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In Vitro Shoot Regeneration and Development of Microcorms of Moroccan Saffron (*Crocus sativus* L.)

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Abstract

***Crocus sativus* L. is a male sterile vegetatively propagated plant. Its flower produces stigmas that when dried, constitute the source of a spice commonly known as Saffron. Slow vegetative propagation and diseases limit the production and the development of saffron. "In vitro" culture could be an effective method to overcome these limitations by improving the quantity and the quality of the planting materials. In this work, *Crocus sativus* L. segments corms of cultivar from the region of Taliouine (Southeast of Morocco) were used for the propagation through indirect organogenesis. To optimize the in vitro growth conditions, we have used the Murashige and Skoog medium (MS medium), supplemented with 2.4-dichlorophenoxyacetic acid (2.4-D) and with 6-benzylaminopurin (BAP) at combination of various concentrations. Our results showed the formation of callus in 85.42% of explants that grow in a culture medium supplemented with 2,4-D combined with BAP, at a concentration of 1mg/l each. In addition, we observed that increasing the concentration of BAP in the culture medium to 1.5mg/l improved the rate of shoots initiation (0.81). In the meantime, we noted that a combination of BAP (8mg/l) and Naphthalene acetic acid (NAA; 2mg/l) has significantly improved the rate of the formation of advanced shoots (6.65). Finally, the shoots that developed were transferred to an induction medium of roots and corms. As a result, we observed that 50% of shoots tested in ½ MS medium supplemented with 2.4-D and of BAP (1 mg/l each) and 5% sucrose, formed corms. Our study provides a first database for in vitro culture of Moroccan saffron cultivars.**

Keywords: *Crocus sativus*; Indirect organogenesis; Microcorm; Moroccan saffron; Plant tissue culture.

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Introduction

Saffron (*Crocus sativus* L.) belongs to the Iridaceae family. Since ancient times, the dried stigmas of its precious flower were known for their aromatic, dyeing and medicinal properties (Theophrastus et al. 1926; Douskos et al. 1980; Negbi et al. 1989). Thus, they were commonly used to color and to give flavor to food (Tirillini et al. 2006). The name of saffron is usually used to refer both to the spice and the plant itself. Some archaeological and historical studies show that domestication of saffron dates back to 2,000-1,500 BC (Grilli Caiola 2004). At present, around 205 tons of saffron is produced worldwide each year; in countries such Iran, India, Greece, Morocco, Spain, Italy, Turkey, France, Switzerland, Pakistan, Azerbaijan, China, Egypt, the United Arab Emirates, Japan and even in Australia (Tasmania). However, 80% of the total annual world production (164 tons) comes from Iran, mostly from the province of Khorasan (Fernández 2004).

Saffron is a sterile triploid (2n=3x=24) plant that grows only by vegetative propagation (Warburg 1957; Mathew 1982). The autotriploid nature of the species renders the improvement of yield and quality of saffron by breeding very difficult (Basker and Negbi 1983). The use of in vitro tissue culture for mass propagation and development of disease-resistant cultivars could be an important alternative to improve the culture of Saffron (Sharifi et al. 2010). That biotechnology is able to produce large quantities and to genetically improve the saffron has been reported in previous studies (Ding et al. 1981; Ilahi et al. 1987; Bhagyalakshmi 1999; Piqueras et al. 1999). The in vitro propagation of saffron through direct and indirect organogenesis from callus cultures was also reported previously (Plessner et al. 1990; Bhagyalakshmi 1999; Sharma et al. 2008; Zeybek et al. 2012).

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The present study reports, for the first time, the induction of calluses, the regeneration and the development of shoots and corms of Moroccan saffron cultivars.

Material and Methods

Plant Media

Murashig and Skoog basal media (MS), containing plant growth regulators (PGRs), was used to induce the callus of Crocus sativus L. The MS media was supplemented with sucrose (30 or 50 g/l for corm induction) and solidified with 0.3 g/l of phytagel. The pH was adjusted to 5.8 with NaOH or HCl (1N) then, the media was autoclaved at 120°C for 20 min and distributed in culture dishes. Depending on the experiment, plant growth regulators were added to the media before autoclaving.

