RESEARCH ARTICLE

Development of an efficient in vitro mutagenesis protocol for genetic improvement of saffron (*Crocus sativus* L.)

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Abstract Saffron (*Crocus sativus* L.) is a triploid ($2n = 3x = 24$), sterile geophyte which can only be propagated by means of underground vegetative corms. Since corm multiplication does not induce genome variations, therefore, the entire saffron population is expected to have a similar genetic makeup. Keeping in view the economic importance of the plant and the factors responsible for its low yield, the present investigation has been undertaken to establish an in vitro ethyl methanesulfonate (EMS) mutagenesis protocol followed by characterization of the induced variability in the advanced generations. The present report is limited to standardization of in vitro mutagenesis protocol only. Among the mutagenic treatments tested, concentrations ranging from 0.1 to 0.5% EMS showed a varied survival of explants. Based on various growth parameters, the LD_{50} was calculated to be 0.3% EMS for 3 h. Among the two types of explants analyzed, the corm explant gave better results for in vitro survival and the growth parameters than callus explant. An average of 57.33 and 92.00 daughter cormlets in all EMS treatments as compared to 47.67 and 57.67 daughter cormlets in control, obtained from callus and corm explants respectively, were transferred to the field. The maximum, average

daughter cormlet weight was obtained in control (3.01 g, corm explant) followed by 0.1% EMS (2.8 g, corm explant). In general, the growth parameters showed decreasing trend with an increase in EMS concentration in both the explants. The present study has been a significant achievement in the sense that the first mutagenesis protocol for *C. sativus* has been standardized.

Keywords Saffron · EMS · Mutagenesis · Callus · Shooting · Cormlet

Introduction

Saffron (*Crocus sativus* L.) as a spice is highly prized for its unique blend of flavor, colouring and aromatic properties of its dry stigmas (Winterhalter and Straubinger 2000; Fernández 2004). Saffron stigmas have been historically used as a spice, dye, perfume, as an ingredient in cosmetics, and for medicinal preparations (Basker and Negbi 1983). Saffron's colour, bitter taste, and aroma are associated with three different molecular features that are, crocin, picrocrocin, and safranal, respectively, and have a great scope in the modern pharmaceutical, food, and textile industries. In addition, saffron has a set of distinctive ecological and physiological properties like low water use capacity, very low harvest potential, growth and development during winter, and a generative phase which is marked by flowering and stigma harvest, followed by the vegetative phase. Different stages in the life cycle of this geophyte have been illustrated (Fig. 1). Saffron production is estimated at about 205 tons per year (Fernández 2004). A large number of flowers (100,000–200,000) are required to make up 1 kg of the spice (about 600,000 dried stigmas). Being labor intensive, farmers find cultivation of saffron

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Fig. 1 Stages in the life cycle of saffron: **a** flowering, **b** stigma harvest, **c** vegetative growth and daughter corm development, **d** senescence of leaves and **e** sprouting of daughter corm

less cost-effective than the rosaceous fruit cultivation in Kashmir. Therefore, there is an urgent need to increase the quality and production of saffron to cope up with growing demand and market specialization. This can be achieved by generating elite germplasm consisting of plants with modified characteristics like, more number of flowers per plant, larger stigmas or stigmas with an increased amount of secondary metabolites.

The origin of *C. sativus* is uncertain. According to one theory, its ancestor has been speculated to be a wild *Crocus*, in which an event of autotriploidy took place (Chichiriccò 1984; Grilli Caiola 2004, 2005). In another study, allopolyploidy through the hybridization of *C. cartwrightianus* and *C. hadriaticus* marks its evolution (Castillo et al. 2005). The identity of saffron's ancestors has not been univocally supported where, on one hand, Brighton (1977) nominated *C. cartwrightianus* and *C. thomasii* and on the other hand, some groups indicated *C. cartwrightianus* as the most probable ancestor (Mathew 1977; Brandizzi and Grilli Caiola 1998; Grilli Caiola 2004). AFLP analysis, however, confirmed that the quantitative and the qualitative traits of *C. cartwrightianus* and *C. thomasii* DNA are compatible with *C. sativus* (Zubor et al. 2004) but, according to Gismondi et al. (2013), different *C. sativus* genotypes might have evolved through independent events stemming from several geographical

