Development of cormogenic nodules and microcorms by tissue culture, a new tool for the multiplication and genetic improvement of saffron

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Original article

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Abstract – In order to develop a continuous system for saffron micropropagation by the induction of nodular cormogenic callus in vitro, different plant growth regulators as well as organic supplements have been evaluated in relation to their capacity to regulate morphogenesis and plant regeneration in cultures of meristematic tissues from saffron corms. Microsurgery of the apical meristematic bud in corms prior to culture increased the induction of cormogenic nodules. High concentrations of BA (2 $mg \cdot L^{-1}$) and low of 2,4–D (0.1 $mg \cdot L^{-1}$) were found to be essential for development and proliferation of cormogenic nodules. The application of pachlobutrazol and imazalil increased the induction rate of adventitious shoots in the nodular cormogenic calli and the growth of microcorms. The corms with adventitious shoots were rooted in medium without growth regulators and were able to generate dormant microcorms in vitro. This technique could be of interest for the multiplication as well as the genetic improvement of saffron crop. (© 1999 Inra/Éditions scientifiques et médicales Elsevier SAS.)

***Crocus sativus* / imazalil / micropropagation / saffron corm / paclobutrazol**

Résumé – Développement de nodules bulbeux et de microbulbes par culture de tissus, un nouvel outil pour la multiplication et l'amélioration génétique du safran. Afin de développer un système pour la micropropagation en continu du safran par l'induction de cals bulbeux nodulaires in vitro, différents régulateurs de croissance et des suppléments organiques ont été évalués en relation avec leur capacité de réguler la morphogenèse et la regénération des plantes dans des cultures de méristèmes provenant de bulbes de safran. La microchirurgie appliquée sur le bourgeon du

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méristème apical du bulbe avant sa mise en culture a vu augmenter l'induction des nodules bulbeux. Des concentrations élevées de AB et faibles, de 2,4 D se sont avérées essentielles pour le développement et la prolifération des nodules bulbeux. L'application de pachlobutrazol et d'imazalil a augmenté la taux d'induction des pousses adventices dans les cals nodulaires de même que la croissance des microbulbes. Les bulbes munis de pousses adventices qui avaient été enracinés en milieu sans régulateurs de croissance ont été capables de générer des microbulbes dormants in vitro. Cette technique pourrait être d'intérêt pour la multiplication ainsi que pour l'amélioration génétique de safran. (© 1999 Inra/Éditions scientifiques et médicales Elsevier SAS.)

***Crocus sativus* / imazalil / micropropagation / bulbe de safran / paclobutrazol**

1. Introduction

Saffron, the dry stigmata of *Crocus sativus* is probably the most expensive food additive [1]. The saffron plant is triploid and sterile, hence its improvement through breeding has not been possible [3]. This plant blooms in autumn and the rest of its growing season constitutes the initiation, filling up and maturing of the daughter corms. As a geophyte it has a slow growth and exclusively vegetative propagation as described before, forming only three to four cormlets each season. This fact in addition to the presence of endogenous infections in many of the corms currently used for production is a strong limiting factor in the application to this culture of new techniques both in the field and in other systems of controlled cultivation. In this context, tissue culture methods offer a great potential for the large scale propagation of saffron and its genetic improvement.

Although several reports on the regeneration of saffron plants in vitro have been made [4, 6, 10, 20], many of them have dealt only with organogenesis or embryogenesis, with scant attention to the production of corms, the most wanted final product from a practical point of view. In this work we present a continuous tissue culture system for the production of saffron corms by the induction of nodular cormogenic callus, plantlet regeneration and microcorms. This tissue culture technique applied to the production of saffron could be used to improve the production of this spice by the large scale propagation of selected pathogen-free ecotypes, the recovery of new genotypes by somaclonal variation or in vitro mutagenesis, and the application of genetic engineering to this crop.

2. Materials and methods

Resting corms of an homogeneous cultivar of saffron (*C. sativus* L.) were collected from local farmers at Albacete, Spain. Healthy resting corms were rinsed in running tap water for 1–2 h, then washed in distilled water, dipped in 80 % ethanol for 25 s and rinsed three times in sterile distilled water. Afterwards, the corms were surface sterilized for 20 min in a 0.8 % solution of sodium hypochlorite under sonication and then rinsed three times with sterile distilled water.