Plant Materials

Healthy saffron corms were collected from the region of Taliouine, situated in the south-east of Morocco in the province of Taroudant, and planted in the experimental plot at the faculty of Sciences (Agadir, Morocco). Samples of new minicorms were separated and used as source of explants.

Sample Sterilization

After removal of protective tunics, the corms were carefully washed with tap water for 30 min, and transferred into a sterile laminar air flow cabinet at room temperature where they were treated with 70% (v/v) of ethanol for 45 sec, followed by a treatment with 0.20% mercuric chloride ($HgCl_2$) solution (w/v) for 20 min. After that, the corms were rinsed four times with distilled sterile water to remove residual $HgCl_2$.

Explants and In Vitro Culture Conditions

After sample sterilization, a cubic section of 5 to 10 mm, from the central meristematic region of each corm, was cut and used as starting explants. A number of 48 explants were used for the experiments of initiation and growth of callus. Thirty six more explants were used for each treatment to perform the other experiments. The explants in each treatment were aseptically placed into sterile Petri dishes or test jars containing either calluses, shoots or corms inducing media. The induction and growth of callus were carried out by their incubation in the dark for 12 weeks in a culture media (MS) supplemented with 4 combinations of PGRs (Table 1). To initiate the multiplication of the shoots, explants with induced callus were transferred into initiation and multiplication medium containing three combinations of BAP and NAA (8 mg/l BAP and 2 mg/l NAA, 2 mg/l BAP and 0.05 mg/l NAA, or 1.5 BAP and 0.00 mg/l NAA); then incubated under a 16H/8H (light/dark) photoperiod (1500 Lux) for 4 months. To evaluate corms formation, the shoots previously obtained were cultured in half-strength MS basal medium containing 5% or 3% of sucrose and 1mg/l of 2,4-D and BAP and incubated in the dark for 3 months. In all the experiments, the explants were incubated in growth room at 25 ± 2 °C and subcultured every four weeks.

Table 1. Different combinations of 2,4-D and BAP tested for callus initiation and growth.

Treatment	Concentration of 2,4-D (mg/l)	Concentration of BAP (mg/l)
0D-0B (Control)	0.00	0.00
0,25D-1B	0.25	1.00
0,1D-1B	0.10	1.00
0,25D	0.25	0.00
1D-1B	1.00	1.00

Recorded Parameters and Statistical Analysis

For each experiment period, the data related to the initiation (callus, adventitious shoots, corms and roots), the growth (callus), the weight (corms), the diameter (corms) and the number (shoots, corms and roots) were collected. The data related to the initiation were collected in terms of presence or absence of a response. By contrast, the data related to the growth (diameter and weight) were visually estimated and measured. The number of shoots, corms and roots were determined by direct counting. The frequency of the initiation of callus, shoots, corms and roots was calculated in percentage of cultured explants that showed a positive response in each experiment. This was followed by the determination of the percentages of averages. Even if the responses obtained were variables, the data collected were transformed into arcsine in order to follow the normal law. This data transformation was not necessary for other variables. Finally, the analysis of variance between averages was calculated, and significant differences were estimated by the Duncan Multiple Range test at $P<0.05$ (Duncan 1955) using a STATISTICA program (version 6).

