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Induced Mutation of *Dendranthemum grandiflora* through Tissue Culture by Ethyl Methanesulphonate (EMS)

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Abstract The induced mutation by ethyl methasulphonate (EMS) was used for new cultivar of chrysanthemum. The calli were regenerated from immature petals of chrysanthemum (*Dendranthemum grandiflora*) 'vivic'. The size of 0.5x.05 cm ray florets were cultured on Murashige and Skoog medium (MS) supplemented with 2 mg/l NAA and 4 mg/l Kinetin for inducing calli. The calli were soaked in 0, 0.5, 1.0, 1.5 and 2% EMS for 60 and 120 minutes to induce mutation. Afterwards, they were cultured in MS medium supplemented with 2 mg/l NAA and 4 mg/l Kinetin to induce shoots for 4 weeks. The LD_{50} were 1.22% EMS for 60 min and 0.72% EMS for 120 min. The shoots were regenerated from callus in control 76.67% but they could not regenerate in the EMS concentration which is higher than 1.5 mg/l. After that they were transferred to the new bottles and cultured with the same medium for every 4 weeks. All shoots regenerated the roots in the MS medium without the plant growth regulator. Rooting plants were transferd to the soil pot plants. The EMS has the efficiency to induce the *in vitro* mutation. There are 3 different mutations characteristic which were found in this experiment. The first were obtained in 0.5% EMS for 60 min which resulted in white stacked ray florets with 2 layers, green disk florets. The second mutation characteristic were found in 1% EMS for 60 min which the flower turned into yellow petal stacked with 2 layers, green disk florets and also the white and yellow ray florets. The last mutation characteristic of 0.5% EMS for 120 min made the ray florets slender white stacked with 2 layers and green disk florets.

Keywords: Chrysanthemum, callus, mutation, ethyl methanesulphonate

Introduction

Chrysanthemum (*Dendranthemum grandiflora*) is one of the world's most important flowering plants. A cut flower is native to China and northern Japan. It has different colors and forms of flowers. Some varieties have the chariateristics of both cut flower and pot plant. Though varied tints and forms are available in chrysanthemum, the ultimate challenge ahead of breeders is to

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develop novel varieties with a really outstanding bloom of ornamental value (Padmadevi and Jawaharlal, 2011). The induction of mutation in chrysanthemum has attracted considerable attention due to the fact that any change in the dominant genes is easily expressed in the first generation and thus, the selection of mutation of directly perceptible characters like flower colour, shape and size is generally very easy and can directly be put to commercial use (Kapadiya *et al.*, 2016). In many countries, mutation induction in chrysanthemum by use of X-ray or Gramma-ray is common due to their easy application and high efficiency. Chemical mutagens like ethyl methanesolphonste (EMS) could also be used and applied successfully where no irradiation facility is available. However they are not widely used principally due to their low penetration into vegetative tissues such as bud shoot and stem which cause a low efficiency and difficulties in reproducing the experiment (Van Harten, 1998). Hence, *in vitro* mutagenesis has the advantage of creating solid mutants in chrysanthemum. Micropropagation is a proven technique for the rapid multiplication and improvement of many ornamental plants, especially for the species like *Dendranthema grandiflora* (Ben Jaacov and Langhans, 1972) The flower tissue for the *in vitro* propagation of chrysanthemum was used to explant type for organogenesis or somatic embrogenesis (Teixeira da Silva *et al.*, 2015).

EMS is a chemical mutagen of the alkylating group and has been widely used in plants because it causes high frequency of gene mutations and low frequency of chromosome aberrations. This mutagen has been used to treat seeds and recently to treat *in vitro* explant of many species (Saxena *et al*. 1990.; Duron, 1992). The studies have shown that *in vitro* mutation of chrysanthemum (*Dendranthema grandiflora* Tzvelev) with EMS in immature floral pedicels. The other brought the flower stalks soaked in 0.77% EMS for 1 hour 45 min make the petals color change from dark pink to pink-salmon, light-pink, bronze, white, yellow and salmon color (Latado *et al.*, 2004). The study found that EMS solution was effective in the mutation of chrysanthemum *in vitro*. It also makes the chrysanthemum color change from the original. Therefore, the objectives of this research to induce mutation in chrysanthemum using EMS in order to solution to obtain morphologically distinct chrysanthemum from unmodified plants.

Materials and methods

Plant material

Chrysanthemum (*Dendranthemum grandiflora)* ‘vivic’ (spay type) was used the ray floret isolated from flower washed thoroughly under running water for 30 min. The ray florets were surface sterilized with 20% clorox (1.2% Sodium hypochloride) tween-20 added 1-2 drop for 20 min and 3 times with

sterilized distilled water for 5 min. The callus was initialed by culturing 0.5×0.5 cm ray floret on MS medium supplemented with 2 mg/l NAA (α-Napthalene acetic acid) and 4 mg/l Kinetin (N-6furfuryladenine). The explants were cultured under cool fluorescent lamps at light intensity 40 $\mu Molm^{-2}$ with a 16 h/day light at temperature 25±2°C. The calli were transferred to the fresh medium after 30 days.

