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Original Article

IN VITRO MICROCORM FORMATION IN SAFFRON (*CROCUS SATIVUS* L.)

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Abstract: Saffron the sterile triploid belongs to Iridaceae family. Due to triploid nature of saffron sexual reproduction is not possible and it is propagated vegetatively by corms. However the multiplication ratio via corms is very low in nature with a ratio of 1:3 thereby increasing the cost of cultivation and making it the most expensive spice in the world. Tissue culture offers a great potential for commercial corm production in saffron. Present investigation was a step towards standardization of effect of different growth regulators ensuring initial bud sprouting, direct shoot regeneration from the base of spouted buds and cormlet production from multiple shoots for establishment of commercially viable protocol. Initial bud sprouting was achieved using NAA (0.5 mg/l) in combination with BAP (1.5 mg/l) and 2,4-D (2 mg/l) in combination with Zeatin (3mg/l) and BAP (1.5 mg/l).Inoculation of sprouted cultures on MS medium supplemented with 2mg/l Zeatin resulted in shooting Significant shoot proliferation was observed on combining cytokinin with auxins. Shoot multiplicationwas achieved from treatment combination 6mg/l Zeatin and 2mg/l 2,4-D. Minicorms of 8g weight were obtained on growth retardant (CCC@0.2 ml/l) in combination with 9%

Key words: Corm regeneration, Saffron (*Crocus sativus* L.)

INTRODUCTION

Saffron a triploid sterile geophyte (2n=3x=24) belonging to family *Iridaceae* is propagated solely by vegetative way with a multiplication index of four to five corms per mother corm. Low multiplication rate and fungal infestation of corms are the bottlenecks for availability of sufficient quality planting material [1]. Ministry of Agriculture, Govt of India launched a mega saffron revolutionary programme -"National Saffron Mission" for promotion of saffron in traditional and non-traditional areas of Jammu and Kashmir State. This is the only saffron producing state with a contribution of about 15 M.T saffron to the global production fetching a total economic returns of Rs 225 crores by involving 16000 farm families from heritage sites of saffron including new areas of Budgam and Kishtwar districts.

To meet the emerging demand of about sixty two thousand M.T of quality corms required for horizontal expansion over 12000 hectares including non-traditional areas of Jammu and Kashmir and tissue culture offers a great potential [2-5]. Although tissue culture protocols for mass propagation of saffron corms are available, smaller size of *in vitro* corms with low survival and flower creating index is the limitation factor and thus warrants further investigation.

Present investigation was thus aimed to develop a commercially viable *in vitro* protocol to address the

issues of non availability of quality planting material ensuring a high rate of multiplication of flowering corms which can increase the production to 79 M.T of dried dark red stigmas of saffron to be used as a spice for flavorings and coloring food, as a perfume and for treatment of many diseases.

MATERIAL AND METHODS

Studies on *in vitro* microcorm formation of saffron (*Crocus sativus* L) were carried out at Tissue Culture Laboratory of Saffron Research Station, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir during 2012-13 and 2013-14. Healthy saffron corms (2cm in diameter) collected from natural sub-populations of Kashmir with productive apical, sub- apical and auxillary meristematic regions were used as explants.

Saffron corms (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 minutes to remove coating layer of micro-organisms ubiquiotously found on them. ii)fungicide dip with Tween-20 for 10 minutes (carbendizime 0.10%, in combination with mancozeb @ 0.2%),iii) sterilant wash in sodium hypochlorite solution (0%, 25%, 50%, 75%) for 10 min and rinsing thrice with sterile distilled water followed by a dip in mercuric chloride solution (0%, 0.8%, 1.6%, 2.4%) followed by final rinsing with sterile distilled water.

The surface sterilized explants were inoculated into test tubes containing the Murashige and Skoog (MS) [6] (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. Different level of auxins (NAA@0,0.5,1.0,1.5,2.0mg/l; 2,4-D@0,1,2,3,4mg/l; IBA@0,0.5, 1.0,1.5,2.0mg/l) and cytokinins (BAP@0,0.5,1.0,1.5,2.0mg/l; Kinetin@0,1.5,3.5,5.5,7.5mg/l; Zeatin@ 0, 1, 2, 3, 4mg/l) were used for bud sprouting. The cultures were incubated in the culture room maintained at 17 ± 2^0C with a photoperiod of 16/8h (light/ darkness) [7]. To increase the base material for shoot induction, sprouting block of three best combinations confirming maximum number of sprouts was established in October, 2012 after inoculation of 900 sterile explants (300 explants/best combination) on MS media supplemented with identified best combination of auxins and cytokinins.