Results

Callus Induction and Growth

In order to study the effect of different combinations of 2,4-D and BAP on the induction and growth of callus from saffron tissues, we tested four combinations of these PGRs in the MS media (Table 1). The initiation of callus was first observed 4 weeks after the incubation (Fig. 1A). The frequency and the size of the callus were found to be different depending on the combination of PGR used (Table 2). No callus initiation was performed in the media without PGR. A ratio of BAP/2,4-D equal to 10 (1 mg/l BAP and 0.1 /l 2,4-D) leads to an initiation and a growth that remain lower than the values of the treatment with the 2,4-D alone at a higher concentration of 0.25mg/l. This last concentration combined with 1 mg/l of BAP had allowed increasing both the initiation and the growth of calluses. After 3 months of culture, our finding showed that 87.5% of explants produced calluses on MS medium containing 1 mg/l of BAP combined with 1 mg/l of 2,4-D. By contrast, the lowest callus production (35.42%) was

found in tissues grown on MS medium containing 1 mg/l of BAP combined with 0.1 mg/l of 2,4-D. The sizes of the calluses obtained were comprised between 0.5 and 2.7cm. The highest size of the callus (2.29cm) was obtained with MS medium supplemented with BAP (1mg/l) and 2,4-D (1mg/l) (Table 2; Fig. 1B). Indeed, as shown in Table 2, we observed that the combination of 1 mg/l of 2,4-D with 1 mg/l of BAP gave the best results for callus initiation and growth.

Effect of BAP and NAA Combinations on Shoots Formation

BAP is one of the most active cytokinin used in plant tissue culture to form adventitious shoots from callus. In order to test different concentrations of BAP and NAA on shoot induction and number from callus of saffron cultivated on MS medium, we compared the BAP alone (1.5 mg/l), a combination of a quite low concentration of BAP (2 mg/l) with NAA (0.05 mg/l) and a combination of high concentration of BAP (8 mg/l) and NAA (2 mg/l).

We observed the formation of shoots with green leaves on corm explants with an intermediate callus structure (Fig. 1C, D). The lowest initiation of shoots was achieved through the combination of 2 mg/l BAP and 0.05 mg/l NAA in which the mean number of shoots was not statistically different when compared to that of BAP alone (1.5 mg/l).

For the adventitious shoot initiation, the statistical analysis (using ANOVA test) indicates that there is no significant difference between the treatment of 1.5 mg/l of BAP and the use of 8 mg/l of BAP combined with 2 mg/l of NAA. In fact, as shown in table 3, these two media were both equally efficient at promoting the adventitious shoot initiation (0.81 and 0.70, respectively). The percentage of shoot initiation with these two treatments was 52.77 and 41.66, respectively. In this experience, the PGR combination able to give the best shoot initiation from saffron callus and the highest mean number of shoots is 8 mg/l BAP and 2 mg/l NAA. For this last combination and in our experimental conditions, shoot initiation occurs primarily between the second and the forth month of culture. If an important adventitious number of shoots is reached in the sixth month of culture with a rate of proliferation of 2.5 of shoots obtained after 4 months of culture, no new initiation was noted after the fourth month on the calluses remained without shoots.

Table 3. Effects of different combinations of BAP and NAA on shoots initiation and number from the callus of saffron.

PGR Combinations (mg/l)	Shoots Initiation*	Mean Number of Shoots
2 mg/l BAP + 0,05 mg/l NAA	0.52 ± 0.11 b	2.75 ± 0.42 b
8 mg/l BAP + 2 mg/l NAA	0.70 ± 0.14 a	6.65 ± 0.20 a
1,5 mg/l BAP + 0,00 mg/l NAA	0.81 ± 0.13 a	2.47 ± 0.51b

Data are means ± SE of six independent experiments. Values in each column marked by different letters represent a statistically significant difference at the P<0.05 level by Duncan's multiple range test. (*Absent = 0 and Present = 1).

Corms and Roots Formation

In order to study the effect of sucrose concentration on saffron cormlet formation and growth, saffron shoots were cultivated on ½ MS medium supplemented by 1 mg/l of BAP and 2,4-D with two different concentrations of sucrose (3% and 5%). After two months of cultivation, we visually observed four cases: i) no cormlet initiation (41,66%), ii) cormlet formation (38,88%), iii) cormlet and root formation (11,11%) and iiii) root formation (8,33%). An example of the formation of the corms with the roots on shoots explants is presented in Fig. 1E, F. Regarding the formation of the corms and the roots and their number, our data showed no statistically significant differences between the concentrations of sucrose used (3% and 5%) (Table 4). However, the 5% concentration of sucrose gave cormlets with a diameter and a fresh weight much higher compared to those obtained with 3% sucrose. In fact, the diameter and the number of corms found in ½ MS media supplemented with 5% of sucrose were about 0.72 cm and 1.45 g, respectively. By contrast, they reached only 0.32 cm and 0.46 g, in MS media supplemented with 3% of sucrose (Table 4).