For the initiation of the cultures, the meristematic tissue of corms was used as the initial explant, dissected vertically as a 1-cm-sided cube and inoculated onto a modified MS [14] culture medium supplemented with 0.1 $mg \cdot L^{-1}$ of 2,4 D (2,4 dichlrophenoxyacetic acid) and 2.0 $mg \cdot L^{-1}$ of BA (bencylaminopurine), according to Piqueras et al. [19]. The pH of the medium was adjusted to 5.8 with 1.0 N HCl/Na OH and solidified with 0.8 % agar and autoclaved for 20 min at 121 °C. The explants were cultured in 25 × 100 mm test tubes each with 10 mL of nutrient medium under 16 h cool-white fluorescent light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25 °C. Under these conditions, the meristematic region of the cultured corms produced nodular cormogenic calli able to develop protocorm-like bodies. After the first subculture, the nodular cormogenic calli were separated from the initial explant and subcultured at 5-week intervals in the same medium (*figure 1a*). These cultures have been used as the plant material for this work. Only one clone was used for all the experiments. For the cultures in liquid medium, 1.5 g of tissue were inoculated in 250-mL Erlehnmeyer flasks containing 100 mL of medium and stirred at 120 rpm.

The nodular cormogenic calli obtained as described before were exposed to different treatments to study their influence on growth and the regulation of morphogenesis in the cultures: several concentrations of MS salts and sucrose (3–7 %) were tested both in solid and liquid cultures as well as adenine sulphate, L-glutamine, casein hydrolisate and sodium di-hydrogen phosphate. To study

the effect on the induction of shoots and the development of corms, several growth regulators (BA, Kin (Kinetin), 2ip(2-isopentenyladenine), 2,4-D, paclobutrazol (PAC) and imazalil (IMA) sterilized by filtration) were added to the medium before adjustment of the pH. The experiments were repeated twice and each treatment was composed of 24 tubes (1 explant/tube), three flasks in the case of liquid medium. The morphogenic response was recorded after 6 weeks in culture.

3. Results

Prior to their inoculation in culture medium, the meristematic apical tissue was completely removed by microsurgery in half of the resting corms used to start the cultures. This explant showed a significantly higher morphogenic response (75 %) compared to controls (15 %) (*figure 1 A* and *B*).

The induction of small shoots in cormogenic calli using the initial level of regulators was higher on $^1/_2$ MS solid medium compared to full strength medium; however, in liquid culture the regeneration of adventitious shoots was prevented. Concerning proliferation, it was clearly improved in liquid cultures up to two times compared to solid ones. A remarkable increase in fresh weight could be observed for the cultures initiated in liquid medium (*table I*). The effect on shoot production of the different citokinins used (BA, kinetin and 2ip) in nodular calli were compared and presented in *table II*. From these results it can be deduced that BA was the most effective citokinin for the induction and differentiation of shoots in the nodular calli with the highest induction rate corresponding to 2–5 mg·L^{-1} BA.

Table II. Effect of different citokinins on morphogenesis and growth of nodular cormogenic calli of *C. sativus*. Significant differences (L.S.D. 95 %) are indicated with different letters (± indicates mean standard deviation).

Citokinin treatment (mg·L^{-1})		Percentage of calli with shoots	Fresh weight (mg)/calli
Control		12.5 c	162.7 ± 28.3
BA	0	12.5 c	
	0.5	25.0 b	588.3 ± 135.7
	1.0	29.2 b	633.4 ± 84.4
	2.0	45.8 a	649.3 ± 65.2
	5.0	50.0 a	661.7 ± 85.9
Kinetin	0	12.5 b	
	0.5	4.2 d	163.7 ± 10.3
	1.0	8.3 c	171.3 ± 29.4
	2.0	10.5 bc	316.3 ± 19.2
	5.0	20.8 a	383.7 ± 105.3
2ip	0	12.5 c	
	0.5	16.7 b	288.7 ± 44.1
	1.0	18.2 b	310.3 ± 26.2
	2.0	25.0 a	504.0 ± 88.2
	5.0	16.7 b	589.3 ± 54.9

** 100 mg fresh weight of callus/tube was the initial inoculum.

Sucrose concentration influenced the induction of adventitious shoots and the proliferation rate of nodular calli; both parameters demonstrated a progressive decrease and the nodular calli became smaller and harder with increasing levels of sucrose in the medium. In liquid medium the cultures showed a significant increase in fresh weight (ten times) compared to those in solid medium (three times) for 3 % sucrose (*table III*). The addition of

Table I. Influence of liquid/solid medium and MS concentration on the proliferation and induction of adventitious shoots in nodular cormogenic calli cultures of saffron (± indicates mean standard deviation).

