Pak. J. Bot., 51(3): 949-955, 2019. DOI: 10.30848/PJB2019-3(19)

EMS INDUCED POINT MUTATIONS IN 18S rRNA GENE OF *HYOSCYAMUS NIGER* L. AN IMPORTANT MEDICINAL PLANT OF KASHMIR HIMALAYA

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Abstract

Hyoscyamus niger contains medicinally important tropane alkaloids such as scopolamine and hyoscyamine. *In vitro* mutagenesis, in combination with molecular marker methods, is a potent technique to produce genetic variability in medicinal plants. The objective of the study was to induce variation in *In vitro* raised *H.niger* using Ethyl Methane Sulphonate (EMS) as a mutagen. For this purpose the callii were treated with 5 different doses of EMS (0.01%, 0.02%, 0.03%, 0.04% and 0.05%) at different time intervals (1/2 hour and 1 hour), and the effect of EMS was observed during morphogenesis. The analysis rvealed that EMS had a profound effect on shoot number and length as well as percentage of shoot formation. 0.03% EMS was seen to be effectively enhancing the average shoot number from 13.5 to 22.0, with 100% callusing response under normal growth regulators. The 18S rRNA gene of the affected plantlets was then amplified and sequenced to observe nucleotide variations, including transitions and transversions, at different concentrations of EMS.

Key words: EMS, 18S rRNA gene, *In vitro* mutagenesis, *Hyoscyamus niger*.

Abbreviations: EMS, Ethyl methane sulphonate; CTAB, Cetyltrimethyl ammonium bromide.

Introduction

Induced mutagenesis is a significant tool to generate genetic inconsistency and variability at a faster rate (Kumar & Singh, 2003) and has been used in improving plant yield and secondary metabolite content (Larik, 1979). Induced mutations play an important role in improvement of medicinally and economically essential traits like secondary metabolite content, high yield, abiotic and biotic stress resistance, etc. in numerous crops (Ahloowalia &Maluszynski, 2001). With the help of this approach more than 3200 mutant cultivars have ben established (Pierre, 2012), either directly as mutants or variants chosen from mutated or altered populations, or indirectly from the combination with stimulated or induced mutants (Jain &Suprasanna, 2011). Many different molecular indicators and markers like sequence tagged sites (STS), Random Amplified polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Simple sequence repeats (SSRs microsatellites) have been established in order to detect genetic diversity and genotype identification(Tilwari *et al.,* 2016). One of the significant methods to induce genetic variation in plants is *In vitro* mutagenesis using EMS. This method can increase genetic variability for development of new varieties (Bashir *et al.,* 2013) as the source of all genetic variations lies in the process of mutation. Chemical mutagens can produce a sequence of mutations by inducing the point mutations (Talebi *et al.,* 2012), and EMS is the most frequently used chemical mutagen in plants (Purnamaningsih & Hutami, 2016). This is a potent alkylating mutagen and sometimes more effective than physical mutagens (Bhat *et al.,* 2005). EMS alkylates Guanine (G), because of which G pairs with Thymine (T) rather than cytosine (C) changing G/C to A/T. In addition to this, EMS can cause small deletions and rearrangements depending on the position of the mutation (Rafi *et al.,* 2016). Due to these properties EMS has been used in many plants to induce variability, which can subsequently improve the quality and quantity of the byproducts in plants like sugarcane (Purnamaningsih & Hutami, 2016) and tomato (Shikata *et al.,* 2016) etc. The EMS induced mutants usuually show improved traits, like increase in metabolite content, resistance to abiotic stress and phenotypic trait variability (Xi-ou *et al*., 2017).

Hyoscyamus niger is an important member of Solanaceae, which is considered as one of the largest drug producing family among plants. This species grows at an altitudinal range of 1500m to 2300m (asl) and is disturbed in India, North Africa, China, South West Asia, Afghanistan, Japan and different parts of Europe (Sajeli *et al.,* 2006). This plant is considered to be a rich repository of alkaloids such as hyoscyamine and scopolamine (Ibrahim *et al.,* 2009). It has been used for the treatment of different ailments like rabies, spasm, ulcers, cough, asthma, motion sickness, bronchitis, inflammation. It also possesses anticholinergic, anaesthetic, spasmolytic, narcotic and antiasthma properties (Hong *et al*., 2012). It is a temperate plant, the seasonal and temperature factors limit its potential to be grown at large scale to meet the increasing market

burdens and demands (Hong *et al.,* 2012). Thus, tissue culture medium is composed of organic and inorganic components (Gollo *et al.,* 2016) supplemented with plant growth regulators which provides plant a medium for its growth and development. Present study, for the first time, was designed to induce EMS mediated variations in the *H. niger*, which was followed by targeting 18S rRNA gene to detect genetic variation between *H.niger* variants in comparison to control plantlets. This variability was also confirmed by DNA sequence analysis.