pressures. Also, historical records and the archeological findings shedding light on the occurrence and spread of saffron and its allied species have been reviewed (Caiola and Canini 2010). Saffron being a triploid ($2n = 3x = 24$), is propagated vegetatively by means of corms. Since corm multiplication does not induce genome variations, therefore, the entire saffron crop is expected to have a similar genetic makeup. The autotriploid nature of *C. sativus* renders the prospects of crop improvement through traditional means, like hybridization, very unlikely (Basker and Negbi 1983). However, there have been efforts by different researchers in this direction using non-conventional breeding techniques such as biotechnological interventions and use of mutagens for induction of genetic variability in saffron (Dhar et al. 1988; Bagheri 1989; Nehvi 2003; Nehvi et al. 2010; Khan et al. 2011). Mutation breeding is an important tool in the improvement of vegetatively propagated crops; particularly in plants with reproductive sterility, where this is the only alternative (Broertjes and Van Harten 2013). The generation of chimera, after physical and/or chemical treatment in vegetatively propagated plants, is a major drawback of conventional mutagenesis. Advances in tissue culture techniques for a wide range of plants have facilitated the use of in vitro mutagenesis for the improvement of both seed and vegetatively propagated plants. In vegetatively propagated crops, in vitro

mutagenesis technique is an efficient method for crop improvement (Novak et al. 1990). Tissue culture makes it more efficient by allowing handling of large populations, enhancing mutation induction efficiency, and also by the possibility of increased mutant recovery. The combination of in vitro culture and mutagenesis is relatively inexpensive, simple and efficient, and several promising results have been achieved in different crop plants (Donini 1982; Donini and Sonnino 1998; Ahloowalia 1998). In vegetatively growing plants like saffron, in vitro mutagenesis of tissue explant, followed by clonal propagation, could be an ideal technique for enhancing genetic variation in the base population and fast multiplication of mutant clones. Using in vitro mutagenesis in saffron may perhaps increase the genetic variability for important characteristics, such as, content of carotenoid metabolites per stigma, variability in morphological and biochemical traits, etc. The micro-propagation of saffron is considered an excellent alternative for its multiplication (George et al. 1992; Ahuja et al. 1994). Keeping in view the economic importance of the plant and the factors responsible for its low yield and lack of interest for cultivation among growers, present investigation has been undertaken to establish an in vitro mutagenesis protocol and identify suitable mutagenic concentrations so as to induce maximum variability in the crop plant followed by characterization of the induced germplasm at morphological, biochemical and molecular levels in the advanced generations. The present report is limited to the standardization of an in vitro mutagenesis protocol only.

Materials and methods

Explant and media

For the collection of plant material, extensive field surveys were carried out in different areas of the Pampore belt in the Kashmir region (Jammu & Kashmir, India): Khrew, Chandhara, Wuyan, and the adjoining areas, during the vegetative phase of the crop. From each site, at least one hundred, healthy and disease free, corms of uniform weight (10–15 g) were collected and then sown in the prepared

beds (2 m²) in the Botanical Garden of the University of Kashmir. From each mother corm, three to four, healthy daughter corms (3–5 g) were excised and any residual parent tissue, if present at the base of the daughter corm, was removed. These daughter corms were then used as the corm explants for mutagenic treatments. Initial callus was obtained from the Tissue Culture Laboratory, Centre of Research for Development (CORD), University of Kashmir, which was then subjected to sub-culturing on CSM (Table 1) in order to generate sufficient amount of callus to be used as the callus explant for mutagenic treatments. Subsequently, fresh callus was also generated from spliced corm explants on the same CSM. All the cultures were kept under 8/16 h dark/light cycle and at 25 ± 2 °C temperature. In each experiment, 180 explants (15 in each treatment of callus and corm explant) were treated and inoculated in triplicates (three explants in each vial). Already available protocols (Quadri et al. 2008, 2010; Parray et al. 2015) were used for the culture conditions with little modifications (Table 1). All chemicals were purchased from HiMedia, unless otherwise indicated.

