African & Mediterranean AGRICULTURAL JOURNAL AL AWAMIA

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# Optimization of the explant disinfection method and *in vitro* multiplication of saffron (*Crocus sativus* L.).

**Tahiri Abdelghani [1], Karra Youssef [1], Ait Aabd Naima [1], Koufan Meriyem [1], Qessaoui Redouan [2], Bouharroud Rachid [3] and Mimouni Abdelaziz [4]**

abdelghani.tahiri@inra.ma

1: Natural Resources and Local Products Research Unit, Regional Center of Agricultural Research of Agadir, National Institute of Agricultural Research, Avenue Enna, BP415 Rabat Principale, 10090 Rabat Morocco.
2: Integrated Crop Protection Unit, Regional Center of Agricultural Research of Agadir, National Institute of Agricultural Research, Avenue Enna, BP415 Rabat Principale, 10090 Rabat Morocco.
3: Research and Development Unit, Regional Center of Agricultural Research of Agadir, National Institute of Agricultural Research, Avenue Enna, BP415 Rabat Principale, 10090 Rabat Morocco.
4: Regional Center of Agricultural Research of Agadir, National Institute of Agricultural Research, Avenue Enna, BP415 Rabat Principale, 10090 Rabat Morocco..

**Abstract**

Saffron (*Crocus sativus* L.), renowned as the world's most expensive spice extracted from its stigmas, is an autumn-flowering, sterile triploid geophyte species (2n=3x=24) belonging to the *Iridaceae* family. It is propagated vegetatively through the development of daughter corms from the mother corm. Low multiplication rates of daughter corms under natural conditions and many biotic and abiotic stresses reduce productivity, thereby restraining the availability of planting material. Thus, the main objective of this work is to improve the vegetative propagation of saffron through tissue culture. The results show that soaking of explants in 0.1% (w/v) mercury chloride ($HgCl_2$) for 10 min is more effective for explant disinfection (95%), with a survival rate of over 85%. Whereas, for the initiation phase, the *in vitro* culture of whole corms on Murashige and Skoog (MS) medium supplemented with 0.5 mg /L of 1-naphthalene acetic acid (NAA) in combination with 3 mg/L of 6-benzylaminopurine (BAP) was more effective, resulting in a sprouting rate of 90% with a maximum of 5 sprouted buds per explant. As for the multiplication phase, the highest number of shoots (4 shoots/single shoot explant) was observed on MS medium supplemented with 8 mg/L of BAP in combination with 0.5 mg/L of NAA. The results of this study can serve as a starting point for establishing an efficient and reproducible *in vitro* regeneration system in order to increase the number of shoots per explant for proliferation and micro-corms formation phases by exploring other culture media with different PGR combinations.

**Keywords:** Saffron, *Crocus sativus* L., corm, growth regulators, *in vitro* culture, organogenesis, explant disinfection.

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# Optimisation de la méthode de désinfection des explants et multiplication *in vitro* du safran (Crocus sativus L.).

**Résumé**

Le safran (*Crocus sativus* L.), reconnu comme l'épice la plus chère au monde obtenu à partir des stigmates, est une espèce géophyte triploïde stérile (2n=3x=24) à floraison automnale et qui appartient à la famille des Iridacées. Il se propage végétativement grâce à la formation de cormes filles à partir du corme mère. Le faible taux de multiplication des cormes filles dans les conditions naturelles conjugué à l'effet de nombreux stress biotiques et abiotiques réduisent la productivité, limitant ainsi la disponibilité du matériel végétal de plantation. Ainsi, l'objectif principal de ce travail est d'améliorer la propagation végétative du safran par la culture des tissues *in vitro*. Les résultats montrent que le trempage des explants dans 0,1 % (p/v) de chlorure de mercure ($HgCl_2$) pendant 10 min est plus efficace pour la désinfection des explants (95 %) avec un taux de survie de plus de 85 %. Alors que pour la phase d'initiation, la culture des cormes entiers sur milieu de Murashige et Skoog (MS) additionné de 0,5 mg/L d'acide 1-naphtalène acétique (ANA) en combinaison avec 3,0 mg/L de 6-benzylaminopurine **(**BAP**)** a été plus efficace puisqu'elle permet d'obtenir un taux de germination de 90% accompagné d'un maximum nombre de bourgeons germés (5 bourgeons/explant). Comme pour la phase de multiplication, le plus grand nombre de pousses (4 pousses/pousse initial) a été observé sur le milieu MS additionné de 8,0 mg/L de BAP en combinaison avec 0,5 mg/L d'ANA. Les résultats de cette étude peuvent servir de point de départ pour établir un système de régénération *in vitro* efficace et reproductible afin d'augmenter le nombre de pousses par explant pour les phases de prolifération et la formation de micro-cormes en explorant d'autres milieux de culture avec différentes combinaisons de PGR.

**Mots clés :** Safran, *Crocus sativus* L., corme, régulateurs de croissance, culture in vitro, organogenèse, désinfection.

