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Tissue culture as an alternative for commercial corm production in saffron: A heritage crop of Kashmir

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The present study aimed at developing a commercially viable protocol for *in vitro* corm production in saffron. Three-step sterilization process involving fungicides and sterilants ensured 94% clean viable cultures. Plant growth regulator (PGRs) ensuring initial bud sprouting, direct shoot regeneration from the base of the sprouted bud and cormlet production from multiple shoots have been standardized. MS Media supplemented with 0.5 mg/l naphthalene acetic acid (NAA) and 1.5 mg/l 6-benzyl amino purine (BAP) ensured maximum bud sprouting in September with direct multiple shoot primordia initiation on 6.5 mg/l BAP in November. 6.5 mg/l BAP + 0.2 mg/l NAA resulted in maximum shoot proliferation (24); however, at higher concentration, the PGRs were detrimental in arresting the growth. Viable shoot clumps established maximum *in vitro* corms in April after sub culturing on growth retardant (CCC) at 0.25% supplemented with 9% sucrose. Subculturing of non flowering *in vitro* corms on growth retardant with sucrose eliminated season dependence of *in vitro* protocols in the 2nd cycle of protocol. Primary and secondary hardening before field transfer ensured 100% corm viability.

Key words: Kashmir, corm, *in vitro*, saffron.

INTRODUCTION

The domesticated saffron (*Crocus sativus* L.), a mutant of *Crocus cartwrightianus* (Deo, 2003) is a triploid sterile geophyte (2n = 3X = 24) belonging to the family Iridaceae (Plessner et al., 1990) and is the most fascinating and intriguing species valued for its colour, taste and aroma (Fernandez, 2004). Dried dark red stigmas of saffron flowers have not only been used as a spice for flavoring and coloring food and as a perfume but also for treating several diseases as it contains more than 150 volatile and aroma yielding compounds and many nonvolatile active components, many of which are carotenoids, including zeaxanthin, lycopene, and various alfa and beta carotenes. However, saffron predominantly contains chemical constituents such as crocin, picrocrocin and saffranal which are responsible for its color, flavor and aroma, respectively. Crocetin glycosyl esters are responsible for its characteristic color and compounds are found in extremely important proportion in stigmas (Sampathu et al., 1984). Saffron also contains flavonoids and one of the general characteristic defining this extensive group of compounds is bitterness. The characteristic bitter taste of saffron has been postulated due to the presence of a glycoside named picrocrocin which is a precursor of safranal, the major compound in saffron aroma.

Saffron is propagated solely by vegetative way using the annual renewal corms. Only four to five corms per mother corm are produced in one growing season through conventional methods. Hence, low multiplication rates and fungal infestation of corms are the bottlenecks for availability of sufficient quality planting material (Kiran et al., 2011). Kashmir India contributes 4.18% (11 M.T) to the global saffron production of 264 M.T. However, National Saffron Mission presently being

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executed by Ministry of Agriculture Government of India for revival of saffron in Kashmir India targets its production to about 79 M.T by 2020 by increasing the area from the present 3785 ha to about 12407 ha besides enhancing productivity from existing 2.50 to 6.37 kg/ha. Tissue culture is a useful method for large scale production of disease free plants using different medium with different ratios of auxin and cytokinin (Ding et al., 1981; Chrungoo et al., 1987; Ilahi et al., 1987; Plessner et al., 1990; Himeno and Sano, 1987; Fakhrai and Evans, 1990; Chen et al., 2003; Majourhay et al., 2007; Jun et al., 2007; Darvishi et al., 2007; Sheibani et al., 2007). However, smaller size of the tissue culture corms with least survival under actual field conditions is the limitation factor with the available tissue culture saffron protocols.

To bridge the gap of 62,035 M.T of saffron corms required for horizontal expansion in Jammu and Kashmir State of India, this present experiment was designed to investigate standardization of commercial protocol for *in vitro* corm production in Saffron with large proportion of bigger size corms weighing above 7 g.

