primer code	Sequence (5'-3')
OP-A 10	5´-GTGATCGCAG-3´
OP-C 12	5´-TGTCATCCCC-3´
OP-D 07	5´-TTGGCACGGG-3´
OP-M 09	5´-GTCTTGCGGA-3´
OP-Q 09	5-GGCTAACCGA-3´

Statistical analysis

The statistical analysis lay-out of the experiments was designed as completely randomized design (Steel and Torrie, 1980). Test of LSD at 5% was used for comparison among means. RAPD analysis, the well resolved and consistently reproducible fragments ranging form 100 bp to 1.5 kb were scored as present or absent.

RESULTS AND DISCUSSION

Effect of chemical mutagenic treatments on shooting behavior

The data in Table (1) and Fig (1) revealed that immersed explants in water, EMS and SA in different concentrations for different times produced significant differences in behavior growth of *P. bipinnatifidum*. For survival percentage, immersed explants in water and EMS for 20, 40 and 80 mg/l for 2, 4 and 8 min and SA 20 mg/l for 2, 4 and 8 min and 40 mg/l for 2 min gave 100% survival percentage. This percentage was decreased to (88.88%) in SA 40 mg/l for 4 and 8 min and 80 mg/l for 2, 4 min. Immersed explants in 80 mg/l for 8 min decreased survival percentage to the lowest value (55.55%).

For shootlet number/explant and shootlet length (cm), immersed explants in 20 mg/l EMS for 2, 4 and 8 min gave higher growth in shootlet number (3.30 and 3.11 shootlet/explant) and shootlet length (3.00 and 2.83 cm). On the other hand, the

explants were immersed in 80 mg/l SA for 8 min reduced shootlet number and length to minimum (1.33 shootlet/explant and 1.0cm).

In leaves number, immersing the explants in water for 8 min and EMS 40 mg/l for 8 min produced higher number of leaves (2.84 and 2.76 leaf/shootlet) in compared to the other treatments. However, the explants immersed in 80 mg/l SA for 8 min gave the lowest number of leaves (1.42 leaf/shootlet).

In conclusion, using 80 mg/l SA for 8 min reducing shooting behaviour. Approving results were reported Endo *et al.* (1997) on *Chrysanthemum*; Han Dongsheny *et al.* (1999) on *Lily connecticut*; Vainola (2000) on *Rhododeron*; Youssef and Abou Dahab (2006) on *Populus alba* and Sayed *et al.*, (2012) on *gardenia jasminoids*.

Table (1): Effect of chemical mutagenic on shooting behaviour of *P. bipinnatifidum*.

	Survival %	Shootlet Number/explant	Shootlet length (cm)	Leaves number
Water 2min.	100	2.11	2.67	2.21
Water 4min	100	1.89	2.50	1.78
Water 8min	100	1.89	1.75	2.84
EMS 20 mg/l 2min	100	3.30	2.83	2.32
EMS 20 mg/l 4min	100	3.11	3.00	2.46
EMS 20 mg/l 8min	100	3.11	2.83	2.17
EMS 40 mg/l 2min	100	2.44	2.00	2.44
EMS 40 mg/l 4min	100	2.78	1.50	1.86
EMS 40 mg/l 8min	100	2.33	1.83	2.06
EMS 80 mg/l 2min	100	2.44	1.67	2.39
EMS 80 mg/l 4min	100	1.89	1.67	2.67
EMS 80 mg/l 8min	100	2.22	1.55	2.76
SA 20 mg/l 2min	100	2.11	1.50	2.38
SA 20 mg/l 4min	100	2.11	1.33	2.11
SA 20 mg/l 8min	100	1.89	1.50	2.21
SA 40 mg/l 2min	100	1.89	1.17	2.05
SA 40 mg/l 4min	88.89	1.44	1.33	2.50
SA 40 mg/l 8min	88.89	2.23	1.50	1.54
SA 80 mg/l 2min	88.89	1.67	1.50	1.53
SA 80 mg/l 4min	88.89	1.78	1.50	1.94
SA 80 mg/l 8min	55.55	1.33	1.00	1.42
LSD at 5%	18.34	0.95	0.75	0.729

