EXPLORING *IN VITRO* CORM INDUCTION POTENTIAL OF *CROCUS SATIVUS* THROUGH DIRECT ORGANOGENESIS

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Abstract. Saffron is an economically high-value crop globally that can give farmers a high income. Its sexually sterile nature demands vegetative propagation and quality planting material. Hence, this study was designed to build a rapid and reproducible procedure for the micropropagation of saffron. We have examined the surface sterilization of corms as explant with fungicides, antibiotics, and 0.1% mercuric chloride, resulting in maximum sprouting (91.07%) with minimum contamination and mortality. As the propagation of saffron corm and its germination is a critical factor for its yield, we have checked different phytohormone concentrations for bud corm germinations. We found that BAP is an efficient cytokinin to initiate buds on corms. MS medium with NAA (0.5 mg L^{-1}) and BAP (3.0 mg L^{-1})led to 97.77% sprouting in an average of 34.67 days. Subculturing of sprouted corms on the increased concentration of BAP enhanced shoot proliferation from the base of the sprouted bud. The MS medium with NAA (0.5 mg L^{-1}) and BAP (6.0 mg L^{-1}) shows maximum mean number of shoots (12.67). The presence of growth retardants like ABA, PBZ, and CCC inhibited shoot growth and led to corm development. PBZ (1.5 mg L^{-1}) significantly affected cormlet induction (80%), with a maximum mean weight of 1.70g. Accumulative sucrose concentration to 90 g L^{-1} further increased mean weight (2.52 g) and size of cormlets (1.08 cm). Our *in vitro* propagation protocol can produce many corms for regular supply to farmers.
Keywords:*ABA, CCC, corm, PBZ, phytohormones, propagation, saffron*

Introduction

Saffron, a golden condiment, is one of the premium spice globally, acquired from dried orange-red stigmas of saffron (*Crocus sativus* L.). In addition to costliest spice

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worldwide, this plant is furthermore used extensively in contemporary medication to treat Alzheimer's disease, cancer, and liver disorders. Environment, nutrition, as well as post-harvest procedures, including storage and drying conditions, all affect the quality of saffron stigma. Despite its high value, saffron cultivation hasn't altered much since thousands of years ago. There are numerous difficulties in growing saffron. The plant reproduces slowly, producing only 3–4 corms per year on average. Further issue is a variety of illnesses, such as recently described viral diseases and quail-specific fungal diseases. Other problems with saffron cultivation include water competition and poor soil nutrition (Chib et al., 2020). Challenges to saffron farming now include soil illnesses, climate change, and a lack of workforce (Moradi 2022). Botanically, it is a small perennial geophyte with underground fleshy corms. It belongs to the Iridaceae family with in excess of 80 species, out of which around 30 are cultivated species worldwide (Lagram et al., 2016). Saffron is an autotriploid (2n=3x=24). It is completely sterile and vegetatively proliferated via corms (Bayat et al., 2018; Nasseer et al., 2018) which usually survive one season, forming only three to four cormlets, thus limiting the multiplication rate.

Besides being a condiment, saffron is also a good source of secondary metabolites having aromatic and pharmaceutical importance (Zengin et al., 2020). Therefore, it is utilised in cosmetics, food, dye and perfumery businesses. Saffrons biochemical constituents consist of monoterpene aldehydes, carotenoids, crocin, crocetin, picrocrocin, and safranal (Sharafzadeh, 2012). These biochemicals have excellent healing features, including anti-tumor and anti-cancer properties (Rezaee-Khorasany et al., 2019). Besides this, saffron's anti-depressant, anti-diabetic, anti-inflammatory, antioxidant and anti-seizure activities have also been reported (Shah et al., 2017).

India enjoys 3rd rank in saffron production internationally after Iran and Spain (Taufique et al., 2017). Kashmir valley in Jammu and Kashmir symbolizes prominent saffron-growing areas globally. It is extensively grown in the upland and Karewa areas of Kashmir valley, especially Pampore and its adjoining areas, along with parts of Kishtwar in the Jammu Division. Saffron is being cultivated over 3715 ha in Jammu and Kashmir with an overall production of 16 MT and 3-4 kg/ha (Anonymous, 2021; Rather et al., 2022). Cultivation of this crop has shrunk over the earlier decade due to high cost of cultivation, low productivity, lack of irrigation facilities, and traditional inappropriate cultivation methods. Large-sized corms yield more flowers than large-sized offspring corms. Therefore, superior corms must be selected for higher stigma yield (Agayev et al., 2009; Rather et al., 2022). However, creating superior clones in sufficient amounts for plantation in vast parts is challenging due to a limited number of offspring corms production.