MATERIALS AND METHODS

Materials for the present study comprised of healthy disease free saffron corms obtained from saffron corm nursery maintained at Dry land Agricultural Research Station, SKUAST-Kashmir. Minicorms and corm sections with apical, subapical and auxillary meristematic regions were used as explants. Explants were sterilized using three step sterilization process involving: i) surface sterilization of corms by first scrubbing the corms gently under running tap water for 10 min to remove coating layer of microorganisms ubiquitously found on them, ii) Fungicide dip with Tween-20 for 10 min (carbendizime at 0.01% and Mancozeb at 0.1%), and iii) sterilants dip using sodium hypochlorite solution (0, 15, 20 and 25%) for 10 min, and rinsing thrice with sterile water followed by a dip in mercuric chloride solution (0.1, 0.4, 0.8 and 1.2%) for 5 min. These steps were carried out under the laminar airflow cabinet. Ex plant segments were cultured on Murashige and Skoog (MS) (1962) media supplemented with different growth regulators (depending upon the growth stage); 3% sucrose, 0.8% agar, autoclaved at 121°C under 15 psi pressure for 20 min. 6-benzyl amino purine (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combinations with naphthalene acetic acid (0.25, 0.50, 0.75, 1.0 and 1.25 mg/l) was used for bud sprouting. Inoculated cultures were maintained through periodic sub culturing onto same media composition.

Sprouted cultures were transferred to MS media supplemented with BAP (4.5, 5.5, 6.5, 7.5 and 8.5 mg/l). The cultures with 2 to 3 clumps of shoots with swollen base were incubated at 17 ± 2°C and were given 16/8 h (light/dark) photoperiodic treatment and subsequently transferred to MS supplemented with BAP (4.5, 5.5, 6.5, 7.5, 8.5 mg/l) in combination with NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg/l). Multiple shoot primordia were sliced and subsequently sub cultured on MS media containing growth retardants chlorocholine chloride (0.125, 0.25, 0.375, 0.5, 0.625%) in combination with 3, 6, 9, 12 and 15% sucrose. For attaining a specific size, microcorms were transferred to MS media containing 3, 6, 9, 12 and 15% sucrose and 0.8% agar. *In vitro*-produced mini corms with average mean weight above 7 g were transferred to coco peat supplemented with vermicompost as a primary hardening medium followed by secondary hardening in growth medium containing sand, vermicompost, FYM and soil in the ratio of 2:3:3:2 and then field transferred in August. Under primary hardening, the cultures were maintained under white fluorescent light of 42 $\mu mol/m^2s^1$ with the photoperiod of 16 h light and 8 h dark at 24 ± 1°C. Mini corms weighing less than desired size were further cultured on MS medium supplemented with CCC (0.25%) and sucrose (9%) as a source of explants with further sub culturing on inoculation medium.

Statistical analysis of data was carried out using 'randomized factorial design'. Interpretation of result was carried out on the basis of critical difference at 0.01 between means.

RESULTS AND DISCUSSION

Surface sterilization

The use of saffron corms as a direct source of ex plant material for the production of 'clean' *in vitro* plantlets presented a major challenge of contamination to the level of 95% (Table 1). Similar results of contamination (100%) with field grown crops especially those with leaf canopy close to the ground have also been reported by Mitchell (2003). To provide clean culture, the three-step sterilization process was undertaken to ensure sterilization against surface and endogenic contamination. Combination of systemic fungicides namely: carbendizime (0.01%) and mancozeb (0.1%) could increase the percentage of clean cultures to 12.5%. Bactericidal action of hypochlorite solutions (bleach) due to both hypochlorous acid (HOCl) and the OCl- ion in presence of systemic fungicides and Tween 20 was significantly more effective (7%) as compared to mercuric chloride in reducing the level of contamination. Combination of systematic fungicides with hypochlorite bleach at 50% for 10 min and mercuric chloride dip at 1.6% for 5 min ensured 94% clean viable cultures. Sterilants (sodium hypochlorite and mercuric chloride) when used at higher concentration (>50 and >2.0%) were phytotoxic resulting in the dehydration and yellowing of the explants. Minimum percentage of clean viable culture (9%) on account of phototoxic effect of sterilants was achieved with 70% NaOCl in combination with 2.4% $HgCl_2$. Most of the times, it appeared that sterilization was achieved, but the endogenic contaminants that were latent in the developing explants reappeared after 3 to 5 weeks of the experiments. The results are in line with the finding of Plessner et al. (1990), Taylor et al. (1998), Ozel et al. (2006) and Karaoglu et al. (2007).