Mutagenesis

Three mutagenic time durations, 3, 6, and 9 h, were tested for ten EMS concentrations ranging from 0.1 to 1.0%. Also, three different methods of treatment were used: (a) Dip method—where the explants were immersed in different concentrations of EMS (b) Inoculation method—where the mutagen was mixed with the culture medium, followed by inoculation of explants (c) Injection method—where the mutagen concentrations were injected, in small volumes, with a sterile needle in and around the meristematic tissue of the explant. Excised daughter corms were thoroughly washed, before treatment, under running tap water followed by washing with 1% Labolene (v/v) to which 2–3 drops of Tween-20 were added. The corm explants were then treated with the mutagen in a non-sterilized environment and kept in a shaker, set at 80 rpm, for different time durations. After treatment the corms were surface sterilized in laminar with 0.1% mercuric chloride (HgCl₂) for 9–10 min, and inoculated on SIM (Table 1). The calli, however, were treated in a sterilized environment

Table 1 Composition of media used for in vitro daughter cormlet generation after mutagenic treatments

Designation	Composition					
	BAP μ M	NAA μ M	PBZ μ M	Sucrose g/l	Agar g/l	Medium
CSM/SIM	20	15	–	30	8	Basal MS
MSM	26.6	–	–	30	8	Basal MS
CIM	–	–	3.4	30	8	Basal MS

CSM callus induction and sub-culturing medium, SIM shoot induction medium, MSM multiple shooting medium, CIM daughter cormlet induction medium, BAP 6-Benzylaminopurine, NAA 1-Naphthaleneacetic acid, PBZ paclobutrazol, MS Murashige and Skoog 1962

Fig. 2 Response of callus explant to EMS treatment: **a** control, **b** 0.1%, **c** 0.2%, **d** 0.3%, **e** 0.4% and **f** 0.5% on CSM/SIM

Fig. 3 Shooting from EMS treated callus: **a** control, **b** 0.1%, **c** 0.2%, **d** 0.3%, **e** 0.4% and **f** 0.5% on CSM/SIM

Fig. 4 Shooting from EMS treated corm: **a** control, **b** 0.1%, **c** 0.2%, **d** 0.3%, **e** 0.4% and **f** 0.5% on CSM/SIM

Fig. 5 Multiple Shooting from EMS treated corm and callus explants: **a** control, **b** 0.1%, **c** 0.2%, **d** 0.3%, **e** 0.4% and **f** 0.5% on MSM

Fig. 6 Daughter cormlet induction with EMS treatment **a** control, **b** 0.1%, **c** 0.2%, **d** 0.3%, **e** 0.4% and **f** 0.5% on CIM

Table 2 Collective growth response of callus and corm explants to EMS mutagen for three exposure durations

Concentration of EMS (%)	Exposure duration during dip method		
	3 h	6 h	9 h
Control	+++++	++	+
0.1	++++	+	–
0.2	+++	–	–
0.3	++	–	–
0.4	+	–	–
0.5	+	–	–

in the laminar and transferred to the shaker (80 rpm) in sealed containers, for different durations of treatment. After mutagenic treatment, the explants (calli) were washed three times with autoclaved double distilled water and transferred to CSM.

Hardening and transplantation

The daughter cormlets once removed from the medium were washed with autoclaved double distilled water and dried on Whatman No. 1 filter paper in the culture room for a period of 1–2 weeks. They were then transplanted onto autoclaved sand-soil mixture (1:1) and kept in temperature and humidity controlled conditions for a period of 15–20 days. During this phase, the well-germinated

cormlets were given 2 h chilling (2–7 °C) treatment, twice a day during morning and evening hours. The stratification was done so that the tissue culture raised population was acclimatized to the winter cold conditions in Kashmir.

Data collection and analysis

Observations were recorded on various parameters like percent survival of explant, regeneration of callus, shoot induction from callus and corm explants, multiple shooting, shoot length, and induction and duration of daughter cormlet formation, etc. Data was recorded from the second day after each inoculation and sub-culture. The recorded parameters from culture conditions were subjected to analysis of mean, and standard deviation, using Microsoft Office Excel 2007. One Way Analysis of Variance (one way ANOVA) and Tukey's HSD post hoc test was performed using the SPSS 16.0 statistical software.