Table 2. Effects of different combinations of BAP and 2,4-D on callus initiation and growth from the corm explants of saffron cultured on MS medium.

PGR_s Combinations (mg/l)	Callus Initiation*	Callus Growth (cm)**
0 mg/l BAP + 0 mg/l 2,4-D	0.00 ± 0.00 d	0.00 ± 0.00 c
1 mg/l BAP + 0.1 mg/l 2,4-D	0.64 ± 0.06 c	1.34 ± 0.28 b
0 mg/l BAP + 0.25 mg/l 2,4-D	0.91 ± 0.12 b	1.42 ± 0.22 b
1 mg/l BAP + 0.25 mg/l 2,4-D	1.11 ± 0.12 a	1.56 ± 0.27 b
1 mg/l BAP + 1mg/l 2,4-D	1.25 ± 0.26 a	2.29 ± 0.12 a

Data are means ± SE of six independent experiments. Values in each column marked by different letters represent a statistically significant difference at the P<0.05 level by Duncan's multiple range test. (*Absent = 0 and Present = 1, ***Minimal = 0.5 cm Maximal = 5 cm*).

Table 4. Effects of two concentrations of sucrose (3% and 5%) on initiation, diameter and weight of cormlet and initiation and number of root obtained, from the shoots of saffron cultured on ½ MS medium supplemented with 2,4-D and BAP.

Studied characters	Sucrose concentrations	
	(30 g/l)	(50 g/l)
Corms initiation*	0.67 ± 0.09 a	0.79 ± 0.11 a
Corms number	0.39 ± 0.09 a	0.79 ± 0.11 a
Average cormlets diameter [cm]	0.32 ± 0.09 b	0.72 ± 0.24 a
Average cormlets fresh weight [g]	0.46 ± 0.11 b	1.45 ± 0.56 a
Roots initiation	0.45 ± 0.08 a	0.45 ± 0.08 a
Roots number	1.25 ± 0.25 a	1.36 ± 0.64 a

Data are means ± SE of six independent experiments. Values in each column marked by different letters represent a statistically significant difference at the P<0.05 level by Duncan's multiple range test. (*Absent = 0 and Present = 1).