For shoot development, the established explants from sprouting block (5 explants/replication /combination) were inoculated on MS medium supplemented with different concentration of cytokinins (BAP@ 0,2.5,4.5,6.5,8.5mg/l ;Kinetin@ 0,3.0,7.0,11.0,15.0mg/l and Zeatin@0,3,6,9,12 mg/l) in October, 2012. 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 medium supplemented with different combinations of cytokinins (BAP@0,2. 5,4.5,6.5,8.5 mg/l; Zeatin@0,2,4,6,8mg/l) and auxins (NAA@ 0,0.25,0.50.0.75.1.0 mg/l and 2,4-D @0,0.5,1.0,1.5,2.0 mg/l) in the month of January, 2013. Viable multiple shoots were sliced and subsequently transferred on MS medium supplemented with 4 concentrations of growth retardant Chlorocholine Chloride (0,0.1,0.2,0.3,0.4 ml/l in combination with 3% sucrose for 6 weeks followed by 9% sucrose in next 7 weeks under corm development.

in vitro microcorms 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 [7] and were then field transferred using randomized block design at Saffron Research Station, Pampore during August, 2012 with a plot size of 1.2 m^2 accom-modating 60 corms in three rows of 2m length with an inter and intra row spacing of 20x10 cm, replicated twice. All the recommended package of practices were adopted to raise the good crop.

Statistical analysis was carried out using Windostat Statistical package 2013. Analysis of variance using factorial randomized block design was used to examine the effects of multiple factors and their interactions (auxins, cytokinins and growth reatardant) on response variables (sterilization, shoot elongation, shoot multiplication and corm development).

RESULTS AND DISCUSSION

For standardization of *in vitro* microcorm formation protocol, data was collected on 19 parameters related

Plate I: Stages of in vitro microcorm formation in saffron

to sterilization, explant sprouting, shoot elongation, shoot multiplication, corm development and field evaluation viz percent aseptic cultures, percent explant survival, percent activation, sprout number, shoot number, shoot length, number of multiple shoots, number of viable shoots, number of corms, initial corm weight, final corm weight, percent field survival, number of flowers per plot, percent flowering, fresh flower weight, pistil length, crocin, safranal and picrocrocin. Mean values of all the characters collected *in-vitro*/in-vivo were subjected to various statistical procedures for drawing the inferences. Analysis of variance (ANOVA) revealed highly significant effects of different levels of sterilization regimes, cytokinins (BAP, kinetin, zeatin), auxins (NAA, 2,4-D, IBA), growth retardants (CCC) and their interactions, on culture asepsis , sprouting, shooting, multiple shooting and corm development. Data on culture asepsis, explant survival, % activation and % flowering were subjected to arc sign transformation for interpretation of results. The results of present investigations are presented under following appropriate heads supplemented with tables and Plate-1

Culture asepsis: Contamination is a serious problem during micropropagation of monocots especially if underground organs such as corms, bulbs, rhizomes and tubers are used as an explant source. Highest mean percentage of aseptic cultures to the tune of 81.51% with highest explants survival (81.51%) was obtained following three step sterilization procedure (carbendizime 0.1% + mancozeb 0.2% in combination with 50% sodium hypochlorite and 1.6% mercuric chloride). Individual effects of sterilants revealed culture asepsis to the tune of 70.57 % accompanied with 65.90 % survival using sodium hypochlorite (50%) and 67.54 % culture asepsis associated with 51.6% survival using mercuric chloride (2.4%). Higher levels of sodium hypochlorite (>50%) and mercuric chloride (>1.6%) were effective in achieving maximum culture asepsis but were lethal in explant survival leading to reduction in survival to 46.43% Combination effect of sterilants together with fungicides increased the explant culture asepsis and survival percentage to 81.51%.(Table-I). The use of saffron corms as a direct source of explant material for the production of 'clean' *in vitro* plantlets presented a major challenge with contamination to the level of 62%. Similar results of contamination (100%) with field grown crops especially those with leaf canopy close to the ground have also been reported [8]. 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 [7]. 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.