Results and discussion

Culture response

Various growth regulators added to the basal MS medium (Murashige and Skoog 1962) were effective in induction of different stages in vitro, eventually resulting in the

Fig. 7 Derivation of LD₅₀ on the basis of two important parameters—survival of explants and mean leaf lengths in EMS treated explants

harvesting of the daughter cormlets. Callus regeneration (Fig. 2), shoot induction from callus (Fig. 3) and corm (Fig. 4), multiple shooting (Fig. 5), and corm induction (Fig. 6) have been achieved on MS medium supplemented with different concentrations of auxin (NAA), cytokinin (BAP) and paclobutrazol, either alone or in combination (Table 1). Parray et al. (2012) observed maximum shooting of in vitro raised corms on MS medium supplemented with BAP (20 μ M) and NAA (15 μ M), although they did not report any callus induction on this medium. However, contrary to our results, where shoots were induced on MS media supplemented with both BAP (20 μ M) and NAA (15 μ M) and multiple shooting using only BAP (26.6 μ M) as a supplement to basal MS media, shoot induction has been reported on MS medium containing only BAP (26.64 μ M) and multiple shooting on MS medium supplemented with both BAP (6.64 μ M) and NAA (1 and 5 μ M) (Devi et al. 2011). Growth retardants have variously been used in culture conditions for the development of storage organs (Sharma et al. 1998; Simco 1993; Nagaraju et al. 2002; Devi et al. 2011) and in case of *C. sativus*, in vitro corm development, paclobutrazol was used, which led to the development of daughter cormlets. The culture response was dependent upon the phenology of the crop, i.e. the shoots/callus formation was delayed during the mitotically dormant months of the year. The findings are in accordance with the earlier reports that saffron's culture response is dependent upon the biological state of the parent plant (Milyaeva et al. 1995; Devi et al. 2011).

Ideal treatment method and optimum EMS dosage

Experiments were designed to study the effectiveness of the chemical mutagen (EMS) on survival and growth parameters of saffron in vitro. To our knowledge, in vitro

EMS mutagenesis in saffron has received no attention so far. Past scientific reports on mutation induction in saffron have been entirely *ex-vitro*, using physical and chemical mutagens (Nehvi et al. 2009; Khan et al. 2011; Salwee and Nehvi 2014). Among the treatment methods tested, the dip method was successful in terms of various growth responses following mutagenic treatment, whereas the inoculation (media enrichment) and direct injection method failed to produce any response. This finding is in line with the general method used for chemical mutagenesis in plant tissues. The failure of growth response in case of media enrichment method is because of sustained exposure of the explant tissue to the mutagen added in the culture medium, whereas, in case of the injection method, it may be due to cell death near and around the shoot tip following infusion of mutagen. In both these treatment methods, the mutagen remained permanently in/around the meristematic tissue, causing significant DNA damage which could not be repaired by the cellular machinery and eventually resulted in cell death, and the lack of any culture response. When different exposure durations were compared, 3 h treatment showed maximum explant survival when compared to 6 and 9 h duration (Table 2). Analysis of the percent survival, number of shoots and leaves showed that EMS-induced mutagenesis imposed a significant impact on in vitro growth and regeneration percentages. From a range of EMS concentrations used in the present study, 0.1–0.5% with 3 h treatment duration produced a dose-dependent growth response whereas, 0.6% and above proved to be inhibitory. The inhibitory effect of higher concentrations of mutagen on various growth parameters has been reported earlier in several crop plants (Pawar et al. 2010; Talebi et al. 2012; Perera et al. 2015; Monika et al. 2016). Depending upon the explant survival and various growth parameters, 0.3% was calculated to be LD₅₀ (Fig. 7). It is

Table 3 Percent responses of callus and corm explants during different stages of induction after EMS treatments