Saffron is a high-value crop grown under specific geographical regions in the world. Therefore it requires technological interventions for growing in non-native conditions through *in vitro* culture. Numerous instances of adequate *in vitro* saffron production have been reported earlier (Rather et al., 2022). However, this technique still has issues, including endogenous sample contamination and employing extremely lethal chemicals to clean up and remove this contamination. This strategy confronts the difficulty of limiting the usage of harmful substances due to the importance of the environment. Hence, much research and study are being conducted to address this issue and enhance production procedures.

Lack of superior planting material is additional constraint that affects saffron plantations and area expansion. Within the last few decades, the intervention of tissue culture techniques has rapidly propagated bulbous and cormous monocotyledons.

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Micropropagation is the best technique to generate many genetically uniform clones (Teixeira da Silva et al., 2016). Since first reports of saffron in-vitro culture (Ding et al., 1979 and 1981), direct shoot induction and micro corm development are the two promising commercial strategies, particularly if in time-bound manner genetically improved saffron corms are to be multiplied (Agayev et al., 2009; Rather et al., 2022). Considering the requirement for a large quantity of quality planting material for increasing saffron production and improving the economy, an attempt has been made to enhance the saffron corms production under *in vitro* conditions.

Materials and Methods

Maintenance, surface sterilization, and establishment of corm explants

Saffron corms were procured from the local farmers of Pampore in Kashmir Valley and Kishtwar district of Jammu in April, June, and August. These corms were maintained in the polyhouse of the School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Jammu and Kashmir, India. Healthy saffron corms were removed from the soil and washed for 30 minutes to remove the soil adhering to them under running tap water. Corms were covered with a tunic which was carefully removed. The dead tissues of the corm were also cut and excised with the help of a surgical blade avoiding any damage to the buds. Corms were washed thoroughly with detergent Tween-20 for 30 minutes, then rinsed under tap water. These washed corms were treated with Streptomycin sulfate, Dithane M-45, and Bavistin (0.1% each) for another 30 minutes. The corms were rinsed to remove the disinfectants. Final sterilization was performed under a laminar airflow chamber with mercuric chloride ($HgCl_2$ 0.1%) or sodium hypochlorite (NaOCl 4%) for different durations ranging from 5-20 minutes and then washed thoroughly with sterile distilled water. Surface sterilized corms were sliced from the lower side and inoculated on Murashige and Skoog medium (1962), augmented with diverse cytokinin (Kinetin, BAP) and NAA concentrations. In addition to whole corms, the corm slices each having a bud, were also used for culture initiation.

Induction of multiple shoots

After the buds sprouted, the old corms were removed by cutting them off, leaving small intact portions, and subculturing on MS medium supplemented with different BAP and NAA (0.5 mg L^{-1}) concentrations for multiple shoot induction.

Cormlet production

The multiple shoot primordia formed directly underneath the bud sprout were used for corm production. The 5-6 shoot-in numbers from the 8-week-old culture were shifted to MS medium with varying concentrations of growth retardants: Paclobutrazol (PBZ), Abscisic acid (ABA), and Chlorocholine Chloride (CCC) to analyze their effect on corm production. After optimization of growth retardant concentration, the result of sucrose concentration on corm size was also studied.

Media preparation and cultural conditions

The nutrient media (MS) used contained 3% sucrose. The pH of MS media was adjusted to 5.8 by adding HCI or NaOH before autoclaving, then 0.8% (w/v) agar was added and dispensed into flasks and sterilized at 121ºC with 1.1 kg/cm^2 pressure for 20

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minutes in autoclave. All experimental explants were subcultured every four weeks and incubated in a growth room at 20±2 °C and 70% relative humidity with photoperiod of 16 hours and provided with 2.5 kilolux light intensity by white, cool, and fluorescent lamps.

Data analysis

All the experimental treatments was performed in triplicate. The completely randomized design was applied for analysis of all the data recorded for different parameters (Gomez and Gomez, 1984). The statistical analysis was performed using the analysis of variance (ANOVA) technique of CRD based on mean values per treatment using angular transformation. The data was analyzed using OPSTAT software.

