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IN VITRO PRODUCTION OF FLORAL BUDS AND STIGMA-LIKE STRUCTURES ON FLORAL ORGANS OF *CROCUS SATIVUS* L.

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Abstract

Floral parts of *Crocus sativus* L., were cultured on MS medium supplemented with various concentrations of kinetin and naphthalene acetic acid. Excised explants from style, perianth, stamen filament and ovary showed different reactions *in vitro*. Stigma-like structures appeared at different frequencies directly or indirectly through meristematic tissue on colourless style and perianth explants. Direct stigma-like structures were frequently produced in the form of bunched groups and intensely pigmented at the basal cut ends of colourless style and perianth explants. Indirect stigma-like structures usually regenerated in large numbers on the surface of the calli and up to 100 per explant were obtained on colourless style explants in medium containing 5 mgl^{-1} of kinetin and 5 mgl^{-1} of naphthalene acetic acid. After successive subculturing or continuous cultures on solid media, complete or incomplete neoformed floral buds formed on colourless style and perianth explants in media with high concentrations of kinetin. At higher concentrations of both kinetin and naphthalene acetic acid many indirect stigma-like structures were also produced on stamen filament and colourful style explants.

Introduction

Saffron that is one of the most expensive spices, is a term used for three branched and red stigma of flowering corms of *Crocus sativus* L. *C. sativus* is a perennial herbaceous and also ornamental plant belonging to Iridaceae family. Because of saffron spice production, this plant is highly important in the economies of countries that have specific agro-climatic conditions for planting and maintenance. Saffron has three main secondary metabolites, including water soluble carotenoid pigments (mainly crocin), bitter taste glycoside (picrocrocin) and spicy aroma (safranal). The proper proportions of these compounds determine the high quality of a saffron. It has a wide variety of uses in food industries as a flavouring and colouring agent and also a herbal medicine in some countries. Therefore it seems that *in vitro* proliferation of saffron stigma should be a potentially useful technique for industrial production of this precious spice (Sano & Himeno, 1987). Besides, establishment of cultures with high regeneration capacity of stigma-like structures is a critical step in order to develop a biotechnological method for saffron production (Fakhrai & Evans, 1990; Sarma *et al.*, 1990; Sarma *et al.*, 1991; Visvanath *et al.*, 1990).

In vitro production of stigma-like organs by using different floral explants of *C. sativus* has been the subject of much attention in the recent years. Attempts have also been made to induce saffron in tissue cultures from ovaries (Himeno & Sano, 1987; Sano & Himeno, 1987; Sarma *et al.*, 1991) and stigmas (Koyama *et al.*, 1988; Hori *et*

al., 1988; Sarma *et al.*, 1990). Fakhrai & Evans (1990) and Han & Zhang (1993) induced stigma-like structures successfully by using different floral explants of *C. sativus*.

Lu *et al.*, (1992) showed the effect of age and variety of explants as well as exogenous hormones on regeneration of style-stigma-like structures in *C. sativus*. The high yields of stigma-like structures and saffron pigments were obtained from corolla tube and ovary explants in a modified MS medium by Otsuka *et al.*, (1992). Recently, Yyongjiong *et al.*, (1996) and Ebrahimzadeh *et al.*, (1996) also reported the regeneration of stigma-like structures from young perianth and style explants with considerable frequencies.

The present report describes the *in vitro* morphogenic and organogenic potentials of different floral explants of *C. sativus* for the production of neoformed organs, specially stigma-like structures.

Materials and Methods

Plant materials: The bulbs of *C. sativus* collected from the saffron field of Gonabad, Khorassan province, Iran were brought into the laboratory in the second half of November 1997.When the flower buds were 5-10 cm long, they were excised from the corms of the plants using microscissors. They were then thoroughly washed under running tap water and sterilized by dipping in 70% ethanol for 5 min., followed by dipping in 1% sodium hypochlorite for 10 min., and rinsed 3 times with sterile distilled water. The flower buds were cut and the floral parts carefully removed and separated into perianths, ovaries, stamens, styles and stigmas. The styles were then separated into two parts by an incision just below the point at which the pigmentation develops. Both the non-pigmented parts (colourless style) and pigmented parts (colourful style) were cultured. The explants were immediately transferred on agar media.