% Response	Callus explant						Corm explant					
	Concentration of EMS (%)											
	Control	0.1	0.2	0.3	0.4	0.5	Control	0.1	0.2	0.3	0.4	0.5
Callus regeneration	90.00 ± 1.4 ^e	73.33 ± 3.6 ^{bc}	66.66 ± 9.5 ^b	53.33 ± 7.3 ^{ab}	43.33 ± 7.3 ^a	33.33 ± 9.0 ^a	–	–	–	–	–	–
Shooting	92.59 ± 7.1 ^{ef}	81.81 ± 4.3 ^{cdef}	70.00 ± 10.7 ^{bcd}	68.75 ± 12.1 ^{bcd}	61.53 ± 6.1 ^{abcd}	60.00 ± 10.4 ^{abc}	100.00 ± 0.0 ^f	90.00 ± 6.9 ^{def}	76.66 ± 7.0 ^{cdef}	56.66 ± 11.1 ^{abc}	43.33 ± 19.1 ^{ab}	36.66 ± 10.4 ^a
Multiple shooting	100.00 ± 0.0 ^b	92.15 ± 6.9 ^{ab}	82.14 ± 12.0 ^{ab}	75.00 ± 10.4 ^{ab}	66.66 ± 10.4 ^a	62.25 ± 14.6 ^a	93.33 ± 6.1 ^{ab}	88.88 ± 9.5 ^{ab}	78.26 ± 11.3 ^{ab}	75.00 ± 17.0 ^{ab}	71.45 ± 15.0 ^{ab}	63.63 ± 15.0 ^a
Daughter cormlet induction	74.28 ± 10.7 ^{bcd}	68.08 ± 15.8 ^{abcde}	56.52 ± 20.5 ^{abc}	46.66 ± 11.0 ^a	50.00 ± 19.5 ^{ab}	40.00 ± 4.27 ^a	100.00 ± 0.0 ^e	90.00 ± 8.8 ^{cde}	85.71 ^{bcd}	73.33 ± 5.6 ^{abcde}	96.23 ± 2.5 ^{de}	60.00 ± 17.8 ^{abcd}

Means followed by the same superscripts are not significantly different at $p \leq 0.05$

Table 4 Mean number of days taken for various growth induction stages after EMS treatment of callus and corm explants

Response in days	Callus explant		Corm explant									
	Concentration of EMS (%)											
	Control	0.1	0.2	0.3	0.4	0.5	Control	0.1	0.2	0.3	0.4	0.5
Callus regeneration	29.80 ± 6.3 ^a	32.90 ± 3.8 ^a	44.30 ± 3.6 ^b	53.60 ± 3.6 ^c	71.30 ± 3.6 ^d	82.80 ± 3.7 ^e	–	–	–	–	–	–
Shoot induction	39.80 ± 6.7 ^a	42.70 ± 3.8 ^{ab}	52.30 ± 6.6 ^{cd}	56.70 ± 6.1 ^{cde}	59.90 ± 6.6 ^{def}	67.60 ± 4.6 ^f	38.10 ± 4.0 ^a	41.70 ± 6.7 ^a	51.00 ± 6.8 ^{bc}	53.40 ± 4.3 ^{cd}	58.50 ± 7.1 ^{cde}	62.80 ± 6.4 ^{ef}
Multiple shoot induction	21.60 ± 3.7 ^a	24.40 ± 3.0 ^{abc}	26.60 ± 5.3 ^{abc}	30.20 ± 5.0 ^{cd}	38.50 ± 5.5 ^e	48.30 ± 4.8 ^f	20.40 ± 3.0 ^a	23.30 ± 2.6 ^{ab}	25.70 ± 5.1 ^{abc}	28.60 ± 5.5 ^{bc}	36.50 ± 4.1 ^{de}	45.70 ± 4.5 ^f
Daughter cormlet induction	28.60 ± 5.0 ^{ab}	34.70 ± 3.4 ^{bc}	38.50 ± 5.0 ^{cde}	43.60 ± 3.5 ^{ef}	50.30 ± 3.2 ^g	60.80 ± 5.4 ^h	26.40 ± 5.0 ^a	33.80 ± 3.1 ^{bc}	36.00 ± 3.0 ^{cd}	42.10 ± 3.1 ^{def}	48.10 ± 4.0 ^g	57.70 ± 5.0 ^h
Daughter corm harvesting	21.20 ± 3.7 ^a	23.40 ± 3.3 ^{ab}	26.90 ± 6.7 ^{abcd}	31.20 ± 5.3 ^{cd}	33.50 ± 4.3 ^{def}	40.40 ± 4.8 ^f	20.20 ± 3.1 ^a	22.50 ± 2.5 ^{ab}	24.90 ± 3.2 ^{abc}	29.10 ± 5.1 ^{bcd}	32.00 ± 3.3 ^{cde}	38.70 ± 5.6 ^{ef}
Total time (months)	4.70	5.27	6.29	7.18	8.45	10.00	3.50	4.04	4.59	5.11	5.84	6.83