Sprouting: Influence of different growth regulator regime on percent sprouting revealed maximum mean percentage of sprouting to the tune of 85.94% when explants were cultured on MS-medium supplemented with NAA (0.5mg/l) in combination with BA (1.5mg/l)-Table-II .Similar effects were also observed with 2,4-D (2mg/l) in combination with BA (1.5mg/l) and 2,4-D (4.0mg/l) in combination with zeatin (3mg/l), whereas lowest percent sprouting to the extent of 23.81% was observed when explants were cultured on MS-media supplemented with 1.5mg/l IBA in combination with BAP 2.0mg/l .Results revealed that higher concentrations of auxins were inhibitory for percent sprouting. Individual effects of auxins and cytokinins on percent sprouting were also observed to be significant but weak. The effects of growth regulators were more pronounced in combinations as compared to individual effects. MS medium supplemented with 2,4-D (4 mg/l) in combination with zeatin @ 3.0mg/l recorded maximum number of sprouts to the tune of 9.10 per explants, followed NAA (0.5mg/l) in combination with BA (1.5mg/l) or 2,4-D (2mg/l) in combination with BAP (1.5mg/l) and recorded 7.65 sprouts per explant, respectively. Among individual effects of auxins and cytokinins, best effects were observed with NAA @ 0.5mg/l (5.17), 2,4-D @ 2mg/l (4.56), IBA @ 1mg/l (3.06), BAP @ 1.5mg/l (4.25), kinetin @ 5.5mg/l (4.26) and zeatin @ 3mg/l (4.24). Absence of growth regulators in MS-medium did not produce any sprout. However addition of auxins/cytokinins had a significant effect on enhancing the sprout number. However higher concentration of auxins and cytokinins have been observed to be inhibitory for enhancing sprout number. Importance of BAP in combination with 2,4-D for bud sprouting at 10°C have been reported[1]

Shooting and multiple shooting: Development of Shoots requires inclusion of cytokinin in the medium. Sub culturing and evaluation of sprouted explants of

Table I: Impact of sterilants on percent in vitro clean cultures and survival in saffron. *values in paranthesis indicate percent explant survival

Sterilants		Mercuric chloride (%)				Mean
		0	0.8	1.6	2.4	
Sodium hypochlorite (%)	0	37.75 (37.75)*	43.56 (43.56)	52.24 (49.31)	58.39 (46.43)	47.98 (44.26)
	30	58.39 (58.39)	61.71 (61.71)	63.43 (63.43)	65.32 (55.25)	62.21 (59.69)
	50	58.39 (58.39)	65.32 (65.32)	81.51 (81.51)	77.07 (58.39)	70.57 (65.90)
	70	56.78 (53.72)	61.71 (58.39)	65.32 (52.24)	69.38 (46.43)	63.29 (52.69)
	Mean	52.82 (52.06)	58.07 (57.24)	65.62 (61.62)	67.54 (51.62)	61.01 (55.63)
		S.E (m)	S.E.(d)	C.D 5%		
	Sodium hypochlorite	0.42 (0.38)	0.59 (0.54)	1.18 (1.09)		
	Mercuric chloride	0.42 (0.38)	0.59 (0.54)	1.18 (1.09)		
	Sodium hypochlorite* Mercuric chloride	0.84 (0.77)	1.19 (1.09)	2.37 (2.18)		

Table II : Effect of growth regulators on per cent activation and sprout number. * Data in paranthesis indicate sprout number