EMS induced mutation

The calli size 0.5×0.5 cm was immersed in 0, 0.5, 1.0, 1.5 and 2.0% EMS for 60 and 120 min on the 80 rpm shaker. The calli were wiped water on sterile paper. They were transferred to the MS medium supplemented with 2 mg/l NAA and 4 mg/l kinetin. They were observed for their growth after 30 days recovery period, after which surviving calli were counted, LD_{50} (half lethal dose) of EMS was estimated. The experiment was 5×2 factorial in randomized complete design, 10 treatments, 3 replications and 10 explants per replication.

Plant regeneration from calli

After culturing the calli, the surviving calli were induced to regenerate plantlets. For shoot regeneration, calli were cultured on MS medium supplemented with 2 mg/l NAA and 4 mg/l kinetin. After shoot formation, they were culture on MS medium free hormone for root development.

Morphological observation

The shoot with roots were transferred to pots containing soil: coconut pieces (1:1) and maintained in greenhouse for 3 months. The plants were observed daily for mutation characteristic.

Statistic analysis

All data were analized using ANOVA and Duncan's multiple range tests at $p \leq 0.05$ by SAS programme.

Results and Discussion

The calli were regenerated from the ray florets culture on MS medium with 2 mg/l NAA and 4mg/l Kinetin after culture for 30 days. Regeneration efficiency in chrysanthemum depends on a large extent on the genotype and the

content of plant growth regurators in the medium (Bankarat *et al.*, 2010). We used the calli from ray florets as the explants in the EMS induce mutation. Ahloowalia (1992) could develop 20 new variants with althered leaf**,** flower shape, petal size and curvature. The callus plants that derived from the leaf did not exhibit the same level of deviation in flower color and shape as those the regenerated from florets (Khalid *et al.*, 1989). Ray florets in particular are useful source of explants for the regeneration of adventitious shoots or somatic embryos in breeding new caltivars of chrysanthemum (Teixeira da Silva *et al.*, 2015). To determine the optimal dose of EMS, the calli were immersed in 0, 0.5, 1.0, 1.5 and 2.0% EMS for 60 and 120 min. There was no calli recovered at 1.5% and 2.0% EMS after 30 days. Therefore subsequence mutation was carried out only with 0.5% and 1.0% EMS. The half lethal dose (LD_{50}) of EMS was found to be 1.22% EMS for 60 min and 0.72% EMS for 120 min (Fig 1). The survival percentage of the calli had decreased when the EMS concentration increased. The untreated with EMS callus produced a 100% survival while the survival percentage of callus at 2.0% EMS was the lowest (Table 1). The shoots were regenerated from callus in control 76.67% and the number of shoot/callus in control 1.73. Samala *et al.* (2014a) studied the effect of EMS for survival rate and stomata characteristics of *in vitro Chrysanthemum morifolium* report LD_{50} of EMS was 1.01% for 60 min in shoot treatment and 0.44% EMS for 90 min. Datta *et al.* (2005) reported that each plant has different LD_{50} depend on the explant, the age of explant and the concentration of mutagen.

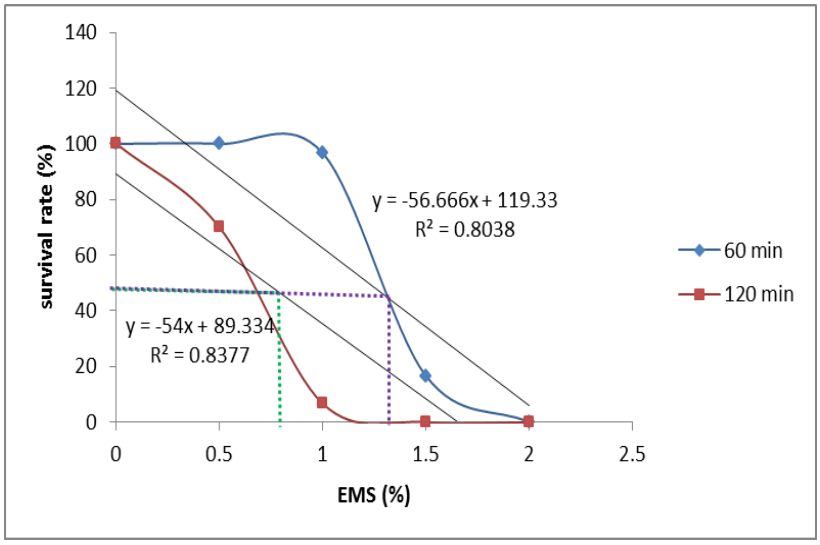

Figure 1 Effect of EMS on survival rate percentage in callus survival.