Type of culture	MS concentration	Percentage morphogenic explants	Proliferation factor	Fresh weight (g)
Solid	$^1/_2$ MS	25.0	1.6	0.161 ± 0.011
	1 × MS	16.7	3.0	0.297 ± 0.060
Liquid	$^1/_2$ MS	20.0	5.3	7.953 ± 0.722
	1 × MS	18.0	7.7	11.546 ± 0.748

** 100 mg fresh weight of callus/tube was the initial inoculum.

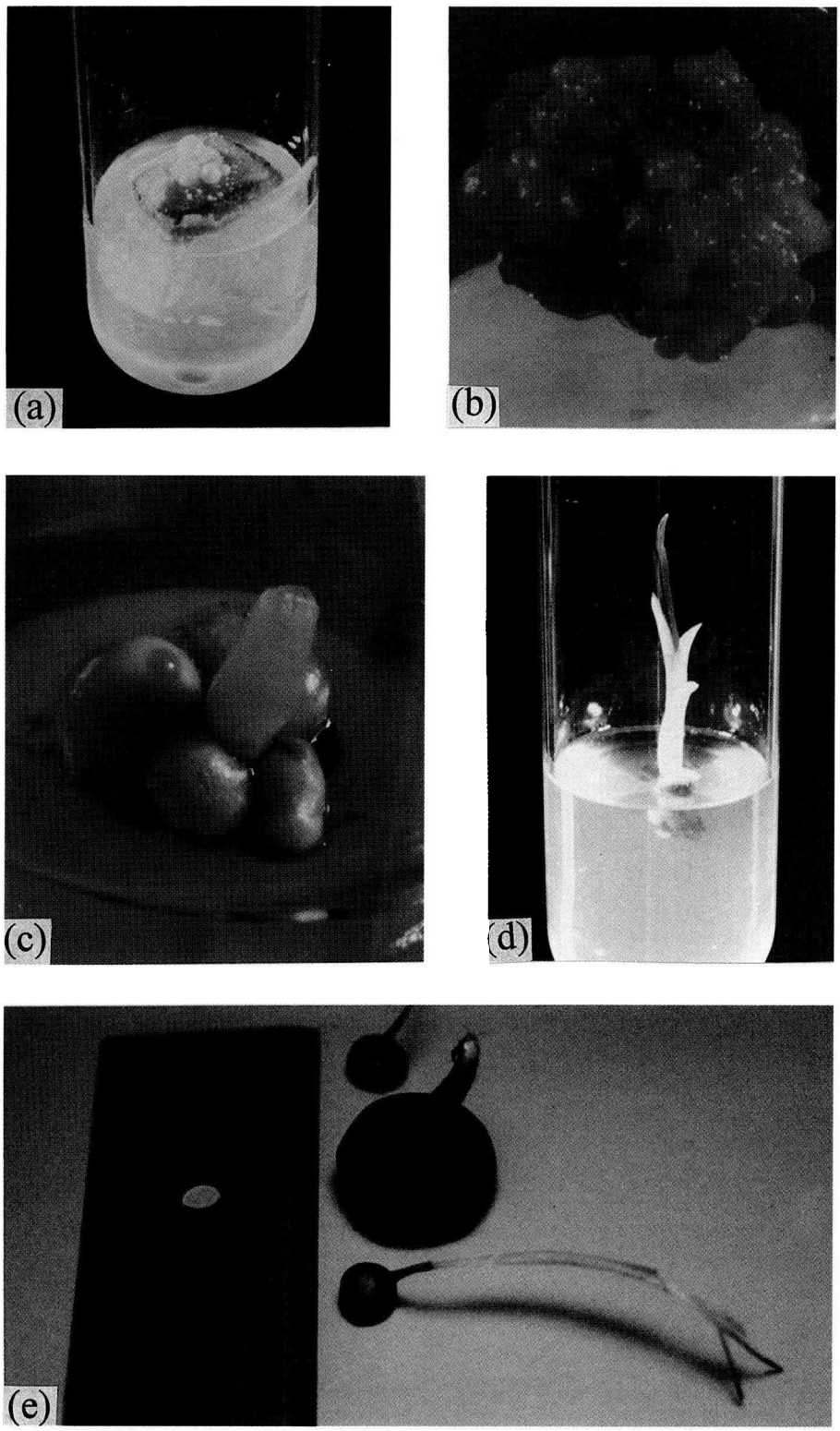

Figure 1. **(a)** Induction of nodular callus on the meristematic zone of a saffron corm cultured on MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg·L^{-1} 2,4 D, after 6 weeks in culture. **(b)** Nodular cormogenic calli used as plant material for the experiments without adventitious shoot formation, cultured in the same medium as (a). **(c)** Cormogenic nodule excised from a nodular calli. **(d)** Saffron plantlet regenerated from a fully developed cormogenic nodule with shoot elongation, normal leaf development and rooting. **(e)** Comparison of field grown corms and mini-corms developed in vitro by tissue culture from micropropagated plantlets.

Table III. Effect of culture type (solid/liquid) and sucrose concentration on growth and morphogenic response of nodular cormogenic calli in *C. sativus* (± indicates mean standard deviation).

Culture type	Sucrose (%)	Percentage of calli with shoots	Proliferation factor	Fresh weight/calli/flask
Solid	3	12.5	3.0	0.302 ± 0.075
	5	9.2	2.3	0.232 ± 0,041
	7	6.3	1.6	0.156 ± 0.019
Liquid	3	6.1	7.2	11.546 ± 0.748
	5	4.6	5.4	8.070 ± 0.119
	7	3.4	3.7	5.585 ± 0.409

* Culture medium supplemented with BA (2.0 mg L^{-1}) and 2,4-D (0.1 mg L^{-1}).
** 100 mg fresh weight of callus/tube was the initial inoculum.

$NaH_2PO_4.H_2O$ (up to 150 mg·L^{-1}) clearly promoted the growth and stimulated shoot development in cormogenic calli compared to the control medium without this compound (*table IV*).