Culture media: The explants were planted on MS semi-solid medium (gelled with 0.8 % microbiological agar-agar (Merck)) supplemented with 3% sucrose, Kin and NAA in 16 different combinations. The pH of the medium was adjusted to 5.7 prior to autoclaving and sterilized at 1.05 kg cm^{-2} for 20 min., and then dispensed into glass Petri dishes. All cultures were maintained in a controlled environment culture room at 25 $\pm$ 2°C in the dark. For each hormonal combination in the medium, there were 30 explants (5 explants in each Petri dish) for each floral part.

After each 45 days (from the date of culture) the explants were subcultured in a similar fresh medium. The number of the fragments that showed a unique morphogenic response were calculated as percent of the total numbers for each explant in each treatment.

Statistical analysis: Statistical analyses were performed using Anova program of the MSTAT-C version 2.1 package. The results were subjected to a statistical analysis at the 5% level using Duncan's Multiple Range Test (DMRT). All graphs were drawn using HG3 package.

Results

Tissue culture of floral explants (perianth, stamen filament, anther, stigma, ovary, colourless and colourful parts of style) on MS media supplemented with Kin (0, 1, 5, 10 mgl^{-1}) and NAA (0, 1, 5, 10 mgl^{-1}) alone or in combinations showed different results. Some explants formed new stigma-like structures and also floral buds in some of the used hormonal combinations.

Colourless style explants: After 45 days, many stigma-like structures were formed directly on the colourless style explants in media containing different concentrations of Kin (1, 5, 10 mg l^{-1}), particularly when NAA concentration in medium was low. These structures mostly originated from the basal cut end of explants in the form of bunched groups and intensely pigmented (Fig. 1). The highest frequency for the production of direct stigma-like structures was observed in medium containing 5 mg l^{-1} Kin and 5 mg l^{-1} NAA (Fig. 9 and Table 1). Maximum number of direct stigma-like structures produced per explants was 20 at a concentration of 10 mg l^{-1} Kin and 1 mg l^{-1} NAA.

With increasing NAA concentration in medium (5 mg l^{-1} or greater) and after 2 months, the formation of indirect-type of stigma-like structures (through meristematic tissue) were observed on colourless style explants. At high concentrations of Kin and NAA the response percentage also increased significantly, so that 5 mg l^{-1} Kin in combination with 5 mg l^{-1} NAA was advantageous to regeneration of these structures. By using this hormonal combination, induction frequency reached to 63% (Fig. 10 and Table 1). Maximum number of these structures were 100 per colourless style explant (Fig. 2).

The most interesting observation in tissue culture of colourless style explants was the formation of floral buds on these explants after 5 months. Complete or incomplete floral buds (eg., flowers with only a three branched stigma and a perianth) were formed on colourless style explants in media containing high concentrations of Kin with considerable frequencies (13% in medium containing 5 mg l^{-1} Kin 1 mg l^{-1} NAA and 17% in medium containing 10 mg l^{-1} Kin 1 mg l^{-1} NAA) (Figs. 3, 11 and Table 1).

Perianth explants: Formation of direct stigma-like structures, normally appeared at the base of perianth explants which occurred at high concentrations of Kin and NAA (Fig. 4). The highest frequency (23%) for the production of these structures was obtained in a medium containing 5 mg l^{-1} Kin and 10 mg l^{-1} NAA (Fig. 9 and Table 1).