Means followed by the same superscripts are not significantly different at $p \leq 0.05$

Table 5 Mean shoot, and leaf number per shoot regenerated in different treatment concentrations from callus and corm explant

Concentration of EMS (%)	Number of shoots (callus-derived)	Number of shoots (corm-derived)	Mean shoot length (cm) (callus-derived)	Mean shoot length (cm) (corm-derived)	Mean number of leaves per shoot (callus-derived)	Mean number of leaves per shoot (corm-derived)
Control	2.92 ± 1.4 ^c	2.17 ± 1.0 ^{abc}	12.32 ± 0.8 ^e	19.90 ± 0.5 ^g	7.00 ± 0.7 ^f	8.40 ± 0.5 ^g
0.1	2.39 ± 1.0 ^{bc}	1.78 ± 0.8 ^{abc}	11.08 ± 0.7 ^e	17.88 ± 1.0 ^f	5.00 ± 1.0 ^{de}	7.60 ± 0.5 ^{fg}
0.2	2.07 ± 0.8 ^{abc}	1.65 ± 0.7 ^{ab}	9.02 ± 0.3 ^d	16.34 ± 1.0 ^f	4.60 ± 0.5 ^{cde}	7.00 ± 0.7 ^f
0.3	1.82 ± 0.7 ^{abc}	1.31 ± 0.6 ^{ab}	6.88 ± 0.4 ^{bc}	11.52 ± 0.9 ^e	3.60 ± 0.5 ^{bc}	5.60 ± 0.5 ^e
0.4	1.50 ± 0.5 ^{ab}	1.14 ± 0.3 ^a	5.34 ± 4.7 ^{ab}	8.84 ± 0.9 ^d	2.80 ± 0.8 ^{ab}	4.80 ± 0.4 ^{cde}
0.5	1.33 ± 0.5 ^{ab}	1.20 ± 0.4 ^a	4.24 ± 1.0 ^a	7.56 ± 1.3 ^{cd}	1.60 ± 0.5 ^a	3.80 ± 0.4 ^{bcd}

Means followed by the same superscripts are not significantly different at $p \leq 0.05$

interesting to note that the callus and corm explants showed a similar trend of percent responses towards concentration and duration of EMS which made both the explants settle with the same LD₅₀.

Effect of EMS on growth parameters

Following EMS mutagenesis, the mean number of days taken for callus regeneration, shoot induction, multiple shooting, and daughter corm induction increased with the increasing concentration, whereas, the percent culture response decreased with respect to control. EMS exposure causes a negative effect on the plant development (Koch et al. 2012; Silué et al. 2013) and a higher mutation frequency has been related to higher cell death (Sung 1976). In general, the callus explant showed lower response percentages in all the parameters studied, except in the percentage of multiple shooting, when compared to the corm explant (Table 3). In case of the callus explant, 0.1% EMS had 73.33% regeneration, whereas, 0.5% EMS had 33.33% regeneration. It was followed by 81.81% shooting with 0.1% EMS which was reduced to 60% with 0.5% EMS. Multiple shooting percentages remained between 92.15% with 0.1% EMS and 62.25% with 0.5% EMS. Finally, the daughter cormlet initiation percentage remained 68.08% with 0.1% EMS and 40% with 0.5% EMS. In comparison, the corm explant had direct shooting and did not involve the callus phase. Shooting percentages remained as high as 90% with 0.1% EMS and 36.66% with 0.5% EMS. This was followed by 88.88% multiple shooting with 0.1% EMS and 63.63% with 0.5% EMS. Finally, the daughter cormlets were induced at 100% with 0.1% EMS and 60% with 0.5% EMS. Another important parameter, showing the effect of EMS on explants, is the number of days taken for culture initiation (Table 4). In case of callus explant, the callus regeneration started after about 32.90 days with 0.1% EMS which increased to as high as 82.80 days with 0.5% EMS. It was followed by shoot induction in the regenerated callus

