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IN VITRO RESPONSE OF CORM AND NEEDLE SEGMENTS OF SAFFRON

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ABSTRACT

To trigger the morphogenetic response, corm and needle segments of saffron were cultured on different concentrations and combinations of phytohormones. Higher concentrations of sucrose (30 – 80 g/l) were also tried. BAP 13.2 µM + suc. 80g/l was found to be the most effective trial for induction and growth of calli from corm segments. However, differentiation of subcultured calli into minicormlets favouring highest cormlet number (13) was recognized on BAP (20 µM) + NAA (15 µM) and maximum multiple shoots were noticed on BAP (26.4 µM) + 50 g/l sucrose. Needle segments showed only non-regenerative moderate callus formation

Key words : Saffron , Corm segments, Needle segments, Callus, multiple cormlets, shoots.

Abbreviations : MS – Murashige and Skoog; BAP – 6 Benzyl- amino purine; NAA Napthalene acetic acid; Kn– Kinetin; 2, 4-D2, 4-di Chloro phenox-yacetic acid; IAA- Indole 3-acetic acid.

INTRODUCTION

The word Saffron has been derived from "azaferon" the Arabic name for this spice. Saffron is a legendary crop and associated as an important component of our culturally rich heritage.

Saffron (*Crocus sativus* L.) or Kesar is one of the most expensive spice. Moreover, it is valued as a medicinal herb, a dye and has a unique aroma for which it has been prized since antiquity. It belongs to the family Irridaceae and genus Crocus of which about 80spp. are so far known. It is a perennial slow growing cormose triploid genophyte unknown in wild state, reproduces only through corm because seeds are unknown (Karaoglu *et al.*, 2007).

Saffron, the world's most expensive spice, consists of the bright red stigmas 25-30^{m} long, which droops over the perianth segments. Saffron is predominantly used to give colour, flavour and aroma to food and specific chemical constituents have been identified. Crocin is responsible for the colour of saffron, whereas picrocrocin and Safranal are responsible for its bitter taste and aroma (Leung, 1980). Historically, it has also been used as an ingredient of fragrance, as a dye and in herbal medicine (Mathew 1983) but the high cost of the spice currently precludes its widespread use in the fields.

Antitumor effect of Saffron and its components *in vivo* and *in vitro* has been reported by a number of workers (Abdullaev and Frenkel, 1999; Anon., 2003). Reports of cancer fighting activity of Saffron have come

from Spain and it was found that it inhibited the growth of human tumour cells – a property that is attributed to carotenoid and crocin. Studies also reveal that metabolic crocin in saffron suppressed the development of colon cancer (Anonymous, 2003).

The major bottle neck of Indian saffron is its low productivity, high cost of production and susceptibility to various diseases (Munshi *et al.*, 1989). Hence development of modern technology for quick propagation methods and high yielding varieties is considered to be important for commercial point of view.

MATERIAL AND METHODS

Corm and Needle segments of Saffron, used as explants, were collected from Pampore (Kashmir) and were thoroughly washed with detergent cedepol (0.5% v/v) and tween 20 (surfactant) under running tap water followed by final rinsing with double distilled water. Subsequently these were surface disinfected with 70% ethanol for 20 sec. followed by 0.1 % $HgCl_2$ for 10 min and finally rinsed 3-4 times with autoclaved double distilled water to remove all traces of sterilant before implanting vertically on the nutrient medium.

The basal medium comprised of the mineral salts and organic nutrients of full and half strength Murashige and Skoog's medium (1962), 3% - 8% sucrose and 0.8% Difcobacto agar. Different concentrations of various phytohormones viz, BAP, NAA, Kn, and 2, 4-D were tried for different morphogenetic responses. All the medium constituents were added together and the pH of the medium was adjusted to 5.2 – 5.8 with 0.1N HCl or 0.1N NaOH and finally dispensed into 100ml Erlenmyer flasks (Borosilicate glass) plugged with non-absorbent cotton. Medium was autoclaved at a temperature of 121°C and 15 lb pressure for 20 minutes. The cultures were maintained at 25 ± 2 °C under 16 hr photoperiod provided by cool white fluorescent tubes (3000 lux).