Results and Discussions

Surface sterilization of saffron corms

Establishing aseptic cultures is a prerequisite for *in vitro* propagation of any plant material. If underground organs used as explant sources such as bulbs, corms, tubers, rhizomes, etc., during micropropagation contamination is primarily a severe problem. In such cases obtaining aseptic cultures becomes cumbersome for establishing successful *in vitro* axenic cultures (Teixeira da Silva et al., 2016). In this investigation, underground saffron corms were utilized as explants to initiate *in vitro* cultures of saffron which are a rich host of various soil-borne microorganisms. Bach and Sochacki (2013) reported that initial explants contamination is one of the key hindrances in the geophytes micropropagation and found that leaving the explants under running tap water (10 min to few hours) was necessary to get rid of microorganisms in all places covering them.

Successful *in vitro* establishment of corms need standardization of duration and concentrations of disinfectants utilized for surface sterilization. Continuous stirring with a few drops of Tween 20 in water resulted in removal of dust or soil particles adhering to corms surface. Inspite of this treatment the *in vitro* cultures were infeted with fungus and bacteria.Further treatment of corms with a combination of fungicides (Bavistin and Diathane M45) and antibiotics (0.1% streptomycin sulfate) before final sterilization eliminated soil-borne contaminants to a large extent. Further, saffron corms were finally sterilized with 0.1% mercuric chloride or 4% sodium hypochlorite. After two weeks, 100% aseptic cultures were obtained when corms were provided treatment with 0.1% mercuric chloride for 20 minutes resulting in the sprouting of 68.83%, while treatment duration of 15 minutes gave maximum sprouting of 91.07% buds on corms with the slightest contamination of 2.20%. Data presented in *Table 1* displays that an increase in duration of exposure to mercuric chloride treatment either delayed sprouting or caused the mortality of buds, following previous observations by Pandey et al. (2009). They reported that increasing exposure of 0.1% $HgCl_2$ from 2.5-10 min improved the disinfection efficiency of *Lilium* bulb scales from 29.3 -96.0% (calculated after one week). At same time, the explants survival in 10-min treatment decreased by more than 14% (after 4 weeks). Taheri-Dehkordi et al. (2020) reported similar observations about treatment duration where 0.1% mercuric chloride was used for 15 minutes to obtain aseptic cultures with 80-87.67% survival rate indicating the concentration and exposure time importance (Hesami et al., 2018). In this study, treatment of corms with NaOCl (4%)

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was ineffective in establishing aseptic cultures even if treatment duration was increased to 20 minutes and cultures were found to be contaminated with microbial infection.

Table 1. *Sterilants and treatment duration effects on sprouting and establishment of aseptic cultures of Saffron*

S. No.	Sterilant	Treatment Duration	Contamination (%)	Mortality (%)	Sprouting (%)
1	$HgCl_2$ (0.1%)	5	68.83 (56.07)*	4.40 (9.92)	26.60 (31.03)
2		10	15.53 (23.10)	35.53 (36.55)	48.87(44.32)
3		15	2.20 (4.96)	8.87 (14.25)	91.07 (75.66)
4		20	0.00 (0.00)	31.07 (33.83)	68.83 (56.07)
5	NaOCl (4%)	5	97.77 (84.98)	4.4 (9.92)	0.0 (0.0)
6		10	77.77 (61.89)	22.2 (28.05)	0.0 (0.0)
7		15	68.83 (56.07)	28.83 (32.43)	2.2 94.960
8		20	48.83 (44.31)	33.3 (35.23)	17.77 (24.83)
	CD		8.31	11.10	9.99

*Numbers in parenthesis represent transformed values

Besides surface contaminants, endogenous contamination (primarily bacteria and fungi) is a far more complicated issue that requires antibiotic therapy (Reed and Tanprasert, 1995). It is not easy to achieve 100% contamination-free cultures. However, treating the explants with antibiotics results in partial contamination control (Karaoglu et al., 2007). Yasmin et al. (2013) described a combination of systemic fungicides alongwith bleach followed by a mercuric chloride (1.6%) treatment for 5 min which resulted in 94% contamination-free saffron cultures. Antibiotics should be cheap, stable, soluble, broadly active, and unaffected by pH or medium components (Teixeira da Silva et al., 2016). Additionally, these should have bactericidal activity and should not be phytotoxic (Falkiner, 1990). Altan et al. (2010) proposed for bacteria that single antibiotics have a narrow target spectrum. Therefore, antibiotic combinations should be preferred to a synergistic effect, i.e., microorganisms control and reduced plant damage. Cavusoglu et al. (2013) compared a combination of various disinfectants (ethanol, Tween 20, NaOCl, and H_2O_2). They reported that a combination of ethanol (70%) and NaOCl (50%) was best with the slightest contamination of corm segments. Chib *et al*. (2020) used NaOCl (4%) in addition to $HgCl_2$(0.1%) and obtained an 86% survival rate for *C. sativus*. Similar results were shown by Yasmin et al. (2013) for treated corm explants with $HgCl_2$ (1.6%) and observed contamination level reduced as the level of $HgCl_2$ increased, resulting in 94% aseptic cultures.