in 42.70 and 67.60 days with 0.1 and 0.2% EMS respectively. The multiple shooting, however, was fast; 24.40 days with 0.1% EMS and 48.30 days with 0.5% EMS. Finally, the daughter cormlets were initiated in 34.70 and 60.80 days with 0.1 and 0.5% EMS, respectively. The corm explant, however, had lower effect of the mutagen and therefore, took fewer days, 41.70 and 62.80 with 0.1 and 0.5% EMS, respectively, to develop direct shoots. Multiple shooting was also fast; 23.30 days with 0.1% EMS and 45.70 days with 0.5% EMS. The daughter cormlets were induced in 33.80 and 57.70 days with 0.1 and 0.5% EMS, respectively.

The effect of the mutagen can also be seen on the growth parameters like mean number and length of shoots, and mean number of leaves (Table 5). Mean number of shoots, per explant, was higher in callus explant because it consisted more number of meristematic regions, but the potential of shoot formation in control callus explant was also reduced due to a long exposure (3 h) to water (set as control) (data not shown). In contrast, the corm explant had few buds and thus produced a lower number of shoots in all concentrations of EMS; 2.39 and 1.78 mean number of shoots, with 0.1% EMS from callus and corm explants, respectively, whereas, 0.5% had produced 1.33 shoots per callus explant and 1.20 shoots per corm explant. The post hoc test has shown the values to be significantly different from each other. The maximum shoot length was seen for shoots regenerated on control corm explants where control had longest shoots (19.90 cm), followed by 0.1% EMS (17.88 cm) and 0.2% EMS (16.34 cm); the shoots derived from the callus explant followed next with control having 12.32 cm, and 0.1% EMS having 11.08 cm, long shoots. The post hoc test, however, did not show any significant difference in these values. The corm-derived shoots had significantly more number of mean leaves than callus-derived shoots. 0.1% EMS had 7.6 and 5.0 mean leaves/shoot, and 0.5% had 3.80 and 1.60 mean leaves/shoot, in corm and callus-derived shoots, respectively.

Table 6 Mean daughter cormlet weight and number of daughter cormlets generated from treatment with different concentrations of EMS

Concentration of EMS (%)	Daughter cormlet number (corm-derived)	Daughter cormlet wt. (g) (callus-derived)	Daughter cormlet wt. (g) (corm-derived)	Daughter cormlets survived during hardening (callus-derived)	Daughter cormlets survived during hardening (corm-derived)	% survival after hardening (callus-derived)	% survival after hardening (corm-derived)
Control	47.67 ± 5.9 ^{de}	2.40 ± 0.3 ^{cde}	3.01 ± 0.5 ^e	42.33 ± 5.0 ^{de}	51.00 ± 2.6 ^f	89.52 ± 12.6 ^b	88.73 ± 7.0 ^b
0.1	25.67 ± 6.0 ^b	2.21 ± 0.4 ^{cd}	2.80 ± 0.5 ^{de}	20.33 ± 9.5 ^{bc}	32.00 ± 5.6 ^{cd}	76.25 ± 19.6 ^b	83.21 ± 5.3 ^b
0.2	14.33 ± 3.2 ^a	2.09 ± 0.5 ^{cd}	2.41 ± 0.5 ^{cde}	11.00 ± 3.6 ^{ab}	24.67 ± 4.5 ^c	75.64 ± 8.4 ^b	87.70 ± 5.3 ^b
0.3	8.00 ± 2.0 ^a	1.79 ± 0.6 ^{bc}	2.24 ± 0.4 ^{cde}	3.33 ± 1.5 ^a	7.33 ± 3.0 ^a	41.67 ± 14.4 ^{ab}	68.69 ± 3.5 ^{ab}
0.4	6.00 ± 2.0 ^a	1.20 ± 0.4 ^{ab}	2.08 ± 0.4 ^{cd}	1.33 ± 0.6 ^a	8.67 ± 2.3 ^{ab}	22.22 ± 4.8 ^a	84.81 ± 12.5 ^b
0.5	3.33 ± 1.5 ^a	0.80 ± 0.3 ^a	1.85 ± 0.3 ^{bc}	0.67 ± 0.6 ^a	2.33 ± 1.2 ^a	17.78 ± 16.8 ^a	58.89 ± 7.5 ^{ab}