All the experiments were carried out in completely randomized block design (CRD). In each treatment, ten replicates were used and each experiment was repeated thrice. Non parametric Kruskal Wallis and Mann- Whitney tests were applied to determine the difference in maximum multiple cormlets & shoots obtained on different concentrations of BAP, NAA, Kn , 2,4-D and sucrose. Significance of results were ascertained at $P < 0.001$ and $P < 0.005$ level.

RESULTS AND DISCUSSION

1. Response of Corm slices

Callus induction

Various degrees of callus induction were observed from corm slices both on MS½ and full strength medium supplemented with a

number of phytohormonal concentrations and combinations i.e. BAP + NAA; Kn + 2, 4 – D; NAA alone and varied conc. of sucrose + BAP (Table 1). Similar type of results were earlier recorded by Ding *et al.*, 1981 from corms of saffron but with higher (83-93%) induction frequency using MS medium containing

Table 1. In vitro culture response of Corm slices of Saffron to different concentrations and combinations of phytohormones.

Medium (Basal)	Sucrose g/l	Phytohormones (µM)		Callus	% Response (callus/ cormlet)	Cormlet No. Mean ± SD
MSx1/2		**BAP**	**NAA**			
	30	2	2	-	---	
	30	4	3	+	40	
	30	5	5	++	50	1.6 ± 0.2
	30	6	4	++	50	1.7 ± 0.2
	30	8	5	++	50	1.7 ± 0.2
	30	10	5	++	50	1.9 ± 0.3
	30	12	6	++	50	1.7 ± 0.2
	30	14	8	++	50	2.1 ± 0.1
	30	15	10	++	50	1.9 ± 0.0
	30	15	12	++	50	2.1 ± 0.4
	30	15	15	++	50	2.0 ± 0.2
	30	18	15	++	60	2.6 ± 0.1
	30	**20**	**15**	**++**	**90**	**4.4 ± 0.6***
	30	25	20	++	60	2.9 ± 0.3
MSx1/2		**Kn**	**2,4-D**			
	30	2	2	-	---	
	30	4	2	-	---	
	30	5	5	+	50	1.0 ± 0.0
	30	8	5	+	50	1.4 ± 0.1
	30	10	5	+	50	1.3 ± 0.2
	30	**15**	**10**	**+**	**50**	**1.5 ± 0.2**
	30	5	---	+	30	---
	30	8	---	+	40	---
	30	10	---	+	40	---
	30	12	---	++	50	---
	30	13.2	---	++	50	---
	30	15	---	++	40	---
	30	20	---	++	40	---
	30	22.4	---	++	40	---
	30	25	---	++	40	---
	30	26.4	---	++	50	---

MS	Sucrose g/l	BAP	NAA	Callus	% Response (callus/ cormlet)	Cormlet No. Mean ± SD
	30	2	---	-	---	—
	30	4	---	-	---	—
	30	5	---	+	30	—
	30	5.37	---	+	40	—
	30	10	---	+	40	—
	30	15	---	+	40	—
	30	2	---	-	---	—
	40	5	---	+	30	—
	40	8.2	---	+	30	—
	50	10	---	+	40	—
	60	13.2	---	+	40	—
	70	13.2	---	++	50	1.4 ± 0.2
	80	**13.2**	---	**+++**	**70**	**3.4 ± 0.4**
	50	15	---	++	50	1.9 ± 0.2
	70	15	---	++	50	1.7 ± 0.2
	80	15	---	++	50	1.7 ± 0.2
	80	20	---	++	40	1.5 ± 0.2
	30	---	2	-	---	
	40	---	5	+	30	1.0 ± 0.0
	50	---	5.37	++	50	2.3 ± 0.2
	60	---	8	+	40	—
	70	---	10	+	40	—
	80	---	15	+	40	—
	80	---	20	+	40	—

Ten replicates/ treatment; Data scored after 7 weeks. ---No response: + low: + + moderate: + + + high
*P < 0.001 Difference between mean values of maximum multiple cormlets obtained on 20µM BAP + 15µM NAA + 30g/l suc. were found to be significantly higher than 15µM Kn + 10µM 2,4-D + 30g/l suc.& 13.2µM BAP + 80g/l suc .using Kruskal Wallis test.