About 46% of saffron soils of kashmir are infested with corm rot pathogens like *Phtophthora* sp, *Fusarium oxysporium* f. sp. *gladioli*, *F. solani*, *F. monliforme* Var *intermedium*, *Rhizoctonia solani*, *Pencillium purpurogenum* and *P. corymbeferum*; and thus, is an emerging threat for availability of disease free materials to be used as a direct source of ex plant material (Mir et al., 2012). Three step sterilization procedure has thus helped in meeting out the challenge of contamination on account of fungal pathogens which is the first step for a successful *in vitro* protocol.

Table 1. Impact of Sterilants on per cent *in vitro* clean cultures.

NaOCl (%)	$HgCl_2$ (%)								Mean
	0	0.1	0.4	0.8	1.2	1.6	2.0	2.4	
0 (without fungicide dip) control	4.6	20.50	39.75	44.5	43.0	42.0	37.0	12.50	30.48
0 (with fungicide dip)	12.50	68.50	75.0	76.50	78.50	84.50	73.50	39.50	63.56
10 (with fungicide dip)	69.50	70.0	72.50	74.50	74.50	79.50	78.50	34.50	69.18
20 (with fungicide dip)	72.50	72.50	77.0	76.50	79.50	85.50	67.50	46.0	72.12
30 (with fungicide dip)	71.0	72.0	74.50	79.0	80.0	86.0	56.50	34.50	69.18
40 (with fungicide dip)	69.50	74.0	79.0	81.50	89.0	88.0	52.50	29.50	70.37
50 (with fungicide dip)	75.0	77.50	83.50	87.50	89.50	94.0	59.0	30.50	74.56
60 (with fungicide dip)	66.50	61.0	47.0	41.0	34.0	32.50	22.50	17.50	40.25
70 (with fungicide dip)	69.50	57.00	45.00	30.00	21.00	15.00	12.50	9.00	32.37
Mean	56.73	63.66	65.91	65.66	65.44	67.44	51.05	28.16	

	SE (m)	SE (d)	CD ($p < 0.01$)
$HgCl_2$	0.38	0.54	1.40
NaOCl	0.38	0.54	1.40
$HgCl_2 \times$ NaOCl	1.08	1.54	3.96