# تحسين طريقة التعقيم وإكثار الزعفران (*Crocus sativus* L.) عبر زراعة الانسجة في المختبر

**عبد الغني الطاهري*، يوسف قارة، نعيمة أيت عابد، رضوان قساوي، مريم كوفان، رشيد بوهرود، عبد العزيز ميموني**

**الملخص**

يعتبر الزعفران (*Crocus sativus* L.) ، أحد أغلى أنواع التوابل في العالم التي يتم الحصول عليها من الوصمات، وهو نوع ثلاثي الصبغيات (2n=3x=24) عقيم ومزهر في الخريف. يتم تكاثر الزعفران عن طريق التكاثر الخضري من خلال تكوين بصيلات جديدة من البصيلات الأم. يعتبر انخفاض معدل تكاثر البصيلات في ظل الظروف الطبيعية وتأثير العديد من الضغوط الحيوية وغير الحيوية من بين العوامل التي تخفظ في الإنتاجية، الشيء الدي يحد من توفر عدد كافي من البصيلات الصالحة للزراعة. وبالتالي، فإن الهدف الرئيسي من هذا البحث هو تحسين التكاثر الخضري للزعفران من خلال زراعة الأنسجة .تظهر النتائج المحصل عليها أن نقع البصيلات في 0.1٪ (وزن / حجم) كلوريد الزئبق ($HgCl_2$) لمدة 10 دقائق أمكن من تعقيم البصيلات بنسبة 95٪ وبمعدل بقاء على قيد الحياة يزيد عن 85٪ . بالنسبة لمرحلة النمو، فزراعة بصيلات كاملة على وسط موراشيج وسكوغ (MS) الذي تم تزويده بـ 0.5 مجم/لتر من هرمون حمض 1- النفثالين أستيك (NAA) اضافة الى 3.0 مجم/لتر من هرمون 6 - بنزيل أمينوبورين (BAP) مكنت من الحصول على اعلى معدل إنبات بنسبة 90٪ مع تكوين عدد من البراعم بمعدل 5 براعم. بالنسبة لمرحلة التكاثر، لوحظ ان أكبر عدد من البراعم (4 براعم / نبات مفرد) تكون على وسط MS الذي تم تزويده بـ 8.0 مجم/لتر من BAP اضافة الى 0.5 مجم/لتر من NAA. يمكن أن تكون نتائج هذه الدراسة بمثابة نقطة انطلاق في إنشاء أنظمة تجديد فعالة وقابلة للتكرار في المختبر من أجل زيادة عدد الشتلات لمراحل التكاثر وتكوين البصيلات من خلال استكشاف وسائط أخرى مع مجموعات مختلفة من الهرمونات.

**الكلمات المفتاحية**: الزعفران، .*Crocus sativus* L، البصيلات ، منظمات النمو ، زراعة الأنسجة، تكوين الأعضاء، تعقيم.

## Introduction

Saffron (*Crocus sativus* L.) has been cultivated since ancient times in many countries of the world for its dried stigmas, which constitute the most expensive spice in the world. It is a perennial sterile triploid geophyte (2n = 3x = 24) plant that grows from an underground stem (corm). It belongs to the genus *Crocus* of the *Iridaceae* family, which includes about 80 species distributed mainly in the Mediterranean and Southwest Asia (Karaoglu et al., 2007; Palomares, 2015). Saffron is very popular for its color (crocine), taste (picrocrocine) and aroma (safranal) (Cardone et al., 2020).

Regarding production, saffron is mostly grown in Iran, Greece, Morocco, India, Spain, China, Turkey, Azerbaijan, and Italy, with a global production of around 418 tons per year. Iran is the leading producer, with 90% of the global production (Cardone et al., 2020). While in Morocco, saffron cultivation is concentrated mainly in Taliouine (province of Taroudant) and Taznakht (province of Ouarzazate), with an average annual production of 4.5T (95% of national production). The area sown by saffron cultivation area is approaching 1680 ha (FIMASAFRAN 2018).

Since saffron flowers are sterile, the propagation of this species is generally achieved vegetatively by the formation of daughter corms from the mother ones. In fact, a saffron corm survives for only one season and can produce up to 5 daughter corms under natural conditions (Parray et al., 2012; Devi et al., 2014). However, the low multiplication rates of daughter corms under natural conditions and many biotic and abiotic stresses reduce productivity, thereby restraining the availability of planting material (corms) (Moradi and Turhan, 2017; Menia et al., 2018). Therefore, biotechnological tools, including *in vitro* tissue culture techniques or micropropagation, could be consideredan appropriate method for the mass production of quality corms under sanitary conditions.