Auxin (mg/l)		Cytokinins(mg/l)															
		BAP(mg/l)					Kinetin(mg/l)					Zeatin(mg/l)					Mean
	0	0	0.5	1.0	1.5	2.0	0	1.5	3.5	5.5	7.5	0	1.0	2.0	3.0	4.0	
NAA	0	4.0 (0.0)*	24.0 (1.4)	24.0 (1.5)	35.2 (1.7)	24.0 (1.4)	4.0 (0.0)	24.04 (1.6)	29.5 (1.2)	40.1 (1.9)	35.2 (2.7)	4.0 (0.0)	54.6 (6.7)	35.2 (1.9)	35.2 (1.9)	24.0 (1.3)	26.5 (1.7)
	0.5	35.2 (2.6)	45.0 (3.1)	65.8 (6.6)	85.9 (7.6)	60.2 (6.3)	24.0 (1.7)	65.8 (7.2)	65.8 (6.7)	75.9 (6.7)	65.8 (6.6)	35.0 (2.8)	60.2 (6.5)	49.8 (3.3)	54.6 (5.5)	45.0 (4.0)	55.6 (5.1)
	1.0	54.7 (3.0)	54.6 (3.3)	50.5 (6.1)	65.8 (2.8)	45.0 (4.7)	45.0 (3.2)	54.6 (5.4)	40.1 (5.1)	60.2 (5.6)	60.1 (5.1)	54.6 (3.9)	54.6 (5.5)	60.2 (5.2)	35.2 (3.4)	49.8 (4.6)	52.3 (4.4)
	1.5	54.6 (2.9)	60.2 (4.2)	49.8 (4.2)	60.2 (6.2)	24.0 (2.6)	24.0 (1.7)	35.2 (3.6)	29.6 (2.4)	65.8 (6.5)	65.7 (6.9)	54.6 (4.8)	60.1 (5.5)	55.4 (5.5)	49.8 (3.9)	60.2 (6.6)	50.0 (4.5)
	2.0	45.0 (2.2)	24.0 (2.8)	54.6 (2.4)	54.6 (4.7)	29.6 (1.5)	29.6 (3.3)	45.0 (3.9)	24.0 (2.2)	49.8 (4.4)	49.8 (5.5)	49.8 (5.9)	65.8 (6.6)	45.0 (3.6)	60.2 (5.5)	49.8 (5.4)	45.1 (4.0)
2,4-D	0	4.0 (0.0)	29.6 (1.2)	29.6 (1.2)	40.1 (1.7)	24.0 (1.4)	4.0 (0.0)	29.6 (1.5)	24.0 (1.1)	35.2 (2.3)	24.0 (1.3)	4.0 (0.0)	24.0 (1.3)	29.5 (1.7)	29.5 (1.5)	24.0 (1.4)	23.7 (1.2)
	1.0	54.6 (2.4)	40.1 (3.6)	55.4 (2.9)	54.6 (3.6)	45.0 (4.9)	29.6 (2.5)	29.6 (2.6)	45.0 (2.6)	65.8 (6.5)	65.8 (5.9)	49.8 (5.2)	60.2 (6.0)	45.0 (4.4)	60.2 (4.2)	54.6 (3.9)	50.4 (4.1)
	2.0	49.8 (2.4)	60.2 (2.7)	65.8 (6.6)	85.9 (7.6)	60.2 (3.5)	35.2 (3.7)	49.8 (5.4)	40.1 (1.4)	60.2 (5.7)	54.6 (4.3)	49.8 (5.9)	60.2 (5.1)	40.1 (4.4)	65.8 (6.2)	49.8 (2.9)	56.2 (4.5)