Bud sprouting and establishment of cultures

The initial signs of establishment of cultures were observed in 2-3 weeks as axillary and apical buds were swelling on all media combinations. MS medium was augmented with different growth hormones (BAP, KIN, NAA) combinations and concentrations. As evident from *Table 2*, augmentation of BAP at the rate of 3 mg L^{-1} led to a higher bud sprouting percentage (97.77) compared to the presence of Kinetin at the same concentration (84.40). It was also observed that cytokinins and auxins in combination significantly affects the regeneration frequency, as reported by Abbas and Quiser (2012), Sivanesan and Jeong (2012), Yasmin et al. (2013) and Mir et al. (2014). BAP is the

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effecient cytokinin for direct shoot bud initiation Sharma et al.(2008); Lone et al.(2016); Kiran et al. (2011); Raspor et al.(2021) emphasized on augmentation of BAP in combination with 2,4-D for bud sprouting. Sharifi et al. (2010) also compared the effect of different concentrations of TDZ and BAP and found TDZ to be better suited in shoot formation. Further, in our study we have observed that shoots in MS media fortified with Kinetin were thin and tender compared to those in BAP fortified medium.

Table 2. *Growth regulators effects on sprouting of corm explants of saffron*

S. No.	BAP (mg L^{-1})	KN (mg L^{-1})	NAA (mg L^{-1})	Sprouting (%)	Days to sprouting
1	1.0	-	-	48.83 (44.31)*	45.33
2	1.0	-	0.5	71.07 (57.46)	45.67
3	2.0	-	-	55.53 (48.16)	42.00
4	2.0	-	0.5	82.20 (65.10)	41.33
5	3.0	-	-	64.40 (53.36)	39.67
6	3.0	-	0.5	97.77 (84.98)	34.67
7	-	1.0	-	42.20 940.48)	48.00
8	-	1.0	0.5	62.20 (52.05)	47.33
9	-	2.0	-	48.83 (44.31)	46.33
10	-	2.0	0.5	71.07 (57.46)	45.67
11	-	3.0	-	64.40 (53.36)	42.33
12	-	3.0	0.5	84.40 (66.80)	42.00
	CD			5.77	

*Numbers in parenthesis represent transformed values

The explants were initiated during May, July, and August to find the most suitable period for culture establishment. It was found that corm explants created in August were more responsive than those initiated in May and July (*Figure 1A-B*). It was observed that all the apical and axillary buds sprouted and developed into healthy shoots with leaves enclosed in cataphylls (*Figure 1C-D*).

Our results align with those of Yasmin et al. (2013), where the sprouting of buds was also observed to be season dependent, and 95% of buds from corm sections were in September. Similar observations were described by Kiran et al.(2011); Renau-Morata et al.(2021). Once corm explants were initiated in May and July, only the apical bud developed into a comparatively weaker shoot and resumed growth as a single shoot throughout the culture period. There were no signs of the emergence of multiple shoots even after six weeks of culture. Gradually, these single shoots started showing senescence, and eventually, the browning of the innermost leaves started; ultimately, the whole plant died.

The culture establishment was also influenced by the size of the corm used as an explant during the initiation of cultures. It was observed that a single shoot developed from the apical bud of a small-sized corm, while axillary buds either failed to develop a shoot or formed weak shoots that eventually turned brown and died. In comparison, all the buds (apical and axillary) oflarge corms ($\geq$2.0 cm) developed into shoots which further grew multiple shoots. Incubating cultures at 20±2°C in the dark was the most suitable culture condition for sprouting apical or axillary buds. Bhagyalakshmi et al.(1999) observed enhanced shoot bud induction from saffron's ovaries under dark incubation. Sharma and Piqueras et al. (2010)and Eftekhari et al.(2023) also recommended that light and temperature influenced direct shoot induction. Temperature

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changes ranging between 15-20°C have also been suggested by Plersner et al. (1990) for better culture establishment.