Materials and Methods

Plant material and establishment of *In vitro* culture: *H. niger* seeds were collected from Kashmir University Botanical Garden (KUBG). After washing with the detergent [Labolene and Extran (0.5%)] supplemented with some droplets of Tween-20, seeds were then treated with $HgCl_2$ (v/v) (0.2%) for 20 min. The seeds were washed three times with double distilled water. These sterile seeds were then inoculated on MS medium (Murashige & Skoog, 1962), maintained at a pH of 5.8. The media was autoclaved for 20 min at 121°C at a constant pressure of 15 psi.

Callus Induction: *In vitro* grown leaf segments, (Approx. 1.5cm^2) from fully grown *In vitro* seedlings and were cultured on MS media improved with different combinations of BAP/NAA/Kn to induce callus formation. Cultures were incubated at 16 hr photoperiod regime, under cool fluorescent tubes with light intensity of 1500-3000 lux and constant temperature (25± 3°C). Relative humidity in the culture room was maintained at 60-70%. Subculturing was done after every two weeks for three months on MS media supplemented with BAP and TDZ.

EMS dosage (%) of callus explants in *Hyoscyamusniger*: 42 days old callus cultures were treated with different concentrations of EMS (0.01%, 0.02%, 0.03%.04% and 0.05%) and were continuously agitated on a shaker at 75 rpm. Room temperature was maintained at 25°C for 1/2hr and 1hr respectively. After EMS treatment callus cultures were rinsed 3 times in double distilled water (DDW) then were inoculated on shoot proliferation medium. At least 3 pieces of callus were placed on each culture vial (150ml). Callus cultures were incubated at 25±3°C, with light intensity of 1500-3000 lux. The proliferation medium for shooting, MS medium was augmented with (30g/L) sucrose, TDZ (16µM) and solidified at 8g/L. After 8 weeks of mutagenic treatment, data on number of shoots, shoot length and shooting % age was recorded.

DNA isolation and PCR analysis: Young leaves from EMS treated plants were used for isolation of genomic DNA using CTAB method (Maniatis *et al.,* 1982) and analysis was done on 0.8% agarose gel. The 18S rRNA gene was targeted by using primers. 5/TGCAGTT AAAAAGCTCGTAG3/ was used as a forward primer and 5/GGTTGAGACTAGGACGG TATCTG 3/ as a reverse primer. PCR amplifications was started in a reaction mixture having a volume of 50µl comprising of 100ng DNA, 0.1µM of reverse and backward primer, 0.2 mM of dNTP, and 0.01 units of Taq/µl DNA polymerase (Sigma) in 1x Taq Buffer at pH 8.7. For amplification, denaturation was done at 94°C for 10 min, followed by denaturation for 30 seconds at 94°C, and annealing at 45°C for 1 minute, followed by extension at 72°C for 1 minute and final extension step at 72°C for 5 minutes. Amplification of the products was examined on 1.5% agarose gel.

DNA sequencing: Sequencing was done commercially from SCIGENOM COCHIN, Kerala, India. For analysis the sequencing DATA in FASTA format were aligned with the reference sequence of 18s rRNA available from NCBI website using Clustal X version 00000000-2 and Chromas Pro version 1.49 beta -2 softwares.

Results

Mutagenic treatment: *In vitro* raised leaves were inoculated on MS media augmented with different phytohormones in different concentrations and combinations of BAP /NAA/ Kn for callus formation. However, best callus response was observed on NAA (10.74 µM) +Kn (1.16 µM) (Table 1) with Green Compact Nodular Callus (GCNC), which was further used for the mutagenic treatment. The callus obtained was treated with different concentration of EMS (0.01% - 0.05%) for 30 min and 60 min and cultured on regeneration medium (MS+TDZ (16 µM). The shoot number showed increasing trend up to 0.03% EMS (22.0) when treated for 30min. However, with further increase in EMS concentration slight decrease in shoot number at 0.04% EMS (18.2) and 0.05% EMS (12.5) was observed (Table 2; Fig. 1-a, b, c).Thus, beyond a thresh hold value of 0.03% EMS shoot number was decreased. Keeping other conditions constant, when time duration was doubled, i.e., increased to 60 min (Table 2, Fig. 1-a, d, e, f), shoot formation was highest in 0.01% and lowest in 0.05 % EMS. Shoot length showed gradual decrease with increase in EMS concentration in both trials.