Several organic compounds were tested (adenine sulphate, L-glutamine and casein hydrolyse) to study their effect on the proliferation and morphogenic response of nodular calli (*table V*). L-glutamine (120 mg·L^{-1}) increased the rate of adventitious shoot induction and proliferation in cultured nodular calli compared to the control. The addition of casein hydrolisate had a positive effect on shoot induction as well as the proliferation of the tissues with increasing concentrations of this product up to 120 mg·L^{-1}. Among the PAC concentrations tested, only 0.1 mg·L^{-1} enhanced the induction of adventitious shoots in cormogenic calli up to two times the control. Higher levels of PAC had a negative effect on growth expressed as the increase in fresh weight (*table VI*). To obtain a similar morphogenic response, higher levels of imazalil were required (0.5^{-1} mg·L^{-1}) in cultured cormogenic calli, this range of concentrations produced the maximum number of shoots per explant and the best growth rate (*table VII*).

For plant regeneration, nodules with adventitious shoots (*figure 1C*) were transferred to solid MS medium without growth regulators where the nodules formed normal leaves, roots and a basal cormlet within 2 months (*figure 1D*). During the third month, a progressive decline of both roots and leaves took place completing the regeneration of the dormant microcorms (*figure 1F*) with an average fresh weight of 0.450 g and 0.96 cm in diameter.

Table IV. Effect of NaH_2PO_4 on morphogenic response and growth of cormogenic nodular calli of *C. sativus* (± indicates mean standard deviation).

NaH_2PO_4 (mg·L^{-1})	Percentage of calli with shoots	Fresh weight/calli
0	12.5	225.0 ± 37.1
50	16.7	285.8 ± 35.0
100	12.5	366.3 ± 55.9
150	25.1	371.6 ± 42.4

* Culture medium supplemented with BA (2.0 mg·L^{-1}) and 2,4-D (0.1 mg·L^{-1}).
** 100 mg fresh weight of callus/tube was the initial inoculum.