Micropropagation of saffron was reported by many authors who described *in vitro* regeneration of the shoot through direct and indirect organogenesis and somatic embryogenesis followed by micro-corms formation under controlled conditions (Gantait and Vahedi, 2015; Hagizade, 2016; Cardone et al., 2020; Khawar et al., 2020; Moshtaghi, 2020; Tahiri et al., 2022). The direct induction of multiple shoots and the formation of multiple micro-corms *in vitro* are of great importance for the propagation of saffron. This method allows the formation of micro-corms with the multiplication of shoots as a continuous propagation system (Sharma and Piqueras, 2010; Devi et al., 2011; Tahiri et al., 2022). However, successful regeneration through organogenesis depends on the age and type of explant, culture medium components, plant growth regulators (PGRs), carbon source, gelling agent, and other environmental factors such as light and temperature (Gantait and Vahedi 2015; Tahiri et al., 2022). Different types of explants were used for *in vitro* regeneration of saffron, including corms, apical and lateral buds, leaves and different floral parts (Ebrahimzadeh et al., 2000; Karaoğlu et al., 2007; Devi et al., 2011; Moradi et al., 2017; Soukrat et al., 2017; Halim et al., 2018; Kareem et al., 2019). Among all the above-mentioned explants, corms and lateral/apical buds were the most used in both direct and indirect organogenesis. The corm was, in fact, more receptive than the other explants for multiple shoot induction (Gantait and Vahedi, 2015).

Tissue asepsis is one of the main conditions for establishing and maintaining *in vitro* plant cultures. However, *in vitro* conditions, designed for optimal plant growth and development, are also ideal for the multiplication of microorganisms (Altan et al., 2010). This is a serious problem of monocots micropropagation, especially if underground organs, such as bulbs, rhizomes, corms, and tubers are used as an explant source (Yildirim 2007; Bach and Sochacki, 2013). In saffron, the contamination rate of corms can reach 95 to 100% (Salwee et al., 2013). To resolve this issue, different types of disinfectants can be used, including hydrogen peroxide ($H_2O_2$), brominated water, formaldehyde (CHO), silver nitrate ($AgNO_3$), mercury II chloride ($HgCl_2$), sodium hypochlorite (NaOCl) or calcium hypochlorite ($Ca(ClO)_2$) (Teixeira da Silva et al., 2016). In addition, the concentration and time of exposure to disinfectants should be optimized depending on explant types (Da Silva et al. 2016; Hesami et al. 2019). Furthermore, many factors can influence the effectiveness of disinfection, including growing conditions and physiological state of the mother plant, size, age and type of explant, type of disinfectant and its concentration, duration, and temperature of exposure. In addition, these factors will not affect only the asepsis of the explant, but also its potential for survival and regeneration, which are essential conditions for the success of an *in vitro* culture protocol (Teixeira da Silva et al., 2016; Tahiri et al., 2022). In saffron, the disinfection protocols reported in the literature are rarely described in detail.

Therefore, the present investigation aims to develop an efficient, easy-to-use protocol for disinfecting explants, initiating and multiplicating saffron shoots, and ultimately achieving the production of micro-corms.

## Materials and Methods

### Plant material

Saffron corms were collected in June 2019 from a plantation carried out within the framework of a research agreement between the National Institute of Agricultural Research (INRA) and the Moroccan Interprofessional Federation of Saffron (FIMASAFRAN). This plantation (planted in October 2017) is in Ifri, Talioune region (province of Taroudant, Morocco), at 1500 m above sea level.

### Explants and surface sterilization

To optimize the sterilization procedure of the explant and achieve improved disinfection rates along with a high survival rate, this study employed four distinct protocols chosen based on those reported by Teixeira da Silva et al. (2016) with slight modifications. Initially, the corms underwent a thorough 30-minute washing under running tap water to eliminate impurities. Subsequently, the corms were subjected to surface sterilization using the following procedures:

- P1: the corms were put in a jar containing filter paper. 1 mL of formaldehyde ($CH_2O$) solution (37%) is then placed on the filter paper. The explants remain in contact with the formaldehyde vapors (37%) for 10 min and are then transferred to the culture medium (Druart 2012).
- P2: the corms were soaked in absolute ethanol for 10 min and then transferred to 50% (v/v) bleach (sodium hypochlorite, NaOCl, 12°) for 30 min. The explants are then

washed three times with sterile distilled water and then transferred to the culture medium (Soukrat et al., 2017).

- P3: the previous protocol (P2) was adjusted by reducing the time of soaking the corms in absolute ethanol to one min (instead of 10 min). The corms were then soaked for 30 minutes in 50% (v/v) bleach containing sodium hypochlorite (NaOCl, 12°) and the explants were washed three times with sterile distilled water before being transferred to the culture medium.
- P4: the corms were soaked in 0.1% (w/v) mercury chloride ($HgCl_2$) for 10 min. The explants are then washed three times with sterile distilled water and then transferred to the culture medium (Ebrahimzadeh et al., 2000).

The disinfected corms were blot-dried with sterile paper and placed on a first culture medium of Murashige and Skoog (MS) (Murashige and Skoog, 1962), supplemented with 0.5 mg/L NAA (1-naphthalene acetic acid), 1.5 mg/L BAP (6-benzylaminopurine), 3% sucrose and solidified with 0.8% of agar. One corm was placed in each glass tube containing 20 mL of medium. A completely randomized design with 10 corms for each treatment was used and repeated three times. The percentages of disinfection [(number of disinfected explants/ total number of explants) *100] and the explant survival rate ([(number of survived explants/ total number of explants) *100] were recorded after two weeks of culture.

### Bud sprouting and shoot initiation

Sterile corms were transferred to MS basal medium supplemented with N6-Benzylaminopurine (BAP), Naphtalene Acetic Acid (NAA), and Kinetin (KIN) at different concentrations as described in **Table 1**.