Molecular analysis

Effect of EMS on 18S rRNA: An effective extraction protocol for DNA from plant samples was obtained in current study (Fig. 2-a). Primers targeting 18S rRNA gene were used for PCR amplification (Fig. 2-b). On the basis of morphological data frequent variations were observed in 60 min treatment as compared to 30 min; thus 60 min treated explants were carried further for molecular analysis. In callus treatment (60 min) the frequent nucleotide variation observed were T to A followed by G to A. (Table 3, Figs. 3 & 4). The result showed nucleotide transitions and transversions from T-A, G-A, T-G, G-T, C-A, C-G at different concentration of EMS.

Table 1. Effect of different phytohormones on callus formation of *Hyoscyamus niger* L. from *In vitro* raised leaves on MS media.

Treatments	Callusing (%)	Degree of intensity of callus formation	Nature of callus
BAP (1 µM)	-	-	-
BAP(2.5 µM)	-	-	-
BAP(5 µM)	30	+	CCC
BAP(10 µM)	30	+	CCC
BAP(12.5 µM)	40	+	CCC
BAP(15 µM)	30	+	CCC
BAP(20 µM)	20	+	CCC
BAP(6 µM)+NAA(3 µM)	80	+++	LGCC
BAP(10 µM)+NAA(5 µM)	70	++	LGCC
BAP(15 µM)+NAA(7.5 µM)	40	++	LGCC
BAP(20 µM)+NAA(10 µM)	30	++	LGCC
Kn(1.16µM)+NAA(2.68 µM)	50	++	LGCC
Kn(1.16 µM)+NAA(5.37 µM)	80	++	GCNC
Kn(1.16 µM)+NAA(8.05 µM)	90	+++	GCNC
Kn(1.16 µM)+NAA(10.74 µM)	100	+++	GCNC
Kn(2.32 µM)+NAA(2.68 µM)	80	++	GCNC
Kn(2.32 µM)+NAA(5.37 µM)	80	+++	LGCC

*Data scored after 42 days of culturing time (n=10), (+) low callus; (++) moderate callus; (+++) intense callus, CCC (Compact Cr Callus), LGCC (Light Green Compact Callus), GCNC (Green Compact Nodular Callus)

Fig. 1. Effect of EMS on *In vitro* regeneration potential of callus of *H.niger* L. on MS + TDZ (16µM). a: Control (0%); b: 0.03% EMS (30 min treatment); c: 0.05% EMS (30 min treatment); d: 0.01% EMS (60 min treatment); e: 0.03 % EMS (60 min treatment); f: 0.05 % EMS (60 min treatment).

Table 2. Effect of EMS on shoot regeneration from *In vitro* raised callus of *H. niger* L. on MS Media supplemented with TDZ (16 µM).

Time duration (hour)	Treatments EMS (% v/v)	Shoot No	Shoot length (cm)	Shooting (%)
Control	0%	^{f}13.5 ± 0.70	^{h}3.2 ± 0.35	100
1/2hr (30 Minutes)	0.01	^{g}14.5 ± 0.69 (7.4%)	^{g}3.0 ± 0.36 (-6.2%)	100
	0.02	^{i}19.2 ± 0.69 (42.2%)	^{f}2.5 ± 0.27 (-21.8%)	100
	0.03	^{j}22.0 ± 0.48 (62.9%)	^{f}2.4 ± 0.23 (-25.0%)	100
	0.04	^{h}18.2 ± 0.73 (34.8%)	^{e}2.2 ± 0.31 (-31.2%)	100
	0.05	^{e}12.5 ± 0.73 (-7.4%)	^{e}2.1 ± 0.31 (-34.3%)	100
1hr (60 minutes)	0.01	^{e}12.0 ± 0.47 (-11.1%)	^{e}2.1 ± 0.31 (-34.3%)	100
	0.02	^{d}10.2 ± 0.0.47 (-24.4%)	^{d}1.9 ± 0.39 (-40.6%)	100
	0.03	^{c}6.7 ± 0.30 (-50.3%)	^{c}1.6 ± 0.36 (-50.0%)	80
	0.04	^{b}3.1 ± 0.48 (-77.0%)	^{b}1.0 ± 0.30 (-69.7%)	80
	0.05	^{a}2.2 ± 0.39 (-83.7%)	^{a}0.5 ± 0.29 (-84.3%)	70