Means followed by the same superscripts are not significantly different at $p \leq 0.05$

Total number and percent survival of daughter cormlets, and mean weight, in each treatment, is given in Table 6. A mean number of 57.33 (callus derived) and 92.00 (corm derived) daughter cormlets from all EMS treatments, and 105.34 daughter cormlets from control, were transferred to the field (Fig. 8). The daughter cormlet survival percentage shows a declining trend with the decrease in the daughter cormlet weight, i.e., smaller the corms, lower is the chance of survival during hardening. Daughter cormlets having an average wt. of 0.8 g (0.5% EMS; callus explant-derived) had 17.78% survival, whereas, those with average wt. of 2.8 g (0.1% EMS; corm explant-derived) had 76.25% survival. Also, lower the percentage of EMS, more number of larger daughter cormlets were produced, e.g., in control 47.67 (callus-derived) and 57.67 (corm-derived), in 0.1% EMS 25.67 (callus-derived) and 38.33 (corm-derived), and in 0.5% EMS, on an average 3.33 (callus-derived) and 4.67 (corm-derived) daughter cormlets were produced. The mean shoot length, mean leaf number/shoot, daughter cormlet weight and survival, show high interdependencies (Fig. 9) which can be manipulated with appropriate in vitro protocols for large scale recovery of corms. The increase in daughter corm weight (corm explant) is probably due to the fact that longer shoots with more number of leaves were produced from the corm explant, which in turn resulted in larger daughter corms. On the other hand, since whole callus being meristematic, produced more number of shoots but these were comparatively smaller and thinner with lower number of leaves. A lower number of daughter cormlets was produced from such shoots, and were also smaller in size. In general, the corm explant proved to be better compared to callus explant on the basis of all growth parameters leading to daughter cormlet production and can thus be exploited for mutant population generation in saffron through EMS mutagenesis. According to Plessner et al. (1990), there is a negative nonlinear relationship between the daughter cormlet weight and leaf length, but the leaf and daughter cormlet dimensions were measured together across different temperature ranges. Also, temperatures greater than 20 °C favored corm development at the expense of leaf growth: whereas below 20 °C favored only leaf elongation on the same medium. In case of in vitro regeneration, the temperature remained constant and separate media were employed for maximum leaf elongation and corm development. The tissue was transferred to the next medium only when the first phase was successfully completed.

Conclusion

The recovery of daughter cormlets following mutagenic treatment of callus and corm explants in saffron has been achieved successfully. Although the number of cormlets

Fig. 8 **a** Hardening, and **b** transplantation, of in vitro raised daughter cormlets

Fig. 9 Correlation between mean shoot lengths, mean daughter corm number and weight, and number of survived daughter cormlets after transplantation (combined data for callus and corm explants on log₁₀ scale)

produced in vitro may not be sufficiently a high number, however, the present experiment is a continuous process till we get a sizeable number of corms for recovery of economically important mutants in saffron. Moreover, to further validate the EMS mutagenesis protocol developed so far in this study, a complete screening of the in vitro raised corms has to be done in the field particularly taking into consideration the floral attributes. Since a 2–3 g corm takes on an average 2–3 years to achieve a proper size of 7–8 g for flowering, we will be expecting first flowering in the coming season for measuring the amount of induced variability in saffron.

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