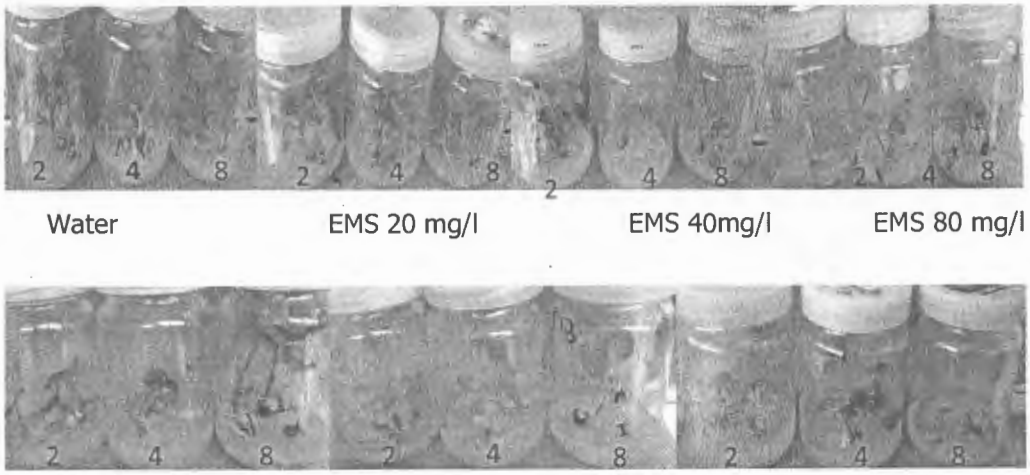

Fig. 1. Effect of water, EMS and SA at different concentrations and different times on shooting behavior

RAPD analysis of chemical mutagenic treatments.

Five random primers (OP-A 10, OP-C 12, OP-D 07, OP-M 09 and OP-Q 09) were screened in RAPD analysis for their ability to produce sufficient amplification products. The results of DNA fingerprints generated by PCR amplification using these primers are presented in figs (2, 3, 4 and 5). RAPD profiles of the mutagenic treatments and the mother plants were non identical.

RAPD marker generated with primer OP-A 10 showed that no amplification differences between mutagenic treatments, while three fragments were detected at 1200, 800 and 300 bp with mutagenic treatments and didn't detected with the mother plants. RAPD marker generated with primer OP-C 12 showed the highest amplification bands. This primer was present differences between mutagenic treatments as well as was presented differences between mutagenic treatments and the mother plant. On the other hand, primer OP-D 07 gave the lowest amplification bands and the difference between the mother plant and mutagenic treatments were very clear. The mother plant was absence 5 bands at 300, 500, 600, 700 and 1100 bp, which detected in the mutagenic treatments. Primer OP-M 09 showed the heights polymorphism amplification differences between the mutagenic treatments. Water treatments at different periods and EMS at different concentrations and different periods showed high similarity but mutagenic treatments with SA were shown differences in polymorphism amplification.

As RAPD markers amplify different regions of the genome, their simultaneous analyses give a better interpretation of the genetic stability of the *in vitro* regenerates (Polambi and Damiano, 2002; Martins *et al.*, 2004). In this respect, RAPDs have been

used for many purposes, ranging from studies at the individual levels (e.g. genetic identity) to studies involving closely related species. Due to their high genomic abundance, RAPDs have also been applied in gene mapping studies. In this respect, RAPD markers have been able to assess the genetic stability of micropropagated plants of *Deutzia scabra* and the effect of slow growth preservation of the same plant almonds (Gabr and Sayed, 2010) (Martins *et al.*, 2004), turmeric (Tyagi *et al.*, 2007), and yams (Ahuja *et al.*, 2002). As RAPD markers amplify different regions of the genome, their simultaneous analyses give a better interpretation of the genetic stability of the *in vitro* regenerates (Martins *et al.*, 2004).