*Data was evaluated by ANOVA using Duncan's multiple range test (SPSS17.0) along with mean ± SD (n=10); the superscript on the values are significant at $p<0.05$. Data scored after 56 days of culturing time

** Percent variation is mentioned in each cell below the main value along the column

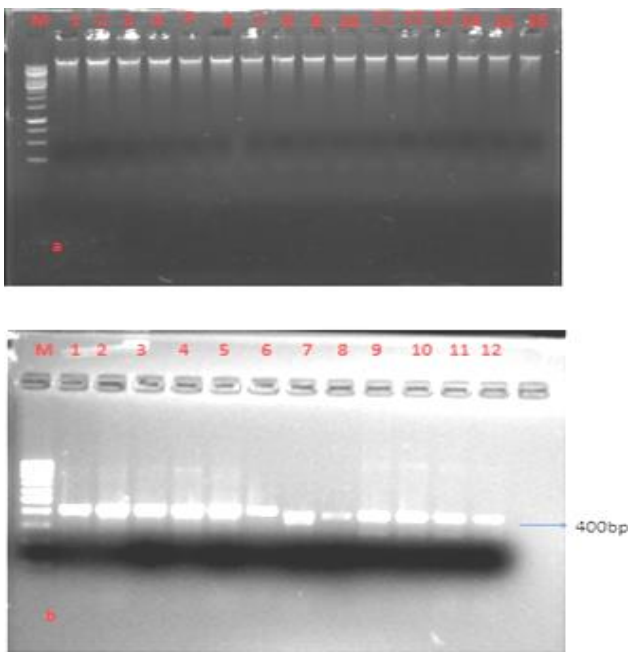

Fig. 2. Representative DNA extraction and PCR analysis of different samples (400bp) of *H.niger* L. treated with different concentrations of EMS. Lane M in both images represent 100bp/1Kb DNA marker a) DNA extraction b) PCR amplification.

Discussion

Our study revealed that best green callus formation was observed at (NAA-10.74µM +K-1.16 µM). Ibrahim *et al.,* (2009) also reported highest callus formation on MS media containing 1 and 2 mg NAA and .05 mg Kn in *H. muticus*. In the current analysis genetic variability was induced in *H. niger* using EMS. At lower concentration of EMS, regeneration potential of *In vitro* raised callus was improved as compared to control, and showed a constant decline with increase in EMS concentration and time duration. This variation at high concentration of EMS may be due to toxicity of EMS at high dosage (Aruna *et al.,* 2010), or it may be due to genotoxic effect of EMS which leads to arrest of cell cycle. It can also result in genetic damage like chromosomal aberrations (Dube *et al.,* 2011). Behera *et al*., (2012) reported that 25 Mm EMS for 1 hour increased the number of shoot , however 4 hour EMS treatments showed significant decrease in the number of shoots under *In vitro* conditions in *Asteracantha longifolia.* Khalil *et al.,* (2015) reported that 15 GY dose of gamma radiations showed increase in the growth parameters (callus biomass) and manufacture of bioactive compounds in *Stevia rebaudiana* under *In vitro* conditions. Furthermore, in M2 generation of *Solanum melongena* physiological variations were reported by Xi-ou *et al*., 2017, when treated with EMS. An increase in the secondary metabolite content (upto ~ 5 to 20 fold) was seen with 20GY irradiation dose in *Capsicum annum* L. and *Stevia rebaudiana* by Vardhan Shukla, (2017). From the EMS mutagenized cucumber line 406 with dark green exocarp, a light green exocarp mutant was discovered (Zhou *et al.,* 2015). Thus, EMS is considered as a potent mutagen to produce genetic variability in plant cells. This was confirmed by our study, as it created genetic variations in *H. niger* which was evident in F_1 generation revealed through molecular analysis.