Like colourless style explants, callus production and subsequently formation of indirect stigma-like structures on these explants was dependent on the increase in the Kin and NAA concentration. At high concentrations of NAA (5 mg l^{-1} or higher) the formation of these structures on perianth explants was greatly enhanced, so that the percentage of responded explants reached to maximum rate (67%) in medium with 5 mg l^{-1} Kin and 10 mg l^{-1} NAA (Fig. 10 and Table 1).

Floral buds were also produced in a higher range of Kin and NAA concentrations on perianth explants. The formation of floral buds on perianth explants was more sensitive to concentration of Kin in the medium (Figs. 5, 11 and Table 1).

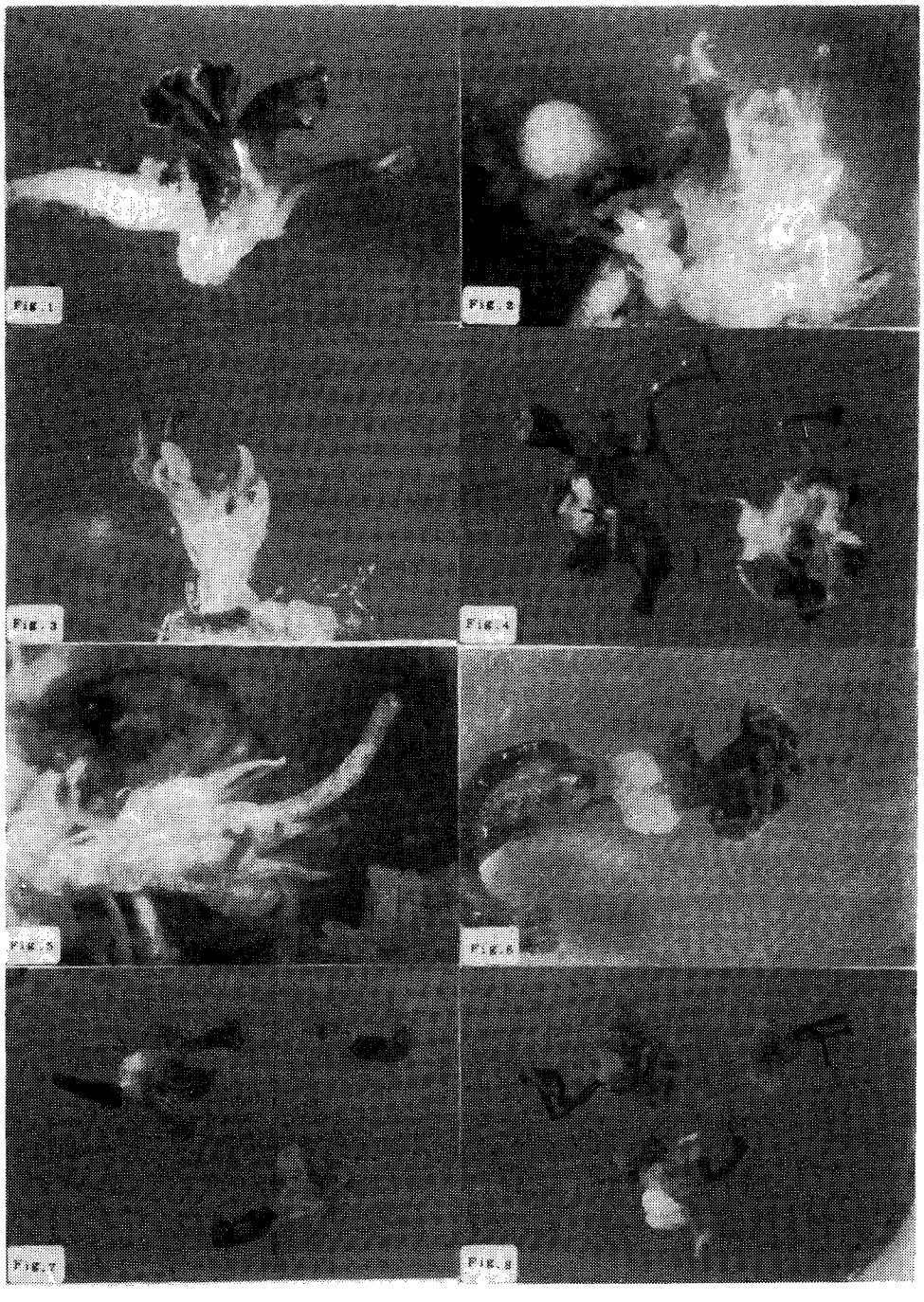

Fig. 1. Production of direct stigma-like structures on colourless style explant after two months on MS medium with 5 mgl $^{-1}$ Kin and 1 mgl $^{-1}$ NAA.

(Contd.)

Fig. 2. Production of indirect stigma-like structures on colourless style explant after two months on MS medium supplemented with 10 mgl^{-1} Kin and 5 mg l^{-1} NAA.

Fig. 3. Production of newly formed flowers on colourless style explant after five months on MS medium supplemented with 5 mg l^{-1} Kin and 1 mg l^{-1} NAA.

Fig. 4. Production of direct stigma-like structures on perianth explants after three months on MS medium supplemented with 5 mg l^{-1} Kin and 1 mg l^{-1} NAA.

Fig. 5. Production of newly formed flower on perianth explant after seven months on MS medium supplemented with 5 mg l^{-1} Kin and 5 mg l^{-1} NAA.