	3.0	35.2 (2.5)	60.2 (3.5)	29.6 (1.8)	60.2 (6.1)	40.1 (1.80	24.0 (1.6)	60.2 (6.5)	35.2 (3.0)	49.8 (4.8)	49.8 (4.5)	45.0 (3.3)	60.3 (5.4)	49.8 (3.5)	65.8 (7.1)	65.8 (1.6)	48.7 (3.8)
	4.0	45.0 (2.6)	40.1 (4.2)	45.0 (3.3)	40.1 (4.7)	29.6 (2.6)	35.2 (2.7)	40.1 (3.2)	24.0 (1.7)	45.0 (5.1)	45.0 (4.8)	54.6 (5.1)	65.8 (5.7)	65.8 (6.3)	85.9 (9.1)	65.8 (6.6)	48.5 (4.5)
IBA	0	4.0 (0.0)	24.0 (1.2)	24.0 (1.4)	24.0 (1.1)	14.0 (1.5)	4.0 (0.0)	35.2 (1.4)	24.0 (1.3)	29.6 (1.7)	24.0 (1.4)	4.0 (0.0)	24.0 (1.3)	29.5 (1.9)	24.0 (1.2)	24.0 (1.3)	20.8 (1.1)
	0.5	29.6 (1.4)	45.0 (3.9)	45.2 (3.8)	45.0 (4.3)	35.2 (3.3)	29.6 (2.2)	29.6 (3.1)	24.0 (1.8)	45.0 (4.1)	35.2 (2.1)	45.0 (4.8)	44.9 (3.4)	40.1 (1.6)	24.0 (2.8)	24.0 (2.1)	36.1 (3.0)
	1.0	29.6 (1.8)	35.2 (3.2)	40.1 (2.8)	40.1 (3.6)	49.8 (2.6)	35.2 (3.6)	35.2 (3.5)	35.2 (2.4)	54.6 (3.7)	24.0 (2.3)	49.8 (6.0)	44.9 (2.1)	45.0 (3.3)	29.6 (3.0)	35.2 (1.7)	38.9 (3.0)
	1.5	29.6 (2.2)	54.6 (3.4)	24.0 (1.7)	49.8 (5.5)	23.8 (1.8)	49.8 (5.3)	29.6 (2.0)	24.0 (1.8)	35.2 (2.8)	40.1 (1.4)	24.0 (1.8)	40.1 (1.6)	40.1 (4.0)	45.0 (5.6)	24.0 (2.2)	36.6 (2.9)
	2.0	24.0 (1.7)	29.6 (2.7)	35.2 (2.3)	29.6 (2.1)	40.1 (1.8)	40.1 (2.5)	24.0 (1.7)	24.0 (1.4)	49.8 (1.7)	44.9 (3.5)	40.1 (2.5)	45.0 (3.8)	44.9 (4.9)	24.0 (2.5)	40.1 (3.3)	35.7 (2.5)
Mean		33.3 (1.8)	41.8 (2.9)	42.6 (3.2)	51.4 (4.2)	36.3 (2.8)	27.5 (2.2)	39.2 (3.5)	32.6 (2.4)	50.8 (4.2)	45.6 (0.0)	37.6 (3.4)	51.0 (4.4)	45.0 (3.7)	45.9 (4.2)	42.4 (3.2)	41.5 (3.3)
		S.E (m)	S.E. (d)	C.D 5%													
Cytokinin		1.0 (0.06)	1.4 (0.09)	2.7 (0.18)													
Auxin		1.0 (0.06)	1.4 (0.09)	2.7 (0.18)													
Cytokinin* auxin		3.8 (0.25)	5.4 (0.36)	10.8 (0.71)													