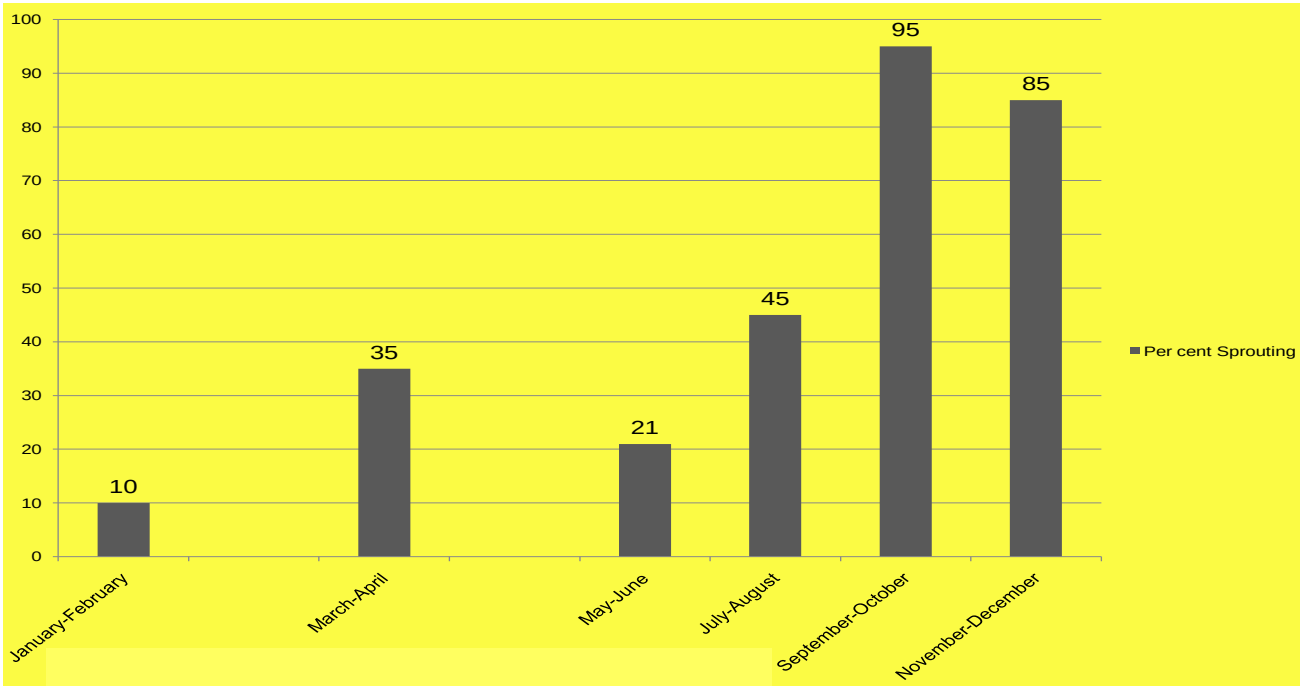

Figure 1. Initial bud sprouting scored over months.

Bud sprouting and shooting

Inoculation of explants on MS media supplemented with different growth regulators over 4 months (May to September) revealed that bud sprouting was season dependent with maximum sprouting (95%) from minicorms/vertical corm sections with apical and auxillary meristematic region in September to October followed by November to December (85%). Minimum sprouts (10%) were observed in January to February. Those buds which sprouted during the months of January to June regained further growth only in the next growing phase, that is, from September to November (Figure 1). Similar report of season dependence of saffron has also been reported by Kiran et al. (2011). Of all the factorial combinations of PGRs used, bud sprouting from corm segments was

Table 2. Effect of different growth regulators on sprout number.

BAP (mg/L)	NAA (mg/L)						Mean
	0.0	**0.25**	**0.50**	**0.75**	**1.0**	**1.25**	
0.0	0.000	0.165	0.225	0.175	0.165	0.15	0.14
0.5	0.715	0.710	1.950	1.650	1.325	0.925	1.21
1.0	1.275	1.425	2.05	1.775	1.825	1.775	1.68
1.5	1.725	1.825	2.475	2.225	2.05	1.575	**1.97**
2.0	1.975	2.025	2.05	1.925	0.675	0.575	1.53
2.5	1.875	1.875	1.8	0.675	0.3	0.275	1.13
	1.26	1.33	1.75	1.40	1.05	0.87	
	SE (m)	**SE (d)**	**CD (p < 0.01)**				
BAA	0.01	0.02	0.05				
NAA	0.01	0.02	0.05				
BAA × NAA	0.03	0.04	0.10				

Table 3. Effect of BAP on *in vitro* shooting.

BAP (mg/L)	Mean number of shoots	Mean shoot length (cm)
0	0.0	0.0
4.5	1.95	6.3
5.5	2.62	7.25
6.5	3.40	9.25
7.5	3.25	8.85
8.5	2.25	6.85
Mean	2.69	7.70
SE (m)	0.17	0.57
SE (d)	0.24	0.81
CD (p < 0.05)	0.41	2.09
CD (p < 0.01)	0.97	3.26

initiated at 15 + 1°C with 16/8h (light/dark) photoperiodic treatment. Different concentrations of PGRs revealed significant differences for sprout number ranging from 0.14 to 1.97 in BAP and 0.87 to 1.75 in NAA. Interaction effects were also observed to be significant. Maximum mean number of sprouts (2.475) were observed in the medium containing 0.5 mg/l NAA and 1.5 mg/l BAP showing an increase of 41.42 and 25.63% over individual best effects of NAA and BAP [Table 2, Plate 1(Stage 2)]. Kiran et al. (2011) reported importance of BAP in combination with 2 to 4 D for bud sprouting at 10°C. Sprouts developed into healthy shoots with three to seven leaves after 16 to 17 weeks of inoculation (November) on MS media supplemented with different concentrations of BAP (4.5, 5.5, 6.5, 7.5 and 8.5 mg/L).

Activated cultures that were repeatedly sub cultured after every 3 weeks on the same media supplemented with 3% sucrose and 0.8% agar revealed maximum shoot number (3.40) and shoot length (9.25 cm) with BAP at 6.5 mg/L [Table 3, Figure 2 (Stage 3)].