**Table 1.** Different hormonal combinations tested for the initiation of saffron cultures

| Medium | NAA (mg/L) | BAP (mg/L) | KIN (mg/L) |
|---|---|---|---|
| **iMS1** | 0.5 | 0.0 | 0.0 |
| **iMS2** | 0.5 | 1.5 | 0.0 |
| **iMS3** | 0.5 | 3.0 | 0.0 |
| **iMS4** | 3.0 | 5.0 | 0.0 |
| **iMS5** | 3.0 | 0.0 | 4.0 |

One corm was placed in each glass tube containing 20 mL of medium. A completely randomized design with 10 corms for each treatment was used and repeated three times. The bud sprouting rate ([(number of sprouted explant/ total number of explants) *100]) and bud (shoot) number were recorded after four weeks of culture.

### Shoot multiplication

After 6 weeks of culture, the initiated shoots were cut into a single shoot (or sprouted bud) and transferred to a multiplication culture medium. Different hormonal combinations of NAA and BAP were tested to study their effect on the multiplication of saffron shoots (**Table 2**). One sprouted bud was placed per jar containing 40 mL of medium. A completely

randomized design with 10 single shoots for each treatment was used and repeated three times. The number of shoots was recorded after two months of culture.

**Table 2**. Different hormonal combinations tested for saffron shoot multiplication.

| Medium | NAA (mg/L) | BAP (mg/L) |
|---|---|---|
| **pMS1** | 0.5 | 0.0 |
| **pMS2** | 0.5 | 2.0 |
| **pMS3** | 0.5 | 4.0 |
| **pMS4** | 0.5 | 6.0 |
| **pMS5** | 0.5 | 8.0 |

### Culture conditions

All culture media were supplemented with supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the culture media was adjusted to 5.7 before autoclaving at 121 °C for 20 min. All established in vitro cultures were incubated and maintained in a growth chamber at 22±2 °C with a 16 h light/8 h dark photoperiod provided by cool white fluorescent light.

### Data analysis

Cultures were observed and regularly evaluated every week. The final data were collected at the end of the experiment and analyzed based on the disinfection rate, the survival rate, the bud sprouting rate, and the bud and shoot numbers. Reported data were recorded from three independent trials (Ten corms per experimental factor). Data were subjected to analysis of variance (ANOVA) and Duncan's Multiple Range Test as a post-hoc analysis at a 5% significance level using SPSS software (version 21). Data are presented as mean ± standard error.

## Results and discussion

### Disinfection efficiency

Four disinfection methods were tested using corms as an explant. A significant difference at a 5% significance level was observed between the four tested protocols for both the disinfection rate ($p = 0.004$) and the survival rate ($p = 0.000$). In the first protocol (P1), the saffron corms were disinfected by exposure to 37% formaldehyde vapor ($CH_2O$) for 10 min. This protocol made it possible to obtain a low disinfection rate (30%) with an explant survival rate not exceeding 11% after 15 days of culture (**Figure 1**). These results are contradictory to those obtained by Druart (1992; 2012), who reported in *Prunus* that exposure of twigs to formaldehyde vapor is easy to apply and very effective for disinfection. While, in the second and third protocols, the corms were first disinfected by soaking in absolute ethanol (ETOH) for 10 min (P2) or 1 min (P3). The corms were then soaked for 30 min in 50% (v/v) bleach (sodium hypochlorite, NaOCl, 12°) before being washed three successive times with sterile distilled water. These two protocols showed their efficacy on explant disinfection with a percentage of 75%, with explant survival rates of 60 and 90% respectively (**Figure 1**). However, treatment of corms with 0.1% (w/v)

mercury chloride ($HgCl_2$) for 10 min (P4) resulted in a high disinfection rate of 95% with over 85% survival rate (**Figure 1**).

The comparison of these methods showed that the third (P3) and fourth (P4) protocols, prove to be more effective since they reduced the average contamination rate by up to 25% and 5% respectively, with an explant survival rate of 85-90%. Furthermore, studies have suggested an additional approach to enhance the disinfection rate by storing corms at low temperatures (1-3 °C) for 9 months. This extended storage duration has been shown to effectively reduce the rate of contamination, as reported by Renau-Morata et al. (2013). Implementing this method can contribute to further improving the overall success of the disinfection process, while Soukrat et al. (2017) recommend storing corms at 35 °C for 4 months in order to achieve a disinfection rate and bud viability of up to 100%.