NAA/IAA/2, 4 - D alone at 1mg/l each. Callus induction has also been reported from pistils of saffron after using BAP + NAA (1mg/l) (Hori *et al*., 1988) but in present study higher degree of callus formation was noticed on BAP 13.2 µM + suc. 80g/l on full strength MS medium (Fig. 1) with 70% induction frequency. It is not only the phytohormones which play a significant role in morphogenetic responses in *in vitro* cultures but it depends on a complex system of endogenous and exogenous interacting factors so is the case with present study where increased sucrose level along with BAP proved fruitful for maximum callus induction.

Fig. 1. Callus formation in corm slices on MS+BAP 13.2µM + suc. 80 g/l.

Fig. 2. Mini cormlet induction in callus raised from corm slices on MS ½ + BAP 20µM + NAA 15µM.

Callus differentiation

Callus cultures after 4-6 weeks time period started showing induction of minicormlets on the same medium in some trials (Table 1), best trial being MS ½ + BAP 20µM + NAA 15µM where highest mean cormlet number 4.4 ± 0.6 was registered with high percentage response (90%) (Fig. 2). Use of Kn with 2, 4- D concentrations in MSx1/2 proved to be less effective for both cormlet induction and cormlet number but only callus induction was observed which are in consonance with Vishvanath *et al.* (1994) who also reported callus induction from floral buds on MS medium fortified with Kn and 2, 4- D. Various BAP and NAA concentrations were also used with higher sucrose level ranging from 40 - 80g/l independently and the most favourable trial found was BAP 13.2µM with 80g/l suc. which resulted in the highest cormlet number (3.4 ± 0.4) with 70% induction frequency (Fig. 3). It seems here that not only phytohormones play a major role in organogenesis but also high conc. of carbon source i.e. Sucrose 80g/l, resulted in highest cormlet number.

Fig. 3. Mini cormlet induction in callus raised from corm slices on MS + BAP 13.2µM + suc. 80g/l.

Fig. 4. Micro cormlet induction in subcultured callus on MS + BAP 26.4µM.

Callus subculture

1) *In vitro* mini cormlet production from callus cultures

Microcormlet development in subcultured callus was observed on various concentrations of phytohormones and sucrose. Callus obtained from corm segments was mainly used for the initiation of microcormlets *in vitro* under the influence of different phytohormones. Low to high callus proliferation was recorded in many cases (Table 2). Cormlet induction from

callus cultures was initially observed as glistering swellings. The cormlets induced were propagated on the same medium for further growth. Amongst these trials MS+BAP 26.4μM has been found most effective favouring 6.4 ± 0.8 mini cormlet induction (Fig. 4). Perusal of literature shows that Blazquez *et al.* (2004) have reported TDZ (0.1mg/l) to be much more efficient chemical for the production of micro corms and helped in the production of 60% regenerants with

Table 2. *In vitro* cormlet induction in sub cultured callus on the various phytohormonal concentrations and combinations.

Medium (Basal)	Sucrose g/l	Phytohormones (μM)	Callus proliferation	Max. Shoot No.	% Response (cormlet)	Mean ± SD Cormlet No.
MS	30	**BAP**				
	30	2	-	---	---	---
	30	4	+	---	20	---
	30	5	+	---	20	---
	30	8	+	---	20	---
	30	10	+	---	20	---
	30	12	+	---	20	---
	30	13.2	+	---	20	---
	30	15	+	---	30	---
	30	20	+	---	40	2.1 ± 0.7
	30	25	++	---	40	2.1 ± 0.3
	30	**26.4**	**++**	**2**	**50**	**6.4 ± 0.8[a]**
MS		**NAA**				
	30	2	-	---	---	---
	30	4	-	---	---	---
	30	5	+	---	---	---
	30	5.37	++	4	60	3.5 ± 0.4
	30	10	+	2	50	2.9 ± 0.4
	30	15	+	2	50	2.3 ± 0.2
MS		**BAP**				
	30	2	-	---	---	---
	40	5	+	---	40	---
	40	8.2	+	---	40	---
	50	10	+	---	40	---
	70	13.2	++	---	30	---
	80	13.2	++	---	30	---
	50	15	++	4	40	1.3 ± 0.1
	70	**15**	**++**	**15**	**60**	**4.6 ± 0.4**
	80	15	++	10	40	2.8 ± 0.3
	80	20	++	4	40	1.3 ± 0.0
MS		**NAA**				
	30	2	-	---	---	---
	50	5	+	2	30	1.6 ± 0.3
	50	5.37	++	12	50	3.7 ± 0.4
	60	8	++	8	40	2.3 ± 0.2