Multiple shooting

Sub culturing of shoot clumps after attaining maximum shoot length on different concentrations of cytokinin (BAP) and auxin (NAA) in November at 17 ± 2°C with 16/8h (light/dark) photoperiodic treatment revealed direct emergence of multiple shoot primordia from the base of an activated buds ranging from 1.25 to 17.91 in BAP and 7.41 to 13.50 in NAA. However, the interaction effects (BAPXNAA) were significantly more profound resulting in maximum number of shoot primordia to the extent of 24 with BAP at 6.5 mg/L in combination with NAA at 0.2 mg/L. Maximum mean number of viable shoot primordia clumps with swollen base having ability to produce corms was reduced to 56.1% in NAA (13.5 to 7.58) and 52.5% (17.91 to 9.41) in BAP. However, among interaction effects, the level of reduction was 46% (24 to 11) achieved in medium containing BAP (6.5 mg/L) and NAA(0.2 mg/L) obtained after 24 to 25 weeks of inoculation [Table 4 and Figure 2 (Stage 4)]. The multi-

Figure 2. Stages of *in vitro* corm production.

Table 4. Effect of PGRs on mean number of shoot clumps.

BAA (mg/L)	NAA (mg/L)						Mean
	0.0	0.1	0.2	0.3	0.4	0.5	
0.0	0.0 (0)	1.5 (1)	2.5 (1)	2.0 (1)	1.0 (1)	0.5 (1.5)	1.25 (0.91)
4.5	8.5 (5.5)	11.5 (6.5)	15 (10.5)	19 (10.0)	14 (9.5)	8.5 (6.5)	12.75 (8.08)
5.5	15 (8.5)	21 (10.5)	21.0 (10.5)	17.5 (10.0)	17.5 (9.0)	15.5 (8.0)	17.91 (9.41)
6.5	15.5 (8.5)	20.0 (10)	24.0 (11)	21.5 (9)	15 (8)	11 (7)	17.83 (8.91)
7.5	8 (3.5)	16 (6.5)	13.5 (9.5)	11 (9.0)	9.0 (6.5)	6 (2.5)	10.58 (6.25)
8.5	5.5 (3.5)	8.5 (4.5)	5.0 (3)	4.5 (3)	4.5 (1.5)	3.0 (3.5)	5.16 (3.16)
Mean	8.75 (4.91)	13.08 (6.5)	13.5 (7.58)	12.58 (7)	10.16 (5.91)	7.41 (4.83)	
	SE (m)	**SE (d)**	**CD (p < 0.01)**				
BAA	0.37 (0.29)	0.52 (0.41)	1.34 (1.06)				
NAA	0.37 (0.29)	0.52 (0.41)	1.34 (1.06)				
BAA × NAA	0.90 (0.71)	1.28 (1.00)	3.31 (2.58)				

Vales in parenthesis are mean number of viable shoot clumps.

plication of shoot primordia were arrested at lower temperatures (<10°C) and at higher concentrations of PGRs (BAP/NAA).

Initiation of multiple shoot primordia at high concentrations of BAP in combination with NAA or BAP in combination with IBA is in conformity with the observations recorded by Chauhan et al. (1999), Majourhay et al. (2007), Raja et al. (2007), Sharma et al. (2008) and Kiran et al. (2011).

In vitro corm production

Slicing and sub-culturing of viable shoots in January on MS medium supplemented with growth retardant (CCC) and sucrose resulted in cormlet development in the form of swelling which started at the base of the shoots after 12 to 14 weeks of multiple shooting (April) and 36 to 39 weeks of inoculation. Different levels of CCC showed significant differences for corm development ranging from

Table 5. Effect of growth retardant (CCC) and sucrose on corm development.

CCC (%)	Sucrose (%)					Mean
	3	6	9	12	15	
0.125	7	8	8	9	8	8.0
0.250	8	8	10	10	10	9.2
0.375	7	8	7	8	9	7.8
0.500	5	6	4	4	5	4.8
0.625	7	4	6	5	5	5.4
Mean	6.1	6.3	6.8	6.8	7	
	SE (m)	**SE (d)**	**CD (p < 0.01)**			
CCC	0.20	0.29	0.75			
Sucrose	0.20	0.29	0.75			
CCC × sucrose	0.46	0.65	1.67			

Table 6. Effect of sucrose on *in vitro* corm development.