The cormogenic nodular calli used for this work have been maintained in culture so far (3 years) without any reduction in their cormogenic potential.

4. Discussion

In relation to the positive effect of removing by microsurgery the apical meristematic tissue of the corms before culture initiation, our observations agreement with those of Plessener et al. [20] who found that microsurgery of the apical bud in saffron corms cultivated in vitro increased both sprouting and corm production. These authors related the positive effect of microsurgery to an increase in ethylene

Table V. Effect of adenine sulphate, L-glutamine and casein hydrolysate on morphogenic response and growth of cormogenic nodular calli of *C. sativus* (± indicates mean standard deviation).

Treatment ($mg \cdot L^{-1}$)		Percentage of calli with shoots	Fresh weight/calli
Adenine sulphate	0	8.3	289.2 ± 37.7
	40	8.3	299.3 ± 46.5
	80	12.5	222.8 ± 18.9
	120	8.2	238.8 ± 40.5
L-glutamine	0	16.7	281.2 ± 33.2
	40	12.5	280.5 ± 42.4
	80	8.3	297.8 ± 54.1
	120	25.0	357.6 ± 65.3
Casein hydrolysate	0	8.3	222.6 ± 31.2
	40	16.7	391.9 ± 58.7
	80	16.3	330.0 ± 41.8
	120	25.0	444.5 ± 57.2

* Culture medium supplemented with BA (2.0 $mg \cdot L^{-1}$) and 2,4-D (0.1 $mg \cdot L^{-1}$).
** 100 mg fresh weight of callus/tube was the initial inoculum.

Table VI. Effect of paclobutrazol (PAC) and imazalil (IMA) on morphogenic response and growth of cormogenic nodular calli of *C. sativus*. Significant differences (L.S.D. 95 %) are indicated with different letters (± indicates mean standard deviation).

Treatment ($mg \cdot L^{-1}$)		Percentage of calli with shoots	Fresh weight/calli
PAC	0.0	16.7 b	261.7 ± 46.6
	0.1	33.3 a	276.1 ± 30.3
	0.5	14.3 c	242.8 ± 41.9
	1.0	15.6 bc	213.4 ± 34.6
	2.0	16.1 b	219.7 ± 37.4
IMA	0.0	12.5 d	281.9 ± 49.8
	0.1	20.8 c	408.6 ± 61.1
	0.5	33.3 b	361.4 ± 58.2
	1.0	41.7 a	323.3 ± 53.0
	2.0	34.1 b	343.6 ± 56.9

* Culture medium supplemented with BA (2.0 $mg \cdot L^{-1}$) and 2,4-D (0.1 $mg \cdot L^{-1}$).
** 100 mg fresh weight of callus/tube was the initial inoculum.

production upon wounding caused by the removal of the meristems since the application of this plant growth regulator to isolated buds has been shown to induce the development of both axillary and adventitious buds in several species as *Iris* [17], hyacinth [18], potatoes [15] and *Lilium* [25].

A high cytokinin/auxin ratio has been considered necessary for shoot induction and development in plant tissue cultures [9, 22] as we have observed in our nodular cormogenic system for *Crocus*. As in other bulb or corm-producing species, such as gladiolus [23], axillary bud proliferation in presence of BA can reduce the formation of callus which may result in ploidy changes. On the other hand, it is critical to use the lowest possible concentration of 2,4 D to minimize the generation of somaclonal variation in the cultures for multiplication purposes, since it has been reported that use of 2,4 D can induce mutations in plant tissue cultures [12]. This circumstance compromised the genetic stability of the cultures but could be of interest for saffron breeding.

Our results are partially in agreement with Daniel et al. [2] who reported that tobacco callus grown on medium with 3 % of sucrose showed the best growth and produced the highest number of shoots, while cultures on media with lower and higher sucrose levels inhibited growth and the formation of shoots. Analogous effects of sucrose on organ differentiation and scale leaf formation were described in cultured tissues of *Lilium* bulb scales by Takayama and Misawa [24].