Table 1. The effect of EMS concentration and duration of soak on survival rate, shoot regeneration, number of shoot/callus and callus size on MS supplemented with 2 mg/l NAA and 4 mg/l Kinetin after cultured for 12 weeks

		Survival rate (%)	Shoot regeneration (%)	No. of shoot/callus	Callus width (cm)	Callus length (cm)
%EMS	0	100.00±0.00a	71.67±11.69a	1.70±0.16a	1.31±0.17a	1.31±0.17a
	0.5	85.00±17.60b	55.00±5.47b	1.35±0.12b	1.17±0.23a	1.17±0.23a
	1.0	51.66±49.56c	46.67±5.16c	1.10±0.10c	0.86±0.56b	0.86±0.56b
	1.5	8.33±9.83d	0.00±0.00d	0.00±0.00d	0.20±0.22c	0.20±0.22c
	2.0	0.00±0.00e	0.00±0.00d	0.00±0.00d	0.00±0.00d	0.00±0.00d
F-test		**	**	**	**	**
Duration (min)	60	62.66±46.36a	36.67±32.87a	0.84±0.74a	0.90±0.61a	0.90±0.61a
	120	35.33±43.40b	32.67±28.90a	0.81±0.72a	0.51±0.53b	0.51±0.53b
F-test		**	ns	ns	**	**
%EMS	Duration (min)					
0	60	100.00±0.00a	76.67±15.27a	1.73±0.20a	1.45±0.12a	1.51±0.13a
	120	100.00±0.00a	66.67±5.77ab	1.66±0.15a	1.17±0.07bc	1.23±0.10ab
0.5	60	100.00±0.00a	56.67±5.77bc	1.36±0.11b	1.34±0.03ab	1.39±0.03a
	120	70.00±10.00b	53.33±5.77cd	1.33±0.15b	1.01±0.23c	1.06±0.26b
1.0	60	96.67±5.77a	50.00±0.00cd	1.13±0.05c	1.32±0.12ab	1.41±0.09a
	120	6.67±5.77d	43.33±5.77d	1.06±0.15c	0.40±0.36d	0.40±0.36c
1.5	60	16.67±5.77c	0.00±0.00e	0.00±0.00d	0.40±0.08d	0.41±0.07c
	120	6.67±5.77d	0.00±0.00e	0.00±0.00d	0.00±0.00e	0.00±0.00d
2.0	60	6.67±5.77d	0.00±0.00e	0.00±0.00d	0.00±0.00e	0.00±0.00d
	120	6.67±5.77d	0.00±0.00e	0.00±0.00d	0.00±0.00e	0.00±0.00d
F-test		**	**	**	**	**
CV%		9.12	17.46	13.73	21.30	20.99

ns = non significant difference ** Significant different at $P \leq 0.01$; Means within column followed by the same later are not significant different as determind by Duncan's multiple range test

After 1 month the shoots were induced from callus, then we cut the internode 0.5 cm and subculture on MS medium by 2 times during 8 weeks. The treatment of 0.5% and 1% EMS in 60 and 120 min soaking had the higher shoots and more leaves than the one in control (Fig 2, Table 2). The same result has shown in research of Samala *et al.* (2014b) which gave the highest growth rate with bigger leaves and thicker roots. Later we transferred the plants with roots to the soil pots and culture them for 4 weeks. The result showed no difference in the height of stem, the number of leaves and the number of flower (Fig 4). They were showed no different in the survival rate, the height of the stem, the number of leaves and the number of flower after 7 weeks (Table 3). However, they were 3 different mutations characteristic from control which were found in this experiment. The first were obtained in 0.5% EMS for 60 min which resulted in white stacked ray florets with 2 layers, green disk florets (Fig 4B, 4F). The second mutation characteristic were found in 1% EMS for 60 min which the flower turned into yellow ray florets stacked with 2 layers, green disk florets and also the white and yellow ray florets (Fig 4C, 4G, 4H). The last mutation characteristic of 0.5% EMS for 120 min made the ray florets slender white stacked with 2 layers and green disk florets (Fig 4D, 4I, 4J).