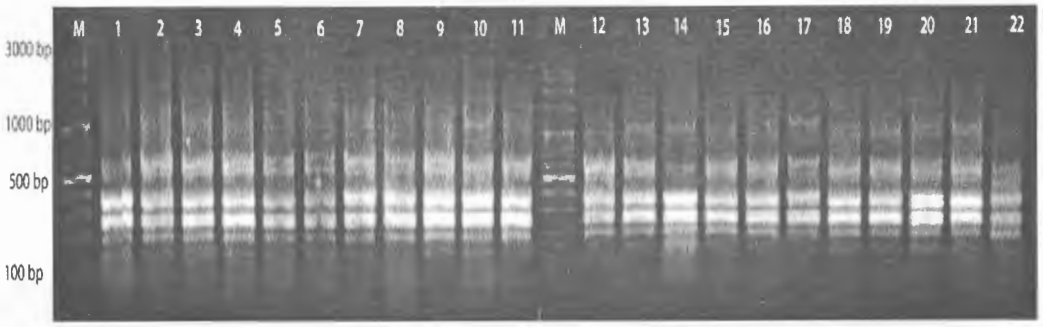

Fig. 2. RAPD DNA amplification pattern obtained for mutagenic treatments and mother plant generated by primer OP-A 10 at 2, 4 and 8 min, respectively: (Lan 1, 2 *and 3) water, (Lan 4, 5 and 6) EMS 20 mg/l, (Lan 7, 8 and 9) EMS 40 mg/l, (Lan 10,* 11 and 12) EMS 80 mg/l, (Lan 13, 14 and 15) SA 20 mg/l, (Lan 16, 17 and 18) SA 40 mg/l, (Lan 19, 20 and 21) SA 80 mg/l, Lan 22 mother plant and M: 100 – 3000 pb DNA Ladder.

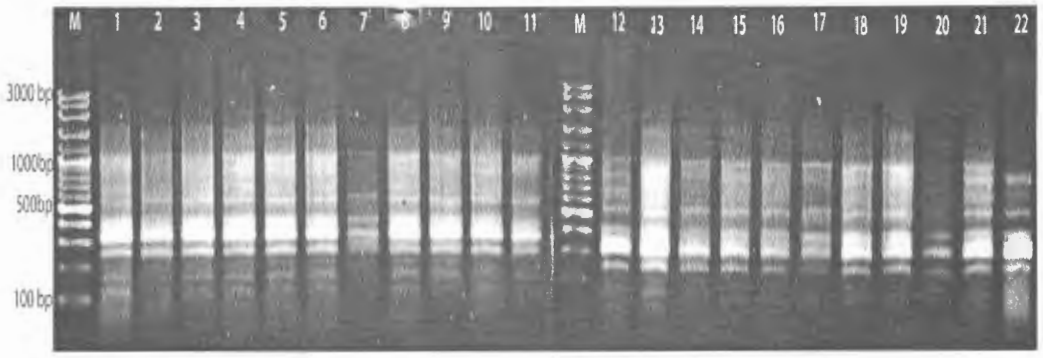

Fig. 3. RAPD DNA amplification pattern obtained for mutagenic treatments and mother plant generated by primer OP-C 12 at 2, 4 and 8 min, respectively: (Lan 1, 2 and 3) water, (Lan 4, 5 and 6) EMS 20 mg/l, (Lan 7, 8 and 9) EMS 40 mg/l, (Lan 10, 11 and 12) EMS 80 mg/l, (Lan 13, 14 and 15) SA 20 mg/l, (Lan 16, 17 and 18) SA 40 mg/l, (Lan 19, 20 and 21) SA 80 mg/l, Lan 22 mother plant and M: 100 – 3000 pb DNA Ladder.