	70	10		++	6	30	1.3 ± 0.1
	80	15		++	4	30	1.4± 0.2
MS		**BAP**	**NAA**	**Callus proliferation**	**Max. Shoot No.**	**% Response (cormlet)**	**Mean ± SD Cormlet No.**
	30	1	1	-	---	---	
	30	2	2	+	---	30	---
	30	3	3	+	---	30	---
	30	4	3	+	---	40	---
	30	5	5	+	---	40	---
	30	10	8	+	---	30	---
	30	15	10	++	---	50	1.5 ± 0.1
	30	20	15	++	---	70	3.3 ± 0.2
	30	25	20	++	---	60	3.4 ± 0.3
MSx1/2		**Kn**	**2,4- D**				
	30	2	2	-	---	---	---
	30	4	2	-	---	---	---
	30	5	5	+	---	30	1.4 ± 0.3
	30	8	5	+	---	30	1.1 ± 0.1
	30	10	5	+	---	30	1.4 ± 0.1
	30	15	10	+	---	30	2.1± 0.2
	30	2	2	-	---	---	---
	30	4	2	-	---	---	---
	30	5	5	-	---	---	---
	30	8	5	-	---	---	---
	30	10	5	-	---	---	---

Ten replicates/ treatment; Data scored after 7 weeks. ---No response:+ low: ++ moderate
[a]P < 0.005 Difference between mean values of maximum cormlets obtained on 26.4µM BAP + 30g/l suc. were found to be significantly higher than 15µM BAP + 70g/l suc. using Mann-Whitney test.

fully developed leaf primordial than BAP (2mg/l) with only 20% regenerants capacity. The major factor influencing the proliferation rate is the interaction of the physiological state of the plant material with the culture medium and its additives. Effect of growth regulators is not specific in most cases. Even different growth regulators belonging to the same class may elicit different morphogenetic response in a given tissue (Bhan, 1998), so is the case with present studies where BAP, Kn belonging to the same class i.e. cytokinin and TDZ being non purine cytokinin like compound differs in their responses. Effect of higher sucrose concentration has also been evaluated either with BAP or NAA concentrations. Amongst these trials maximum cormlet number induced was 3.7 ± 0.4 on MS + NAA 5.37 µM + suc. 50g/l with high percent response (80%) (Fig. 5).

Fig. 5. Micro cormlet induction in subcultured callus on MS + NAA 5.37µM + suc.50g/l.

Fig. 6. Multiple shoot induction in subcultured callus on MS + BAP 26.4µM + suc. 50g/l.

2) Multiple shoot induction

Organogenetic potential of saffron callus cultures varied on different phytohormonal trials. Callus proliferation continued in many trials (Table 3). Multiple shoot induction from subcultured callus raised from corm slices was obtained in a number of cases. In many cases it was also accompanied by cormlet induction. Highest number of multiple shoot induction 4.6 ± 0.4 was recorded on MS+BAP 26.4µM + suc. 50g/l with 60 percent induction response (Fig. 6). It was followed by NAA 5.37µM + suc. 50g/l, here the number was 3.7 ± 0.4 with 50 % induction frequency (Table 3). It seems here that while using BAP and NAA alone it resulted in maximum multiple shoot induction but at the same time in both cases elevated concentration of sucrose was used and it is not only the phytohormones but combined interaction of both phytohormones and high carbon level which plays a significant role in getting maximum multiple shoots. Alicchio *et al.* (1982) held the opinion that organogenesis in *in vitro* cultures depends on a complex system of endogenous and exogenous interacting factors which is also depicted in present observations.

2. Response of Needle bases

Needle bases from actively growing corms were also used to study their callogenic and regenerative potential on different growth regulators (Table 4). Callus induction was found in few trials and the degree of callus formation ranged from low to moderate. MS ½ + Kn10µM + 2, 4-D 15µM + suc. 30g/l favoured moderate callus formation and the percent induction was 60% (Fig. 7). In contrast to corm slice, no organogenesis in the form of cormlet or shoot induction was recorded. The callus after subculturing on MSx1/2 basal medium failed to show any organogenetic potential and continued to be unorganized type of callus only.