It has been earlier reported that most commonly used chemical mutagen in plants is Ethyl Methane Sulphonate that leads to alkylation of purine bases like Guanine (G) resulting in mismatch between nucleotides. As a result alkylated G pairs with T rather than C, which results in G/C to A/T transitions (Bhat *et al.,* 2007). Our study has also revealed that the point mutations caused by EMS are single-base substitutions, which may arise due to transitions (purine into purine

or pyrimidine into pyrimidine) and transversions (pyrimidine to purine) and vice-versa (Bhat *et al*., 2007). Similar analysis was carried out by Suprasanna *et al*., 2008, who used SCAR marker in a Multiplex PCR with dwarf primer and 18S rRNA primers for characterization of tissue culture derived dwarf types in banana cultivar Robusta. They observed an amplified fragment of 1500 bp in the normal, semi dwarf type but not in the dwarf types. This banding pattern was predictable as defined earlier by Ramage *et al*., (2004) compared to our study, which showed nucleotide variations because of EMS mutagenesis. Genetic variability of M× g putative mutants which raise from the EMS actions including control was assessed using ISSR markers. However, the polymorphisms recorded through ISSR examination indicated the incidence of genetic alterations between plants which were regenerated from the EMS treated calli in comparison to control (Perera *et al.*, 2015). Our findings also showed genetic nucleotide variation in the form of transitions and transversions and point mutations (T-A, G-A, T-G, G-T, C-A, C-G) are caused by EMS.

Fig. 3. Multiple sequence alignment of samples generated from 18SrRNA amplification showing various base pair changes (shown by arrow head). The FASTA sequences of samples were aligned with the reference sequence of 18SrRNA available from NCBI website using Clustal X software.

Table 3. Types of point mutations induced by EMS in 18S rI gene of *Hyoscyamus niger*.

Time (hours) explants	Sample (EMS concentration %)	Wild type	Variation type
1hr (60 min)	A 8	No change	
	A 7	T	A
	A9	T	A
		C	A
		T	A
		C	G
	A10	T	G
		G	A
		G	A
		T	G
		G	A
	A11	T	A
		T	A
	A12	T	A
		T	A

A 8 (control), A 7 (0.01%), A9 (0.02%), A10 (0.03%), A11 (0.04%), A12 (0.05%) (60 min treatment of Callus)

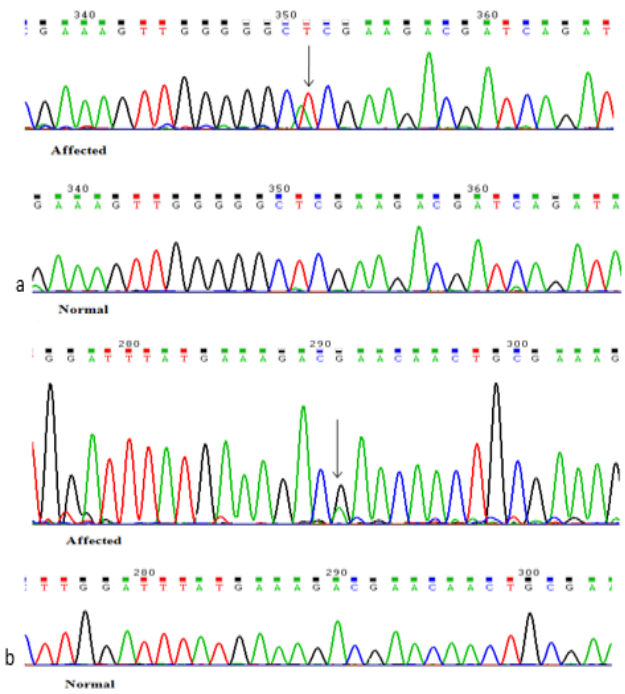

Fig. 4. Representative sequencing chromatogram of 18S rRNA gene. Arrow shows overlapping peaks indicating mutation a. Mutation T to A; b. Mutation G to A

Conclusion

18S rRNA gene was first time characterized as a model gene following EMS mutagenesis in *H. niger* and transition and transversions type of alterations were recorded in this gene. Therefore, it is believed that similar mutations might have occurred in alkaloid producing genes of *H. niger*. However, the mutagen treated *In vitro* raised plantlets have been transferred to the field and the incidence of selection of any suitable mutant line is possible only after thorough screening of M2 and M3 generations.

Acknowledgement

S. Durdana was supported by Research grant from University of Kashmir, Srinagar (Grant No. F (RS. Scholarship- CORD) DFBS/KU/15). Research infrastructure was collectively provided by Centre of Research for Development (CORD) University of Kashmir and Department of Botany, University of Kashmir. Part of the molecular analysis was performed at Scigenom Laboratory Kochin, India.

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(Received for publication 16 February 2018)