Fig. 6. Production of completely differentiated direct stigma-like structures from the base of stamen filament explant after three months on MS medium supplemented with 5 mg l^{-1} Kin and 1 mg l^{-1} NAA.

Fig. 7. Production of semi-organized calli and indirect stigma-like structures on stamen filament explants after three months on MS medium supplemented with 1 mg l^{-1} Kin and 5 mg l^{-1} NAA.

Fig. 8. Formation of semi-organized calli and indirect stigma-like structures on colourful style explants on MS medium with 5 mg l^{-1} Kin and 5 mg l^{-1} NAA.

Stamen filament explants: Formation of direct stigma-like structures on stamen filament explants was only restricted to two hormonal combinations. This probably was due to the establishment of a suitable hormonal ratio in these media (Figs. 6, 9 and Table 1). In contrast to direct stigma-like structures, the formation of indirect ones was completely dependent on the type of hormonal combination. Stamen filament explants produced semi-organized calli at their bases when they were cultured on media containing high concentrations of NAA. Indirect stigma-like structures formed subsequently on the surface of these calli. The best results were obtained on fragments that were cultured on media containing 1 mg l^{-1} Kin 10 mg l^{-1} NAA and 1 mg l^{-1} Kin 5 mg l^{-1} NAA with frequencies of 50% and 30%, respectively (Fig. 10 and Table 1). These stigmata synthesized a lot of intense orange pigments (crocin) and grew to 2.5-3 mm long after 3 months (Fig. 7).