Fig. 7. Callus formation in needle bases on MS ½ + Kn 10µM + 2, 4-D 15µM.

Legends 1-7. In vitro culture response of corm slices and needle segments of Saffron.

Fig. 1. Callus formation in corm slices on MS+BAP 13.2µM + suc. 80 g/l.

Fig. 2. Mini cormlet induction in callus raised from corm slices on MS ½ + BAP 20µM + NAA 15µM.

Fig. 3. Mini cormlet induction in callus raised from corm slices on MS + BAP 13.2µM + suc. 80g/l.

Fig. 4. Micro cormlet induction in subcultured callus on MS + BAP 26.4 µM.

Fig. 5. Micro cormlet induction in subcultured callus on MS + NAA 5.37µM +suc. 50g/l.

Fig. 6. Multiple shoot induction in subcultured callus on MS + BAP 26.4µM + suc. 50g/l.

Fig. 7. Callus formation in needle bases on MS ½ + Kn 10µM + 2, 4-D 15µM.

Table 3. Multiple shoot/ cormlet induction in subcultured callus of Saffron

Medium (Basal)	Sucrose g/l	Phytohormones (µM)		Callus proliferation	Max. Cormlet No.	% Response (shoot)	Shoot No. Mean ± SD
MS ½		**BAP**	**NAA**				
	30	2	2	-	---	---	
	30	4	2	+	---	30	
	30	5	5	+	---	50	1.6 ± 0.0
	30	8	5	+	---	40	1.7 ± 0.0
	30	**10**	**5**	**+**	**---**	**50**	**1.8 ± 0.4**
	30	15	10	++	---	40	1.2 ± 0.2
	30	20	15	++	---	40	1.5 ± 0.3
	30	20	20	++	---	50	1.7 ± 0.0
MS		**BAP**					
	30	2	---	-	---	---	---
	40	5	---	+	---	40	---
	40	8	---	+	---	40	---
	50	10	---	+	---	40	---
	50	13.2	---	++	6	30	---
	50	15	---	++	6	30	---
	50	20	---	++	6	40	1.3 ± 0.1
	50	**26.4**	**---**	**++**	**10**	**60**	**4.6 ± 0.4***

	60	26.4	---	++	6	40	2.8 ± 0.3
	70	30	---	++	4	40	1.3 ± 0.0
MS		**NAA**					
	30	2	---	-	-	---	---
	40	5	---	+	6	30	1.6 ± 0.3
	50	**5.37**	**---**	**++**	**8**	**50**	**3.7 ± 0.4**
	60	10	---	++	8	40	2.3 ± 0.2
	70	15	---	++	6	30	1.3 ± 0.1
	80	20	---	++	6	30	1.4± 0.2

Ten replicates/ treatment; Data scored after 7 weeks. ---No response: + low: ++ moderate
***P < 0.001 Difference between mean values of maximum multiple shoots obtained on 26.4µM BAP + 50g/l suc. were found to be significantly higher than 5.37µM NAA + 50g/l suc.& 10µM BAP + 5µM NAA + 30g/l suc .using Kruskal Wallis test.**

Table 4. Callus induction from needle segments under the effect of growth regulators

Medium (Basal)	Sucrose g/l	Phytohormones (µM)		Callus	% Response
MS		**BAP**	**NAA**		
	30	2	2	-	---
	30	5	5	-	---
	30	10	5	-	---
	30	15	10	-	---
	40	20	15	-	---
	50	0	5.37	-	---
MS ½		**BAP**	**NAA**		
	30	2	2	-	---
	30	5	2	-	---
	30	5	2	+	30
	30	10	5	+	20
	30	15	10	+	40
	30	20	15	++	50
MS ½		**Kn**	**2,4-D**		
	30	2	1	+	20
	30	4	5	+	40
	30	5	8	+	40
	30	10	15	++	60
	30	15	10	+	40

Ten replicates/ treatment; Data scored after 7 weeks- no response: + low: ++ moderate

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