The same positive effect was observed in micropropagated *Hosta decorata* [16] where the highest production of shoots was achieved in medium supplemented with 170 $mg \cdot L^{-1}$ of $NaH_2PO_4.H_2O$. The positive impact of the supplement with $NaH_2PO_4.H_2O$ on the culture development could be explained by the depletion in P that has been reported for cultured tissue media in vitro where limiting mineral deficiencies have been observed after 2 or 3 weeks of culture [28]. P is also the most rapidly depleted element in cell suspension cultures [5]. In our case, the addition of $NaH_2PO_4.H_2O$ could compensate for a deficiency of PO_4 in the original medium that could limit the growth of the cormogenic calli. Concerning casein hydrolysate, our results are

Table VII. Effect of IMA on adventitious shoot regeneration and growth of cormogenic nodular calli of *C. sativus*. Significant differences (L.S.D. 95 %) are indicated with different letters (± indicates mean standard deviation).

Treatment ($mg \cdot L^{-1}$)	Total no. of shoots/calli	Shoots longer than 0.5 mm	Fresh weight (mg)/calli
0.0	3.17 ± 0.51 c	2.08 ± 0.48	351.8 ± 39.1
0.1	3.82 ± 0.75 bc	2.45 ± 0.61	365.4 ± 54.0
0.5	6.17 ± 0.96 a	4.67 ± 0.77	472.3 ± 71.2
1.0	6.09 ± 0.64 a	4.36 ± 0.62	480.6 ± 53.6
2.0	4.70 ± 0.67 b	3.30 ± 0.65	422.2 ± 45.5

* Culture medium supplemented with BA (2.0 $mg \cdot L^{-1}$) and 2,4-D (0.1 $mg \cdot L^{-1}$).
** 100 mg fresh weight of callus/tube was the initial inoculum.

in agreement with those of Plessner et al. [20] who were able to increase significantly the development of corms on isolated apical buds of saffron.

As far as we know, paclobutrazol (PAC) and imazalil (IMA) have been used in saffron tissue culture for the first time in the present study. Some of our observations are in agreement with Ziv et al. who reported an enhanced bud and protocorm proliferation in *Gladiolus* [29] and *Philodendron* [30]. According to these authors, increased bud proliferation mediated by PAC, a growth retardant, could be the result of impaired apical dominance or changes in sink and source correlation. Growth retardants were also found to inhibit ethylene production [8] and to stimulate the translocation of assimilates to growing seeds [13]. Some of the effects described for growth retardants are considered to be mediated by the elevated concentration of cytokinins [8, 11], as in our culture medium for *Crocus*. We consider that the increased cytokinin and the reduction in ethylene are factors involved in the amplification of bud development, mediated by PAC, in *C. sativus*.

Imazalil (IMA) is an imidazole fungicide although it is not a growth retardant. It shares structural features common to this family of compounds, and their target enzymes are monooxygenases [21] so they might affect several biochemical pathways. A synergistic action of imazalil and BA on adventitious bud formation has been recently observed by Werbrouck and Debergh [26] in micropropagated *Spathiphyllum* plants that resembled some of our observations in *Crocus*. The mode of action proposed for IMA is not well understood, it only induces shoots in the presence of exogenous cytokinins such as BA. This fact indicates some alteration caused by IMA of BA metabolism. When cultured on a medium containing BA *Spathipyllum* mainly converts BA into large amounts of [9G]BA, which is considered a storage or a detoxification product and into [9R]BA, which still has cytokinin activity [27]. IMA might influence the concentration of the active BA metabolites and could also have an inhibitory effect on their catabolic enzymes. On the other hand an effect on GA metabolism for IMA can not be excluded, because of the parallelism in structure between imazalil and growth retardants.

In the present study we have developed a micropropagation protocol for *C. sativus* by the induction of nodular cormogenic masses from meristems of resting corms. The effect on the regenerative potential of these tissues of key components in the culture medium have been evaluated. This technique for saffron micropropagation has produced plantlets and microcorms and is currently been used for to obtain transgenic saffron plants at our laboratory. This methodology could be used to speed up saffron breeding coupled with somaclonal variation and in vitro mutagenesis.

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