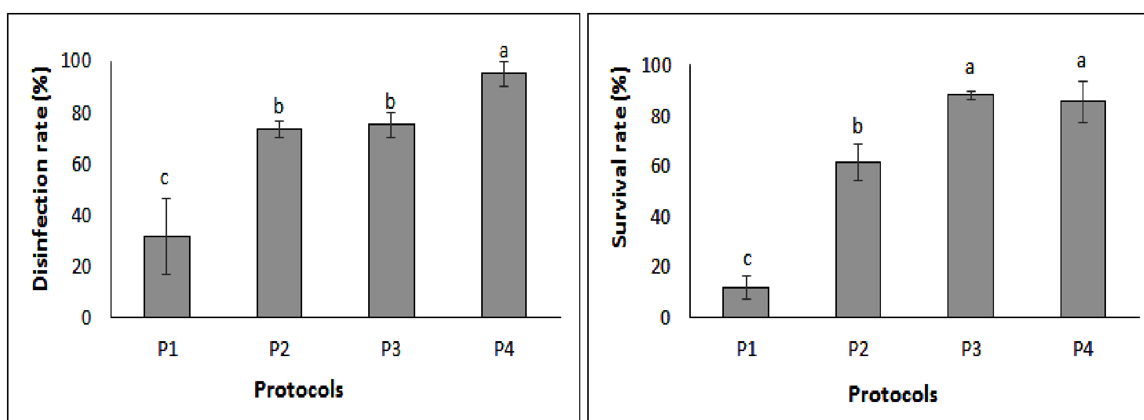


**Figure 1.** Disinfection and survival rates of *Crocus sativus* corms sterilized with different disinfection protocols. P1: formaldehyde vapors (37%) for 10 min. P2: absolute ethanol (10 min) + 50% (v/v) bleach (sodium hypochlorite, NaOCl, 12°) for 30 min. P3: absolute ethanol (1 min) = 50% (v/v) bleach (sodium hypochlorite, NaOCl, 12°) for 30 min. P4: 0.1% (w/v) mercury chloride ($HgCl_2$) for 10 min. Histograms with the same letters are not significantly different.

## Cultures initiation and bud sprouting

The effect of cytokinin type, concentration, and their interaction was found to be significant ($p < 0.05$) on bud sprouting rate and number (**Figure 2**). The sprouting rate varied between 70% in the medium without cytokinin (BAP) and 100% in the iMS4 medium containing 3.0 mg/L NAA and 5.0 mg/L BAP. On the other hand, the sprouted bud number after 6 weeks of culture was significantly affected by the concentration of PGRs (**Figures 4a and 4b)**. Indeed, the combination of NAA (0.5 mg/L) and BAP at a concentration of 3 mg/l significantly ($p = 0.021$) increased the number of sprouted buds (4.5 buds/corm). Similar results were reported by Salwee and Nehvi (2014), with a bud sprouting rate of 85% and a higher number of bud /corms (8). It is well known that the combination of different growth regulators has been reported to have strong effects on shoot regeneration in saffron. However, the success of direct regeneration of saffron shoots generally depends on the explant used, the combination of growth regulators, and the incubation temperature (Vahedi et al., 2014; Tahiri et al., 2022).

From these results, it is clear that the concentration of cytokinin, especially BAP, affects bud regeneration in the saffron plant. These results were consistent with the findings of Piqueras and Fernandez (2004) who indicated that BAP is the most efficient cytokinin for shoot initiation and proliferation from saffron meristems. This has also been confirmed by other researchers (Sarma et al., 1990; Loskutov et al., 1999; Sarhan et al., 2013; Simona et al., 2013). While the other combination did not show any significant effect compared to the combination of 0.0 mg/L BAP + 0.5 mg/L NAA, which yielded an average of 2 sprouted buds/corm.

Interestingly, for the initiation of aseptic cultures, the effect of season and temperature on the response to bud sprouting has been reported. Indeed, maximum bud sprouting was observed between November and December and a minimum between May and August (Devi 2013). To overcome this problem, Soukrat et al. (2017) recommend storing corms at 35 °C for 4 months in order to achieve bud viability of up to 100%. In this work, the corms used are harvested in August and stored at room temperature (25 ° C).

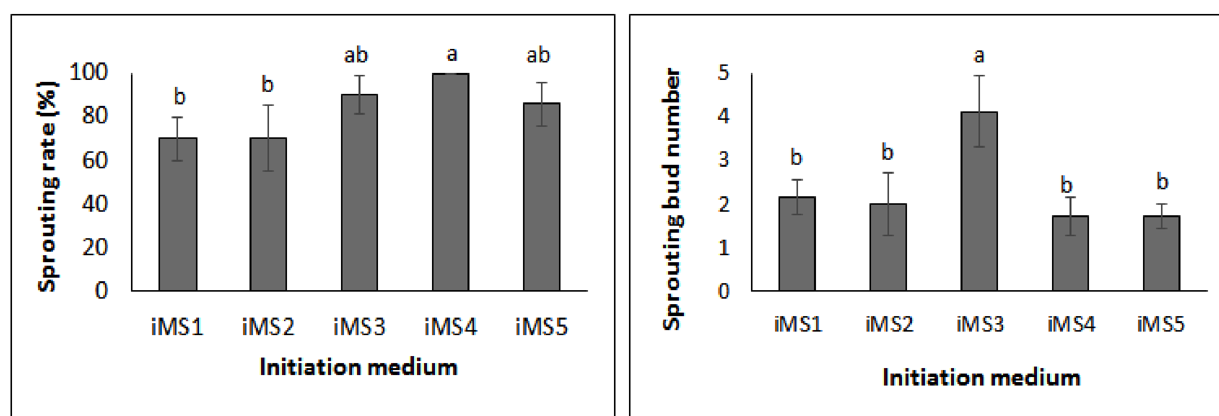


**Figure 2**. Effect of different hormonal combinations on saffron bud sprouting rate and number *in vitro*. iMS1 (0.5 NAA); iMS2 (0.5 NAA+1.5 BAP); iMS3 (0.5 NAA+3.0 BAP); iMS4 (3.0 NAA+5.0 BAP); iMS5 (0.5 NAA+4.0 KIN). Histograms with the same letters are not significant.