Figure 1. *Initiation (A&B) and establishment (C&D) of saffron corms on MS medium with BAP (3.0 mg L^{-1}) and NAA (0.5 mg L^{-1})*

Multiple shoot induction and proliferation of cultures

Regeneration of multiple shoot primordia was observed from the base after 4 weeks in the multiplication media when the sprouted bud, along with some part of the corm attached to it, was shifted to MS medium augmented with a high BAP concentration along with auxin (*Figure 2A-F*). The maximum mean shoots number (12.67) was observed in presence of BAP (6 mg L^{-1}) and NAA (0.5 mg L^{-1}), which was at par with shoots (12.33) attained in medium augmented with BAP (6.5 mg L^{-1}) and NAA (0.5 mg L^{-1}). As evident from *Table 3*, an addition in BAP concentration directly influenced multiple shoot initiation. After emergence of multiple shoots, the old corm left attached started showing senescence and was entirely removed. MS medium comprising BAP (3-3.5 mg L^{-1}) failed to multiply and finally died. Kiran et al. (2011) suggested fortification of a high concentration of BAP in the multiplication medium. Sharma et al. (2008) also obtained multiple shoots in the existence of a high concentration of BAP (6 mg L^{-1}), resulting in an average of 9 shoots per corm, per the observations recorded in the present investigation. In their studies, Sharifi et al. (2010) and Chib et al. (2020) observed that cytokinins like BAP, Zeatin, and Kintin prerequisite auxins to excite multiple shoot

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induction in saffron. Mir et al. (2014) also emphasized the importance of NAA and BAP on the shoot regeneration of saffron. However, they attained a maximum shoot numbers (11.6) on MS medium with a comparatively low concentration of BAP (22.2µM) along with NAA (21.6µM).

Figure 2(A-F). *In vitro shoot multiplication and proliferation saffron on MS medium with BAP (6.0 mg L^{-1}) and NAA (0.5 mg L^{-1})*

Multiple shoot induction was observed under *in vitro* conditions occurred when the plant was in active growth under natural/*in vivo* conditions. Yasmin et al. (2013) described the emergence of multiple shoots from the base of activated buds on MS media with BAP and NAA in November at 17±2ºC. They observed that low temperature and

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high concentration of growth regulators arrested multiplication of shoot primordial, as shown in our results.

Table 3. *Different Concentration effects of growth regulators on in vitro multiplication of saffron shoots*

S. No.	BAP (mg L^{-1})	NAA (mg L^{-1})	Mean shoot number per corm	Mean days for multiple shoot induction
1	3.0	0.5	1.00	59.33
2	3.5	0.5	2.33	59.33
3	4.0	0.5	4.33	58.00
4	4.5	0.5	5.00	57.67
5	5.0	0.5	7.67	57.33
6	5.5	0.5	8.33	57.00
7	6.0	0.5	12.67	52.33
8	6.5	0.5	12.33	52.67

In vitro cormlet formation

A cluster of 5-6 multiple shoots was shifted to MS medium augmented with different growth retardants concentrations viz. CCC (50-150 mg L^{-1}), PBZ (0.5-2.0 mg L^{-1}), and ABA (1-3 mg L^{-1}) to analyze their effect on cormlet induction. Using growth retardants like CCC and PBZ positively impacted corm development as they inhibited shoot growth. Fortification of MS medium with PBZ (1.0 mg L^{-1}) caused highest mean number of cormlets (3.0) and maximum cormlet induction percentage (80.00), which was significantly different from all other medium combinations, as shown in *Table 4*. In MS medium devoid of growth retardants, no cormlet induction was observed. The least cormlet induction of 26.6% was observed in the presence of ABA at 1.0 mg L^{-1} concentration. CCC in the medium was not as effective as PBZ for cormlet induction. Cormlet production started as swelling at the base of shoots after 4 weeks of culture due to growth retardant, especially PBZ (1.0 mg L^{-1}), as shown in *Figure 3(A-D)*.