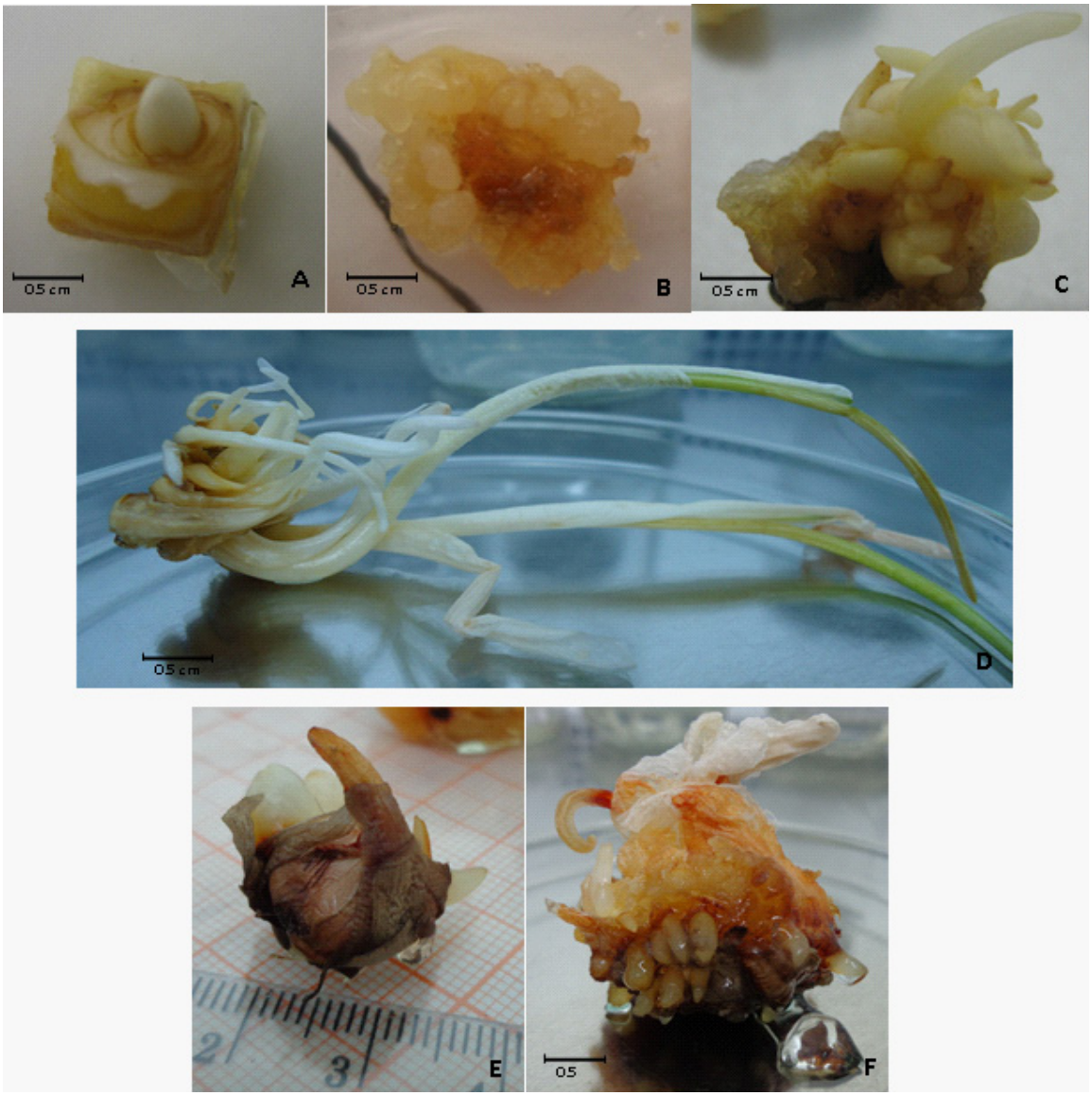

Fig. 1. Induction of callus, adventitious shoots and corms via indirect organogenesis of saffron (C. sativus). (A): early stage of callus formation on explant. (B): explant completely transformed into callus on MS medium with 1 mg/l 2,4-D + 1mg/l BAP, (C): adventitious shoots formation from callus tissue on MS medium supplemented with 1,5mg/l BAP. (D): adventitious shoot with green leaves formed in MS medium supplemented with 8 mg/l BAP + 2 mg/l NAA. (E): corm formation on ½ MS medium supplemented with 3% (w/v) of sucrose and 1mg/l BAP + 1 mg/l 2,4-D. (F): corm with roots formed in ½ MS medium supplemented with 5% (w/v) of sucrose and 1 mg/l BAP + 1 mg/l 2,4-D.

Discussion

Indirect organogenesis of saffron could be an important way to quickly propagate the selected cultivars of this species. Saffron calluses represent a permanent and inexhaustible source of plant material. The initiation and growth of callus, the induction of shoots and the formation of saffron cormlets are the key steps of indirect organogenesis and their control and improvement are imperative prior to undertaking any propagation through indirect organogenesis. The purpose of this study is to optimize conditions for the "in vitro" shoot regeneration and the development of microcorms of Moroccan saffron, by using different concentration and combination of plant growth regulators and culture media.