## Shoot proliferation

To improve the regeneration and multiplication of saffron shoots, a single shoot primordium was transferred to a multiplication medium (MS) containing different concentrations of BAP (0.0; 2.0; 4.0; 6.0 and 8.0 mg/L) in combination with NAA (0.5 mg/L). These hormones are known to stimulate cell division and the development of axillary buds by inhibiting apical dominance (Rubio-Moraga et al., 2014). Regeneration of shoots was observed in all media and the number of shoots increased significantly ($p = 0.000$) with increasing the concentration of BAP, except in a medium containing only NAA (0.5 mg/L) (**Figure 3**).

The concentrations of BAP had a significantly superior effect on shoot number after 4 weeks of culture. The highest number of shoots (4 shoots/single shoot) was observed on MS medium supplemented with 8.0 mg/L of BAP in combination with 0.5 mg/L of NAA

with a normal appearance (color green) (**Figure 4d)**, while the lowest number (1 shoot/single shoot) was observed on MS medium containing only NAA (0.5 mg/L) with a white appearance (**Figure 4c**). The maximum number of shoots obtained in this work (16 shoots/corm) is slightly higher compared to that obtained by other researchers with the same concentrations of BAP (Cavusoglu et al., 2013; Devi, 2013; Salwee et al., 2013; Soukrat et al., 2017).

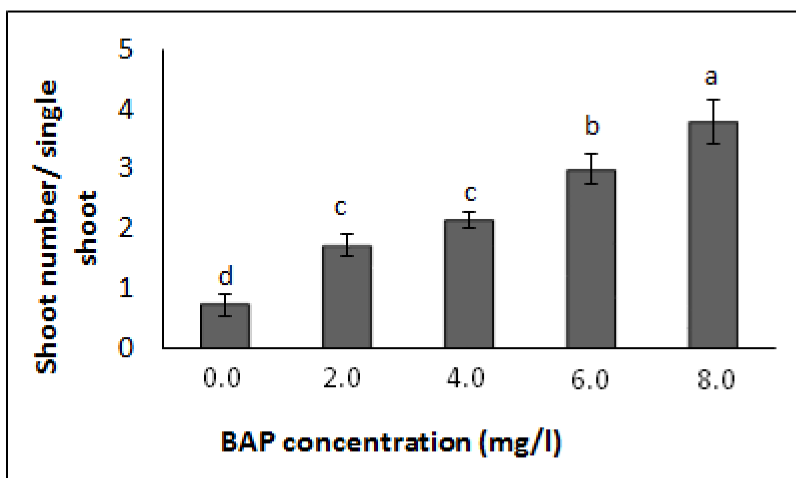


**Figure 3.** Effect of the concentration of BAP (0.0; 2.0; 4.0; 6.0 and 8.0 mg/L) in combination with 0.5 mg/L of NAA on the number of saffron newly formed shoots per single shoot. Histograms with the same letters are not significantly different.

Numerous studies have evaluated the effect of the combination of cytokinin and auxin, and more particularly BAP and NAA, on the regeneration of saffron shoots (Piqueras and Fernandez, 2004; Salwee, 2010; Cavusoglu et al., 2013; Simona et al., 2013; Vahedi et al., 2014). These studies reported that BAP is the most effective cytokinin for shoot proliferation from saffron meristems. Indeed, higher concentrations of BAP alone or in combination with a low concentration of NAA probably favored the production of lateral buds, leading to maximum shoot proliferation in saffron (Salwee 2010). The high concentrations of cytokinin are used to overcome the apical dominance of shoots, which improves the growth of lateral buds (Vahedi et al., 2014).

The maximum number of shoots obtained in this work (16 shoots per initial corm) was slightly higher compared to that obtained by other researchers with the same concentrations of BAP. Devi (2013) obtained a maximum number of shoots (15 shoots) on MS medium supplemented with 8 mg/L of BAP and incubated at 15 ± 2 °C. Maximum shoot initiation (96.7%) was obtained using MS medium supplemented with BAP (6 mg/L). However, a low percentage of shoot and leaf formation was obtained with MS medium supplemented with BAP at a low concentration (1 mg/L) or a very high concentration (10 mg/L) (Cavusoglu et al., 2013). While Salwee et al. (2013) obtained a maximum number of new buds at the base of the shoots (12) on a medium without auxin but supplemented with 6.5 mg/L of BAP in combination with 0.2 mg/L of NAA. However, the latter obtained a number of shoots ranging from 3 to 6 using 6 to 8 mg/L of BAP in combination with 0.5 mg/L of NAA. These differences may be related to the cultivation conditions and mainly