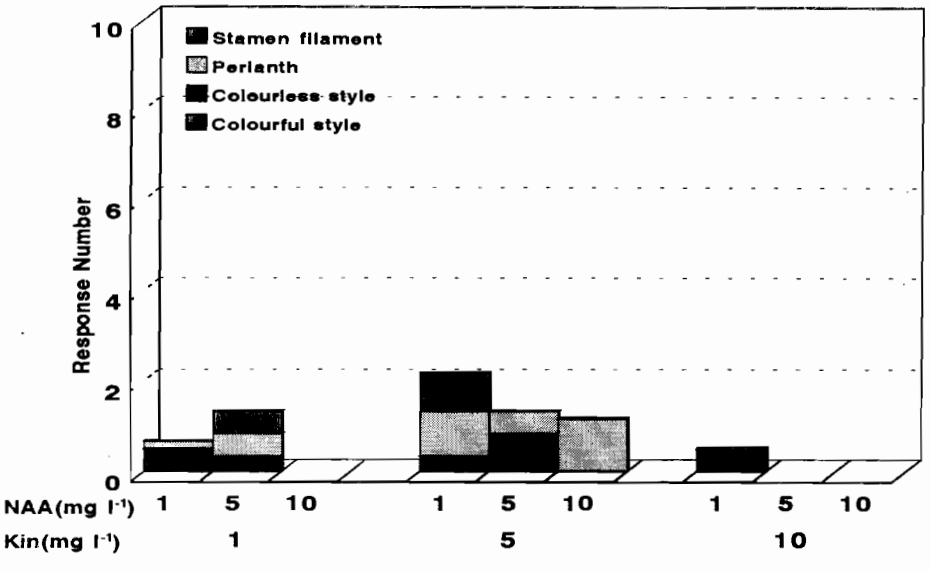

Fig. 9. Effect of different concentrations of Kin and NAA on the formation of direct stigma-like structures the floral explants. The values reported are the mean of six replicates.

Table 1. Effect of different hormonal combinations of Kin and NAA on morphogenic responses of floral explants of *C. sativus* L. cultured on MS medium. The values represent both the percentage and the mean numbers of responded explants.

	Morphogenic Response											
	Direct stigma				Indirect stigma				Floral bud			
Explant* Growth Regulator* (mg l^{-1})	cfS	csS	P	SF	cfS	csS	P	SF	cfS	csS	P	SF
Kin + NAA												
0+0	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
0+1	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
0+5	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
0+10	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
1+0	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
1+1	$0^{e}(0)$	$10^{bc}(0.5)$	$3.3^{de}(0.17)$	$0^{e}(0)$	$0^{K}(0)$	$10^{i-k}(0.5)$	$6.6^{i-k}(0.33)$	$3.3^{ik}(0.17)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
1+5	$0^{e}(0)$	$6.6^{cd}(0.33)$	$10^{bc}(0.5)$	$10^{bc}(0.5)$	$33^{c-f}(1.67)$	$17^{g-i}(0.83)$	$27^{fg}(1.33)$	$33^{c-f}(1.67)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
1+10	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$47^{cd}(2.33)$	$10^{i-k}(0.5)$	$17^{g-i}(0.5)$	$50^{bc}(2.5)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
5+0	$0^{e}(0)$	$3.3^{de}(0.17)$	$0^{e}(0)$	$0^{e}(0)$	$0^{k}(0)$	$0^{k}(0)$	$0^{k}(0)$	$0^{k}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
5+1	$0^{e}(0)$	$6.6^{cd}(0.33)$	$20^{a}(1)$	$17^{ab}(0.83)$	$33^{c-f}(1.67)$	$10^{i-k}(0.5)$	$33^{c-f}(1.67)$	$20^{f-h}(1)$	$0^{e}(0)$	$13^{ab}(0.67)$	$3.3^{de}(0.17)$	$0^{e}(0)$
5+5	$0^{e}(0)$	$17^{ab}(0.83)$	$10^{bc}(0.5)$	$0^{e}(0)$	$43^{c-e}(2.17)$	$63^{ab}(3.17)$	$30^{ef}(1.5)$	$17^{g-i}(0.83)$	$0^{e}(0)$	$0^{e}(0)$	$10^{bc}(0.5)$	$0^{e}(0)$
5+10	$0^{e}(0)$	$0^{e}(0)$	$23^{a}(1.17)$	$0^{e}(0)$	$30^{d-f}(1.5)$	$17^{g-i}(0.83)$	$67^{a}(3.3)$	$10^{h-k}(0.5)$	$0^{e}(0)$	$0^{e}(0)$	$6.6^{cd}(0.33)$	$0^{e}(0)$
10+0	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{K}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
10+1	$0^{e}(0)$	$10^{bc}(0.5)$	$0^{e}(0)$	$0^{e}(0)$	$33^{c-f}(1.67)$	$23^{fg}(1.16)$	$33^{c-f}(1.67)$	$20^{f-h}(1)$	$0^{e}(0)$	$17^{a}(0.83)$	$3.3^{de}(0.17)$	$0^{e}(0)$
10+5	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$10^{h-k}(0.5)$	$10^{h-k}(0.5)$	$13^{g-j}(0.67)$	$23^{ef}(1.16)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$
10+10	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$33^{c-f}(1.67)$	$6.6^{i-k}(0.67)$	$0^{k}(0)$	$6.6^{i-k}(0.67)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$	$0^{e}(0)$