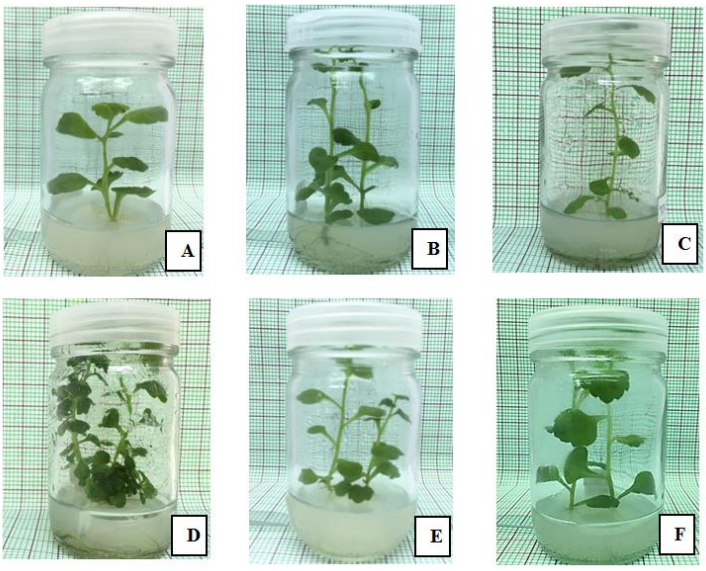

Figure 2. The chrysanthemum after treating with various concentration of EMS and cultured on MS medium for 4 weeks (A) control 0% EMS, 60 min (B) 0.5% EMS, 60 min (C) 1.0% EMS, 60 min (D) control 0% EMS, 120 min (E) 0.5% EMS, 120 min (F) 1% EMS, 120 min.

Table 2. The effect of EMS concentration and duration of soak on height of shoot and the number of leaves on MS medium for 8 weeks

		Height of shoot (cm)	No. of leaves
%EMS	0	2.80±0.05b	5.23±0.08b
	0.5	3.92±0.06a	7.03±0.45a
	1.0	3.92±0.05a	7.41±0.19a
F-test		**	**
Duration (min)	60	3.57±0.54a	6.58±1.00a
	120	3.53±0.57a	6.53±1.08a
F-test		ns	ns
%EMS	Duration (min)		
0	60	2.84±0.01b	5.26±0.05b
	120	2.77±0.06b	5.20±0.10b
0.5	60	3.94±0.08a	7.13±0.20a
	120	3.91±0.03a	6.93±0.66a
1.0	60	3.93±0.03a	7.36±0.15a
	120	3.91±0.08a	7.46±0.25a
F-test		**	**
CV%		1.64	4.76

ns = non significant difference ** Significant different at P ≤ 0.01; Means within column followed by the same later are not significant different as determind by Duncan's multiple range test

Figure 3. The chrysanthemum after transfer to soil for 4 weeks (A) control 0% EMS, 60 min (B) 0.5% EMS, 60 min (C) 1.0% EMS, 60 min (D) control 0% EMS, 120 min (E) 0.5% EMS, 120 min (F) 1.0% EMS, 120 min.

Table 3. The effect of EMS concentration and duration of soak on survival rate, the height of stem, the number of leaves and the number of flower after transferred to soil 7 weeks

		Survival rate (%)	Height of stem (cm)	No. of leaves	No. of flower
%EMS	0	97.61±21.55a	20.61±6.31a	17.26±4.67a	6.88±3.58a
	0.5	100.00±0.00a	21.56±3.12a	17.83±1.69a	7.40±2.54a
	1.0	97.61±0.00a	21.63±4.59a	17.78±3.51a	7.45±2.74a
F-test		ns	ns	ns	ns
Duration (min)	60	96.82±17.67a	21.43±6.45a	17.52±4.53a	7.42±3.09a
	120	100.00±0.00a	21.10±4.25a	17.73±2.38a	7.06±2.82a
F-test		ns	ns	ns	ns
%EMS	Duration (min)				
0	60	95.23±17.67a	20.01±7.96a	17.42±6.12a	6.95±3.98a
	120	100.00±0.00a	21.21±3.86a	17.09±2.16a	6.80±2.47a
0.5	60	100.00±0.00a	22.69±2.85a	17.95±1.78a	8.14±2.38a
	120	100.00±0.00a	20.43±2.90a	17.71±1.53a	6.66±2.50a
1.0	60	95.23±0.00a	21.61±5.65a	17.19±4.78a	7.19±2.22a
	120	100.00±0.00a	21.66±3.15a	18.38±1.46a	7.71±2.74a
F-test		ns	ns	ns	ns
CV%		12.80	26.33	20.74	41.18

ns = non significant difference
Means within column followed by the same later are not significant different as determind by Duncan's multiple range test

It come to the conclusion that using EMS has no effect to the size and number of leaves but it can change the colours of the flower and length of petal which also appeared to the other variety of chrysanthemum (Latado et al., 2004, Fang, 2011 and Van Harten, 1998).

Figure 4 The chrysanthemum after transferred to soil for 11 weeks (A,E) control 0% EMS (B,F) 0.5% EMS 60 min (C,G,H) 1% EMS 60 min (D,I,J) 0.5% EMS 120 min.

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