Table 4. *Different concentration effects of growth retardants on in vitro cormlet development*

MS medium	mg L^{-1}	Mean no. of cormlets	Cormlet induction (%)	Mean weight of cormlets (g)
Control (Basal)	-	-	-	-
ABA	1.0	1.3	33.33 (34.99)	0.53
	2.0	1.6	46.67 (43.06)	0.57
	3.0	1.6	60.00 (50.74)	0.70
PBZ	0.5	2.3	53.33 (46.90)	0.83
	1.5	3.0	80.00 (63.40)	1.70
	2.0	2.6	60.00 (50.74)	1.50
CCC	50	1.6	46.67 (43.06)	0.73
	100	2.6	60.00 (51.12)	1.16
	150	2.3	53.33 (46.90)	0.90
CD			10.51	

*Numbers in parenthesis represent transformed values

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Figure 3(A-D). *Corm induction under in vitro conditions on MS medium*

Devi et al. (2011) also used CCC and PBZ for cormlet production from multiple shoots and found PBZ better than CCC. On the contrary, Yasmin et al. (2013) found CCC (0.25%) along with sucrose (9%) in MS medium effective for cormlet development. In addition, increased concentration of CCC had a detrimental effect on cormlet production. Mir et al. (2014) used Paclobutrazole (1.5 mg L^{-1}) in both MS and G_5medium along with BAP and NAA and obtained a maximum of 10 micro-corms on MS medium fortified with NAA (0.5 mg L^{-1}) and BAP (2 mg L^{-1}).

The development of cormlet requires a large amount of energy reserve provided by carbohydrate sources. In this study, the optimized medium was supplemented with different sucrose concentrations (30-120 g L^{-1}) to observe its influence on the size and weight of cormlets. It was observed that increasing sucrose concentration increased the size of the cormlet and the weight of the cormlet, as evidenced by *Table 5*.

Table 5. *Effect of sucrose concentration on in vitro cormlet development*

Sucrose concentration (g L^{-1})	Mean weight of cormlets (g)	Mean size of cormlets (cm)
30	1.60	0.53
60	1.92	0.70
90	2.52	1.08
120	2.18	1.04

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The maximum weight of 2.52 g was attained in a medium with 90 g L^{-1} of sucrose, followed by a 2.18 g weight gained in a medium with 120 g L^{-1} of sucrose. A full cormlet size of 1.08 cm was achieved in medium with 90-120 g L^{-1} of sucrose. Cormlets with well-developed tunics were formed after 12-14 weeks of culture (*Figure 4A-B*).

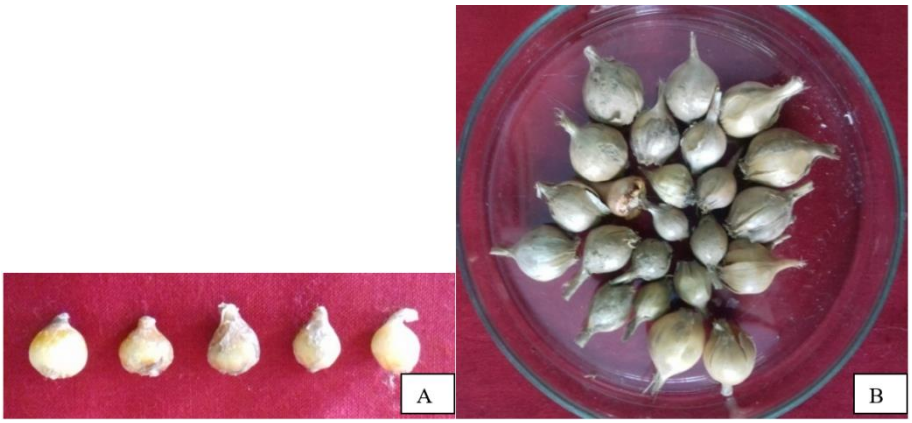

Figure 4. *In vitro-producedcormlets*

Zaffar et al. (2014) suggested an improved protocol for developing viable cormlets. Sharma et al. (2008) compared different carbon sources and found sucrose (80 g L^{-1}) essential forcormlet induction over mannitol. Samaha (2018) also deliberate the effect of carbon sources like sucrose, fructose, and glucose on corm regeneration. Therefore, we have standardized the in-vitro propagation protocol for corm production in saffron that could be utilized for better yield.

Conclusions

Saffron being the costliest condiment and spice, warrants quality planting material. One of the best ways to produce the vegetatively propagated crop is *in vitro* propagation with corm as an explant. Our study standardized the *in vitro* protocols for saffron corm *in vitro* propagation in MS media with different hormonal concentrations. We have found that maximum shoot proliferation was observed in the MS medium with BAP (6.0 mg L^{-1}) and NAA (0.5 mg L^{-1}). PBZ (1.5 mg L^{-1}) had a significant effect on cormlet induction. Similarly, we have standardized the sucrose concentration for maximum cormlet growth in saffron. Increasing sucrose concentration to 90 g L^{-1} increased the cormlets' mean weight and size. Therefore, our *in vitro* propagation protocol standardized can produce many corms for regular supply to regional farmers.

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