*Abbreviations: cfS-colourful style, csS-colourless style, P-perianth, SF-stamen filament, Kin-kinetin, NAA-α-naphthalene acetic acid.

The means in columns that are not followed by the same letters are significantly different at the 5% level using Duncan's Multiple Range Test (DMRT).

Values in parentheses represent the mean numbers of responded explants in each treatment.

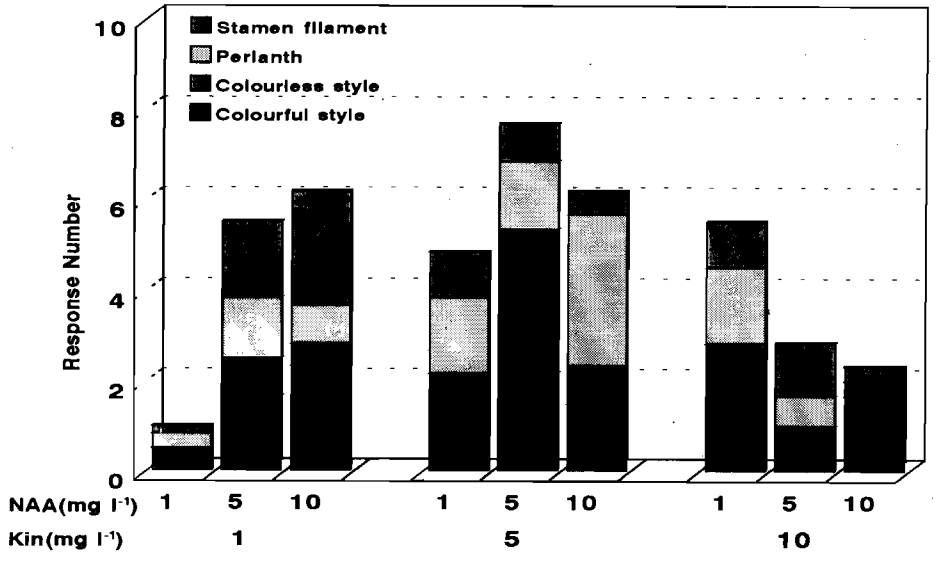

Fig. 10. Effect of different concentrations of Kin and NAA on the formation of indirect stigma-like structures on the floral explants. The values reported are the mean of six replicates.