the incubation temperature. Indeed, Devi (2013) and Soukrat et al. (2017) used an incubation temperature of 15 ± 2 ° C under continuous light (38 ± 2 μmolm-2s-1), while in this work we used an incubation temperature of 22 ± 2 ° C with a photoperiod of 16h/8h (light/dark). Based on these results, it would therefore be necessary to re-evaluate shoot multiplication for an additional 4 weeks. It would also be interesting to test the combination of high concentrations of BAP (6 and 8 mg/L) with different concentrations of NAA (0; 0.1; 0.2; 0.5 mg/L) to increase the rate of multiplication of saffron *in vitro*. Further subculture of the obtained shoot on the free-PGR MS medium supplemented with 9% sucrose showed regeneration of one micro-corms/shoot (data not shown, **Figure 4e**).

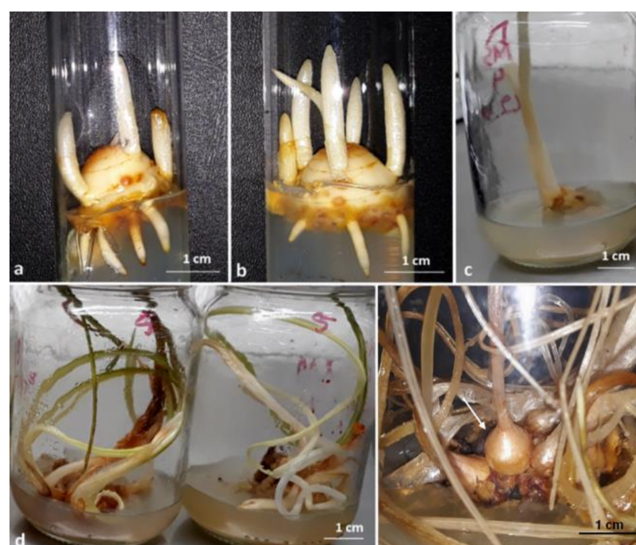


**Figure 4**. *In vitro* shoot regeneration and micro-corms formation of *Crocus sativus* L. (a) bud sprouting on MS + 0.5 mg/L NAA; (b) bud sprouting on MS+ 0.5 mg/L NAA+3.0 mg/L BAP; (c) shoot proliferation on MS + 0.5 mg/L NAA; (d) shoot proliferation on MS + 0.5 mg/L NAA + 8.0 mg/L BAP; (e) micro-corm formation on free-PGR MS + 9% sucrose.

## Conclusion

Micropropagation offers a promising alternative for large-scale multiplication, enabling rapid multiplication of plants. However, the success and cost of micropropagation largely depend on the survival percentage of the seedlings under field conditions. Saffron is a geophyte plant that is difficult to handle in *in vitro* culture. Thus, the present work aimed to optimize the disinfection and *in vitro* multiplication of saffron. It has been found that soaking explants in 0.1% (w/v) mercury chloride (HgCl2) for 10 min is more effective for explant disinfection (95%) with a survival rate of over 85%. During the initiation phase, the cultivation of whole corms on MS medium supplemented with 0.5 mg/L of NAA in combination with 3 mg/L of BAP proved to be more effective since it made it possible to obtain a sprouting bud rate of 90% with a number of 5 shoots per explant. For the multiplication phase, the highest number of shoots (16 shoots/corm) is observed on MS medium supplemented with 8.0 mg/L of BAP in combination with 0.5 mg/L of NAA. Hence, more investigations should be carried out to develop efficient and reproducible *in vitro*

regeneration systems in order to increase the number of shoots per explant for proliferation and micro-corms formation phases by exploring other culture media with different PGR combinations.

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## References

Altan F., Bürün B. et Sahin, N. (2010). Fungal contaminants observed during micropropagation of *Lilium candidum* L. and the effect of chemotherapeutic substances applied after sterilization. African Journal of Biotechnology, 9. p. 991–995. DOI: 10.5897/AJB08.090

Bach A. et Sochaki D. (2013). Propagation of ornamental geophytes: physiology and management systems. In: Kamenetsky R., Okubo H. (eds.) Ornamental Geophytes from Basic Science to Sustainable Production. CRC Press, Boca Raton. p. 261–287.

Cardone L., Castronuovo D., Perniola M., Cicco N. et Candido V. (2020). Saffron (*Crocus sativus* L.), the king of spices: An overview. Scientia Horticulturae, 272. 109560. https://doi.org/10.1016/j.scienta.2020.109560

Cavusoglu A., Sulusoglu M. et Erkal S. (2013). Plant regeneration and corm formation of saffron (*Crocus sativus* L.) *in vitro*. Research Journal of Biotechnology, 8. p. 128-133.

Devi K., Sharma M. et Ahuja P.S. (2014). Direct somatic embryogenesis with high frequency plantlet regeneration and successive cormlet production in saffron (*Crocus sativus* L.). South African Journal of Botany, 93. p. 207–216. https://doi.org/10.1016/j.sajb.2014.04.006

Devi K., Sharma M., Singh M. et Ahuja P.S. (2011). *In vitro* cormlet production and growth evaluation under greenhouse conditions in saffron (*Crocus sativus* L.)- A commercially important crop. Engineering in Life Sciences, 11. p. 189-194. https://doi.org/10.1002/elsc.201000080

Devi K. (2013). Studies on *In vitro* Propagation and Genetic Transformation in Saffron *Crocus sativus* L. PhD thesis, Guru Nanak Dev University.