Table III: Effect of cytokinins on shoot number and shoot elongation under *in vitro* conditions

Cytokinin	Concentration (mg/l)	Number of shoots	Shoot length (cm)
BAP	0	1.2	2.1
	2.5	3.2	5.2
	4.5	3.5	5.6
	6.5	5.1	7.9
	8.5	4.3	6.2
Kinetin			
	0	1.6	2.3
	3	3.2	4.9
	7	4.3	6.0
	11	3.8	4.9
	15	3.8	5.8
Zeatin			
	0	1.5	1.9
	3	4.9	6.5
	6	5.3	8.2
	9	4.5	6.3
	12	4.3	6.8
Mean		3.6	5.4
S.E(m)		0.07	0.09
S.E.(d)		0.10	0.13
C.D 5%		0.22	0.26

Table IV: Effect of growth regulators on number of multiple shoots and number of viable shoots under *in vitro* conditions. * *Data in paranthesis indicate number of viable shoots*

Auxin (mg/l)		Cytokinins (mg/l)										Mean
		NAA (mg/l)					2,4-D (mg/l)					
	0	0	0.25	0.50	0.75	1.0	0	0.5	1.0	1.5	2.0	
BAP	0	5.5 (2.6)*	11.3 (7.4)	11.6 (7.7)	7.9 (6.0)	10.4 (5.9)	5.4 (2.2)	10.6 (7.1)	15.1 (9.6)	18.7 (12.2)	12.8 (7.9)	10.93 (6.86)
	2.5	5.0 (2.0)	12.6 (9.9)	12.2 (7.7)	9.0 (6.9)	10.2 (6.1)	5.5 (2.5)	7.9 (4.0)	11.2 (7.1)	18.5 (11.9)	12.2 (8.6)	10.43 (6.67)
	4.5	5.4 (2.5)	20.3 (11.3)	13.1 (7.7)	10.3 (7.6)	12.9 (8.3)	5.3 (2.5)	14.7 (10.5)	13.5 (8.7)	24.2 (14.3)	10.9 (7.9)	13.06 (8.13)
	6.5	5.4 (2.4)	29.9 (20.5)	20.8 (11.7)	11.8 (8.0)	13.1 (9.7)	5.3 (2.2)	13.8 (8.9)	19.0 (12.3)	29.2 (20.1)	16.4 (12.4)	16.4 (10.8)
	8.5	5.1 (2.0)	24.8 (15.8)	14.1 (8.2)	18.4 (12.5)	12.7 (8.5)	11.3 (5.2)	5.6 (3.2)	12.7 (9.1)	26.0 (16.7)	23.6 (16.0)	15.4 (9.7)
Zeatin	0	5.2 (2.6)	23.1 (13.7)	14.7 (9.2)	11.0 (5.9)	15.1 (8.7)	5.2 (2.5)	13.12 (6.5)	15.6 (10.1)	21.1 (14.5)	18.4 (10.8)	14.2 (8.4)
	2	9.8 (5.3)	15.3 (10.8)	16.2 (11.4)	9.6 (6.5)	18.7 (12.6)	5.1 (2.2)	5.3 (10.1)	20.2 (14.3)	26.5 (14.6)	25.4 (17.2)	15.2 (10.5)
	4	10.0 (6.6)	14.7 (9.1)	13.3 (9.0)	9.5 (5.6)	15.0 (9.8)	5.1 (2.2)	12.6 (7.6)	20.9 (14.1)	27.6 (16.4)	27.9 (17.4)	15.6 (9.7)
	6	7.6 (5.0)	13.8 (8.0)	16.7 (8.9)	12.7 (7.7)	9.1 (4.9)	7.9 (3.8)	10.4 (5.1)	21.3 (13.0)	32.5 (21.8)	25.0 (18.0)	15.65 (9.6)
	8	8.2 (4.8)	14.2 (8.8)	11.6 (7.0)	16.4 (12.1)	13.8 (9.5)	7.9 (4.3)	12.8 (7.5)	18.1 (10.9)	20.9 (13.6)	22.6 (13.3)	14.6 (9.1)
Mean		6.7 (3.6)	18.0 (11.5)	14.4 (8.8)	11.7 (7.9)	13.1 (8.4)	6.4 (3.0)	11.7 (7.0)	16.7 (10.9)	24.5 (15.65)	19.5 (12.95)	14.2 (8.9)
		SE(m)	S.E. (d)									
Cytokinin		0.07 (0.05)	0.10 (0.07)									
Auxin		0.07 (0.05)	0.10 (0.07)									
Cytokinin* auxin		0.10 (0.07)	0.14 (0.10)									

Table V: Effect of CCC on corm development under *in vitro* conditions

CCC (ml/l)	Number of corms	Initial weight of corms (g)	Final weight of corms (g)
0	0.0	0.0	0.0
0.1	6.3	3.2	6.4
0.2	7.5	4.1	8.0
0.3	6.2	3.2	6.2
0.4	5.5	2.7	5.8
Mean	5.1	2.6	5.3
SE(m)	0.10	0.10	0.08
S.E.(d)	0.15	0.14	0.12
C.D 5%	0.33	0.30	0.26

Table VI: Field evaluation of *in vitro* corms for % survival , floral and quality attributes

CCC (ml/l)	% Survival	Number of flowers	% Flowering	Fresh Flower Weight(g)	Pistil Length(cm)	Crocin $E^{1\%}_{1cm}$	Picrocrocin $E^{1\%}_{1cm}$	Saffranal $E^{1\%}_{1cm}$
0	4.0	0.0	4.0	0.0	0.0	0.0	0.0	0.0
0.1	59.4	22.0	36.5	260	5.2	295	120	60
0.2	85.9	54.3	74.3	300	5.5	310	122	65
0.3	56.4	32.5	47.5	290	4.9	280	119	63
0.4	54.0	14.0	28.7	260	5.0	290	121	61
Mean	51.9	24.5	38.6	222	4.12	235	96.4	49.8
S.E(m)	0.34	0.96	1.03	1.20	0.09	1.30	1.10	0.90
S.E.(d)	0.48	1.37	1.46	1.60	0.13	1.58	1.15	1.10
C.D 5%	1.0	2.94	3.13	3.40	0.31	3.30	2.20	2.30