Sucrose (%)	Gain in weight (g)
0	0.500
3	1.400
6	2.900
9	3.450
12	3.000
15	3.300
SE (m)	0.61
SE (d)	0.86
CD (p < 0.01)	3.46

5 to 9 corms/viable shoot clump, whereas, differences for sucrose and interaction effects were non significant. Medium supplemented with 0.25% CCC and 9% sucrose showed maximum mean number of corms (10) per viable shoots [Table 5 and Figure 2 (Stage 5)]. Higher concentration of CCC was detrimental in making viable shoot clumps least effective in corm proliferation and mean corm number was reduced to 7 in MS medium supplemented with CCC at 0.625%. The corms were sub cultured on different concentrations of sucrose to enhance the initial corm weight. Maximum gain in mean corm weight (3.450 g) was achieved in MS medium supplemented with 9% sucrose in 6 to 7 weeks [Table 6 and Figure 2 (Stage 6)]. The shoots dried up once the corms were formed with well-developed tunics.

The results reveal a corm proliferation capacity of 110 corms/ex plant with a proportion of corms weighing above 8 g to the tune of 46 and 53% of non flowering corms with average corm weight ranging from 2.5 to 6.8 g [Figure 2 (Stage 11)]. Chlorocholine chloride and paclobutrazol has also been reported to favour corm initiation and development (Sharma et al., 1998; Simco, 1993; Raja et al., 2007; Kiran et al., 2011). Corm development was ensured on 17 ± 2°C and was given 16/8h (light/dark) photoperiodic treatment. However, the use of low temperature and continuous light for cormlet production has been reported by Milyaeva et al. (1995) and Rout et al. (2001). Sub culturing of non flowering corms on CCC supplemented with 9% sucrose served as explants for next generation corm development [Figure 2 (Stages 8 to 10)]. The use of *in vitro* corms as ex plant could help in early sprouting and thus eliminated season dependence of *in vitro* protocols. Another advantageous proposition is direct multiple shoot primordia induction from the base of buds without an intervening callus phase, thereby reducing the chances of variability.

Hardening and field evaluation

In vitro corms were subjected to primary hardening on 24± 1°C under white fluorescent light of 42 µmol/m^2s^1 with the photoperiod of 16 h light and 8 h dark for 8 to 10 weeks under laboratory aseptic conditions using coco peat and vermin compost as medium for growth followed by secondary hardening for 4 to 5 weeks under ambient conditions using sand (20%), Vermi compost (30%), FYM (30%) and soil (20%) as medium for growth supplemented with water spray after every 3 to 5 days [Figure 2 (Stage 12)]. The *in vitro* corms were planted under actual field conditions on 20th August with recommended INM dose at Saffron Research Station and rice straw was used as mulch till the corm sprouts were above ground. Evaluation has confirmed 100% survival of *in vitro* corms. Further evaluation for morphological, floral and quality attributes revealed that performance of *in vitro* corms for flower creating index and other related traits was comparable to what is achieved from field corms used as control (Table 7). *In vitro* corms on an average observed 1 to 3 flowers/corm weighing 240 to 795 mg on fresh weight basis. Pistil length ranged from 3.5 to 5.5 cm with dry pistil weight ranging from 9 to 11 mg/corm suggesting a productivity level of 4.5 to 5 kg/ha with a population density of 5 lakh corms/ha.

Quality evaluation for colour, flavour and bitterness

Table 7. Performance of *in vitro* corms under field conditions for floral, morphological and quality attributes.

Status	Number of flowers/corm	Fresh flower weight/corm (mg)	Pistil length (cm)	Dry pistil weight/corm (mg)	Leaf length	Number of leaves/corm	Crocin	Saffranal	Picrocrocin
In vitro corms	1-3	240-795	3.5-5.5	9-11	35	18	298	121	65
Field grown corms	1-3	215-280	3.6-5.4	10-11	37	19	280	120	68
SE(m)	0.02	3.97	0.08	0.50	1.45	2.58	5.99	3.42	5.95

as per ISO 3632 standards did not reveal any deviation for the quality. The carotinoid level of *in vitro* corms was similar to what is achieved from field grown corms. Study thus confirmed that the present protocol is not only profitable in achieving very high multiplication rate of disease free corms but also ensures stability of the saffron genome without any change in morphological, floral and quality attributes.

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