In the initiation and growth of saffron calluses step, we focused our experiments on two PGRs; the BAP as a most active cytokinin used in plant tissue culture and the 2,4-D as a strong auxin. Himeno et al. (1987), Loskutov et al. (1999) and Sarma et al. (1990) have demonstrated the requirement of BAP as cytokinine for direct organogenesis of saffron. The use of MS medium supplemented with different combinations of these two PGRs, showed that the combination of 1 mg/l BAP and 2.4-D gave the best results of calluses initiation and growth and contains an equal concentration of cytokinin and auxin. This result is consistent with previous reports that showed a positive effect of different combinations of 2,4-D and BAP on the initiation of

Crocus sativus L. callus (Ilahi et al. 1987; Plessner et al. 1990; Chen et al. 2003; Zeybek et al. 2012). Comparing different combinations of PGRs, it should be noted that when the combination is more favorable to the initiation of callus, the appearance of callus is earlier. The percentage of callus formation after one month culture of explants is 52%, 60%, 86,84% and 90% for PGRs combination of 1 mg/L BAP – 0,1 mg/L 2,4-D, 0 mg/L BAP – 0,25 mg/L 2,4-D, 1 mg/L BAP – 0,25 mg/L 2,4-D and 1 mg/L BAP – 1 mg/L 2,4-D respectively.

Particular attention was paid to shoots initiation from callus of C. sativus and also to their number by focusing on the BAP and NAA effect. BAP alone at 1,5 mg/l is among the treatments able to initiate a high number of shoots. It does not show a statistically significant difference in terms of shoots number when compared to the combination of NAA and BAP (0.05 mg/l and 2 mg/l respectively). In these tests, the combination of 8 mg/l BAP and 2 mg/l NAA remains the best combination since it generates both the best rate of shoots initiation and the maximum number of formed shoots. This combination particularly concentrated on BAP must be followed taking into account the possible somaclonal variations. The major part of shoot initiation occurs between the second and the fourth month of callus culture and the calluses initiated for shoot formation subsequently develop more and more shoots. However, calluses that do not show shoot formation after the fourth moth of culture remained without initiation even after 6 months of culture. More recently, Sharifi et al. (2010) showed that the high rate of shoot regeneration per explants (19.55) was observed on B5 medium supplemented with 0.4 mg/L of NAA and 0.6 mg/L of BAP. The highest average of shoots initiation (0.81) was found in MS medium supplemented with 1.5 mg/l of BAP. This rate is higher than the value (0.33) obtained by Zeybek et al. (2012). In our study, the highest number of shoots was obtained in MS medium containing 8 mg/l of BAP and 2 mg/l of NAA. In these conditions, the average size of shoots formed was about 6.65. This average is less than that obtained in reports by Sharifi et al. (2010), and higher than that obtained by Zeybek et al. (2012).

The importance of a high concentration of sucrose in the in vitro production of saffron corms was reported by of Sharma et al. (2008) and Zeybek et al. (2012). Our results showed that the use of 5% sucrose, allowed the formation of corms on 50% of explants. This rate is higher than that obtained by Zeybek et al. (2012). In addition, our statistical analysis supports the lack of significant difference between the two concentrations of sucrose used (3% and 5%) regarding the initiation and the number of corms formed. These results are consistent with those reported by Zeybek et al. (2012), but inconsistent with those reported by Sharma et al. (2008). In fact, the average weight we found (0.46 g) in corms initiated in the MS medium supplemented with 3% of sucrose was less than that obtained by Sharma et al. (2008). Interestingly, in MS medium containing 5% of sucrose this average (1.45) was higher than that obtained by the same authors (1.10) in MS medium supplemented with 8% of sucrose.

Abbreviations

BAP: 6-benzylaminopurin
2,4-D: 2,4-dichlorophenoxyacetic acid
NAA: Naphthalene acetic acid
MS: Murashige and Skoog edium (Murashige and Skoog, 1962)

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