3 best combinations (4mg/l 2,4-D in combination with 3mg/l Zeatin, 0.5mg/l NAA in combination with 1.5mg/l BAP and 2mg/l 2,4-D in combination with 1.5mg/l BAP) on MS medium containing different level of cytokinins revealed progressive increase in average number of shoots with increase in concentration of cytokinins up to 6.5mg/l for BAP (5.18), 7.0mg/l for kinetin (4.30) and 6.0 mg/l for Zeatin (5.38).Maximum number of shoots (5.3) with an average shoot length of 8.2 cm was revealed by 6 mg/l zeatin ,followed by 6.5 mg/l BAP exhibiting 5.12 shoots with an average shoot length 7.9 cm (Table-III). Inclusion of Kinetin exhibited weak response to shooting and shoot elongation and thus could not be considered for multiple shooting. Similar results of shoot elongation for BAP has been reported [7].Efficient shoot multiplication is important from biotechnological point of view [8].As such optimization of conditions which improve shoot multiplication and result in better shoot quality are highly desirable [9]. Shoot proliferation and their development was significantly influenced by hormonal treatment during the course of investigation. Significant shoot proliferation was observed in combination effect of cytokinin (BAP, Zeatin) and Auxin (NAA, 2,4-D) associated with MS basal salts .Perusal of Table-IV revealed maximum number of multiple shoots (32.5) combined with maximum number of viable shoots (21.8) by using 6.0mg/l zeatin in combination with 1.5 mg/l 2,4. Similar effects for multiple shoots with more number of viable shoots was pronounced with 6.5 mg/l BAP in combination with 0.25mg/l NAA (29.92) and 6.5 mg/l BAP in combination with 1.5mg/l 2,4-D (29.25) with 20.5 and 20.10 average number of viable shoots respectively. Absence of auxins or cytokinins in MS-medium was inhibitory for multiple shooting and number of viable shoots and recorded minimum number of multiple shoots ranging from 5.20 to 5.50. However addition of auxins revealed more pronounced effect in enhancing multiple shooting as compared to cytokinins. Individual effects of auxins revealed significant increase in number of multiple shoots up to 0.25mg/l NAA (18.04) and 1.5mg/l 2, 4-D (24.5). Similarly, amongst cytokinins BAP at 6.5mg/l and zeatin at 6.0mg/l recorded maximum significant effect on enhanced multiple shooting. The influence of cytokinins alone or in combination with low levels of auxins has been demonstrated during de-nova shoot bud organization in many species [10]. Available information in saffron points towards the role of plant growth regulators on shoot morphogenesis and support results of the present investigation [1,3,7]

Microcorm formation in vitro: Corm formation was characterized by four stages of growth after

transferring excised viable shoots in corm inducing medium (MS media + CCC) with 3-9% sucrose. These stages were defined as shoot culture development, elongation of basal portion of shoots, corm enlargement and corm maturation after 12 to 14 weeks of multiple shooting (April). Maximum number of corms (7.5) with initial weight of 4.1g after 6 weeks of sub-culturing on CCC media which advanced to 8.0 g after 7 weeks were observed with 0.2 ml/l CCC supplemented with 3% sucrose for first 6 weeks followed by 90 % sucrose to ensure gain in weight .Absence of CCC in MS-medium did not reveal any base swelling thereby indicating no corm development. Least number of corms (5.5) were observed with 0.4mg/l CCC (Table-V). Sucrose medium influenced corm formation and development indicating that the availability of sucrose as an energy source is one of the limiting factor the corm developm-ent in *in vitro* saffron. Higher concentration of sucrose favored higher frequency of flowering corms due to increase in efficiency of auxin moment [10] which in turn helps in tuberization. *in vitro* corm formation in saffron has been reported by several workers [1,7].

Field Evaluation: In vitro corms were subjected to primary hardening on 24± 1°C under white fluorescent light of 42 µmol/m2s1 with the photoperiod of 16 h light and 8 h dark for 8 to 10 weeks under laboratory aseptic conditions using coco peat and vermicompost as medium for growth followed by secondary hardening for 4 to 5 weeks under ambient conditions using sand (20%), vermicompost (30%), FYM (30%) and soil (20%) as medium for growth supplemented with water spray after every 3 to 5 days [7]. Evaluation of *in vitro* corms ss under actual field conditions of Saffron Research Station, Pampore with recommended INM dose revealed maximum survival percentage of 85.9 % with 74.3% flowering corms. Further evaluation for 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-VI). Pistil length ranged from 3.5 to 5.5 cm associated with high quality saffron confirming ISO grade 1 values for crocin, picrocrocin and saffranal.

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