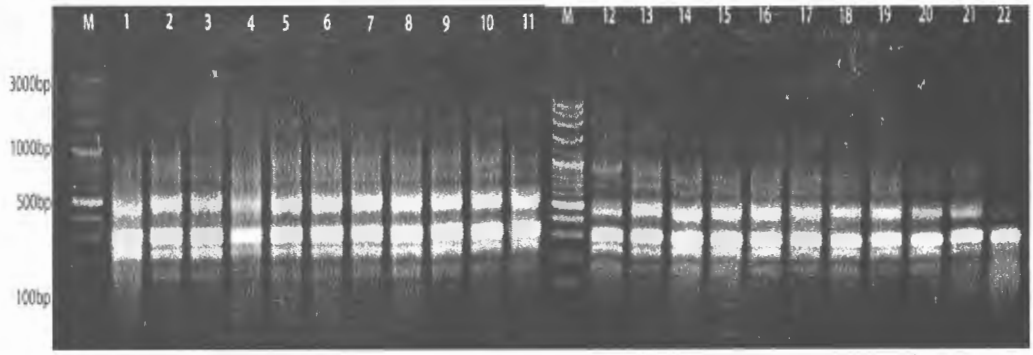

Fig. 4. RAPD DNA amplification pattern obtained for mutagenic treatments and mother plant generated by primer OP-D 07 at 2, 4 and 8 min, respectively: (Lan 1, 2 and 3) water, (Lan 4, 5 and 6) EMS 20 mg/l, (Lan 7, 8 and 9) EMS 40 mg/l, (Lan 10, 11 and 12) EMS 80 mg/l, (Lan 13, 14 and 15) SA 20 mg/l, (Lan 16, 17 and 18) SA 40 mg/l, (Lan 19, 20 and 21) SA 80 mg/l, Lan 22 mother plant and M: 100 – 3000 pb DNA Ladder.

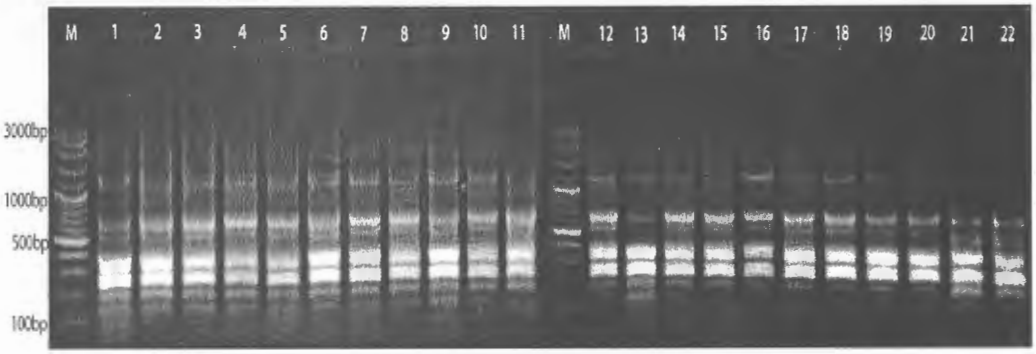

Fig. 5. RAPD DNA amplification pattern obtained for mutagenic treatments and mother plant generated by primer OP-M 09 at 2, 4 and 8 min, respectively: (Lan 1, 2 and 3) water, (Lan 4, 5 and 6) EMS 20 mg/l, (Lan 7, 8 and 9) EMS 40 mg/l, (Lan 10, 11 and 12) EMS 80 mg/l, (Lan 13, 14 and 15) SA 20 mg/l, (Lan 16, 17 and 18) SA 40 mg/l, (Lan 19, 20 and 21) SA 80 mg/l, Lan 22 mother plant and M: 100 – 3000 pb DNA Ladder.

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التأثير المطفرلمادتى الإيثيل ميثايل سلفونات والصوديوم أزيت على مزارع الأنسجة لنبات الفلودندرون

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فى هذا البحث تم دراسه تأثير المطفرات بتركيزات مختلفة من مادتى الميثايل إيثايل سلفونات والصوديوم أزيت على مزارع الأنسجة بهدف احداث طفرات لنبات الفودندرون.

أوضحت النتائج المتحصل عليها أن معظم المعاملات أعطت بصفة عامة أعلى نسبة بقاء بإستثناء تلك المنفصلات المعاملة بالصوديوم أزيت بتركيز ٨٠ مليجرام/لتر لمدة ٨ دقائق حيث أعطت أقل نسبة بقاء وهى ٥٥.٥٥%. وأعطت معظم المعاملات نتائج جيدة فيما يتعلق بطول النبات وعدد النباتات وعدد الأوراق فيما عدا نقع الاجزاء النباتيه فى تركيز ٨٠ مليجرام/لتر صوديوم ازيد لمده ٨ دقائق اعطى اقل النتائج لطول الافرع وعددها وعدد الاوراق. ومن ناحية أخرى وجد أن نتائج التوصيف الجزئى باستخدام تقنية ال RAPD-PCR اعطت إختلافات بين النباتات المعاملة كيماويا بالمطفرات وبين النبات الأم فى حين أن النتائج لم تظهر إختلافات بين التركيزات المختلفة للمواد المطفرة.