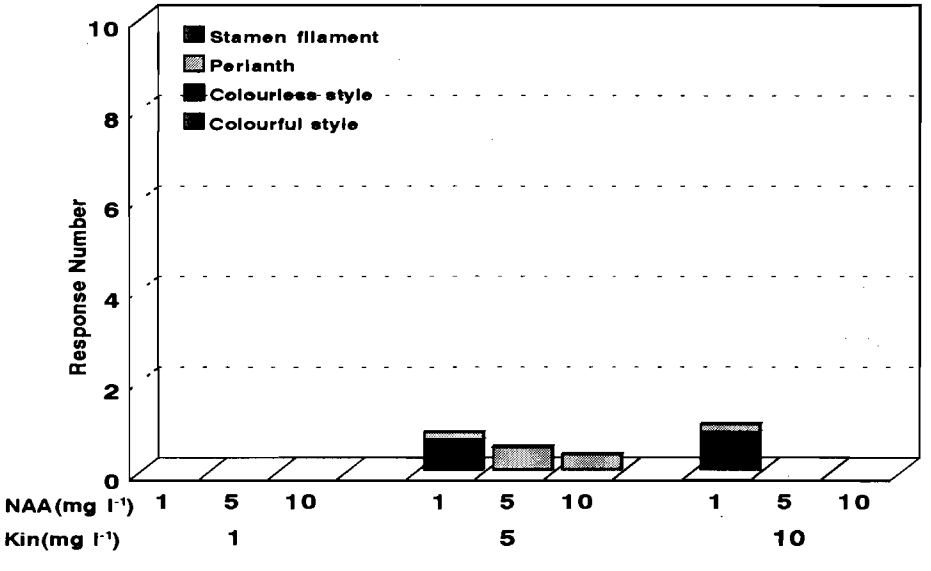

Fig. 11. Effect of different concentrations of Kin and NAA on the formation of neoformed floral buds on the floral explants. The values reported are the mean of six replicates.

Colourful style explants: The young colourful style explants produced pigmented and semi- organized calli when cultured on the media containing high concentrations of Kin or NAA or both. After a few weeks the formation of fully pigmented stigma-like structures were observed on these callus-like tissues. Although a large number of these stigma-like structures were produced on each callus, the length of these structures did not exceed 2 mm. The highest number of stigma-like structures produced per explant was 50 for a medium containing 5 mgl^{-1} Kin and 5 mgl^{-1} NAA (Fig. 8).

The frequency of morphogenic responses of colourful style explants in MS media with different hormonal combinations is shown in Fig. 10 and Table 1.

Only callus production was observed on intact ovaries in all of the hormonal combinations used. The rate of callus production increased at higher concentrations of Kin and specially in NAA.

Discussion

The results showed that at higher concentrations of NAA, callus production increased markedly on all of the explants that represented a morphogenic response. The importance of NAA as a growth regulator for induction of callus on corm and some floral explants of *C. sativus* has also been demonstrated by some workers (Ding *et al.*, 1981; Sano & Himeno, 1987; Sarma *et al.*, 1990).

In spite of some similarities in frequency and type of neoformed organs among the colourless style and perianth explants, they were different in size, shape and rate of pigmentation on each explant. The length of directly formed stigma-like structures on colourless style explants was much longer than that of those on perianth explants. The stigma-like structures that were formed directly on colourless style explants became intensely pigmented and grew up to 5 or 6 mm long (usually one-fourth to one-third of naturally grown stigmas). They resemble the natural ones in appearence.

With increasing the NAA concentration in the medium, callus formation and subsequently regeneration of indirect stigma-like structures were promoted on colourless style and perianth explants. Callus formation, mainly pigmented calli and regeneration of them to large numbers of indirect stigma-like structures were also observed at high frequencies on stamen filament and colourful style explants in many of the media, specially those with high concentrations of Kin and NAA.

It is interesting to note that subsequent to successive subcultures and with continuous culture on solid media, some newly formed floral buds regenerated on 5 or 6 months old colourless style and perianth explants in media containing high concentration of Kin. It seems that some internal and external factors such as physiological age, establishment of a suitable hormonal balance in explants, the type of explant and also existence of high concentrations of exogenic kinetin were effective factors in the regeneration process of neoformed flowers on these explants. The stimulant effect of some growth regulators specially kinetin for the regeneration of floral buds in *C. sativus* plant has previously been proved by Azizbekova *et al.*, (1978). These results show that flowering in this plant can be promoted by the use of exogenic kinetin.

Our results indicate that most of the floral parts have inherent potentials for the production of neoformed organs specially, direct and indirect stigma-like structures.

This potential can be successfully utilized for *in vitro* production of these structures with greater frequencies.

No major response was observed when mature anther explants and fully pigmented stigmata were cultured with different hormonal combinations. This probably was due to their specific physiological age.

The major advantage of this approach is the high *in vitro* production frequencies of stigma-like structures and the formation of floral buds on some of the floral explants, an observation which has not been reported previously.

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