Druart Ph. (2012). Micropropagation of prunus species relevant to cherry fruit production. In: Lambardi M, Ozudogru EA, Jain SM (eds.) Protocols for micropropagation of selected economically important horticultural plants. Methods in Molecular Biology, 994. p. 119-136. doi: 10.1007/978-1-62703-074-8_9.

Druart P. (1992). *In vitro* culture and micropropagation of plum (*Prunus* spp.). In : High-Tech and Micropropagation II. Springer, Berlin, Heidelberg, 1992. p. 279-303.

Ebrahimzadeh H., Karamian R., Noori-Daloii M.R. (2000). Somatic embryogenesis and regeneration of plantlet in saffron, Crocus sativus L. Journal of Sciences, Islamic Republic of Iran, 11. p. 169–173

FIMASAFRAN, 2018. Etat d'avancement du contrat-programme de la filière du safran, FIMASAFRAN, Octobre 2018.

Gantait S. et Vahedi M. (2015). *In vitro* regeneration of high value spice *Crocus sativus* L.: a concise apprais*al*. Journal of Applied Research on Medicinal and Aromatic Plants, 2. p. 124–133. https://doi.org/10.1016/j.jarmap.2015.07.003

Hagizade A. (2016). Optimizing Propagation of Saffron (*Crocus sativus* L.) Corms by *in vitro* direct regeneration (M.Sc. thesis). Ferdowsi University of Mashhad, Iran

Halim R., Akyol B., Gürel A. et Bayraktar M. (2018). *In vitro* callus induction of saffron (*Crocus sativus* L.). International Journal of Innovative Science and Research Technology, 3(11). p. 696–700.

Karaoglu C., Cocu S., Ipek A., Parmaksiz I., Sarihan E., Uranbey S., Arslan N., Kaya M.D., Sancak C., Ozcan S., Gurbuz B., Mirici S. C. et Khawar K.M. (2007). *In vitro* micropropagation of saffron. Acta Horticulturae, 739. p. 229–234. https://doi.org/10.17660/ActaHortic.2007.739.28.

Kareem M., Nabi B., Kashtwar M., Wani A.A. et Dhar, M.K. (2019). Preliminary report on development of proper stigmas and stigma-like structures in saffron under *in vitro* conditions. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 89(4). p. 1213–1217. https://doi.org/10.1007/s40011-018-1037-2

Khawar M.K., Yildirim M. et Sarihan E. (2020). *Ex vitro* macropropagation of saffron (*crocus sativus* l.) Corms. In: Sarwat M et Sumaiya SBT-S, eds. Academic Press. p.45-56. doi:https://doi.org/10.1016/B978-0-12-818462-2.00004-8

Loskutov A.V., Beninger W., Ball T.M., Hosfield M., Nair M. et Sink K.C. (1999). Optimization of *in vitro* conditions for stigma like structure production from half ovary explants of *Crocus sativus* L. In Vitro Cellular et Developmental Biology - Plant*,* 35. p. 200-205.

Menia M., Iqbal S., Zahida R., Tahir S., Kanth R.H., Saad A.A. et Hussian A. (2018). Production technology of saffron for enhancing productivity. Journal of Pharmacognosy and Phytochemistry*,* 7(1). p.1033-1039.

Moradi M.M. et Turhan S. (2017). The importance of Saffron plant in Afghanistan's agricultura. Journal of Biological et Environmental Sciences*,* 11(33). p.165-169.

Moshtaghi N. (2020). Tissue and cell culture of saffron. In: Koocheki, A., Khajeh-Hosseini MBT-S, eds. *Woodhead Publishing*, 2020:229-246. https://doi.org/10.1016/B978-0-12-818638-1.00014-9.

Murashige T. et Skoog F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiology Plant*,* 15. p. 473-479.

Palomares C. (2015). Le safran, précieuse épice ou précieux médicament. Thèse de doctorat, Université de Lorraine, France

Parray J.A., Kamili A.N., Hamid R. et Husaini A.M. (2012). *In vitro* cormlet production of saffron (*Crocus sativus* L. Kashmirianus) and their flowering response under greenhouse. GM Crops Food Biotechnology in Agriculture and the Food Chain*,* 3(4). p. 289–295. https://doi.org/10.4161/gmcr.21365

Piqueras A. et Fernandez J.A. (2004). Phase change from dormancy to continuous shoot proliferation in cultured meristems of saffron corms. *In Vitro* Cellular et Developmental Biology – Animal, 40. 74A.

Renau-Morataa B., Moyáa L., Nebauera S.G., Seguí-Simarrob J.M., Parra-Vegab V., Gómezc M.D. et Molinaa R.V. (2013). The use of corms produced under storage at low temperatures as a source of explants for the *in vitro* propagation of saffron reduces contamination levels and increases multiplication rates. Industrial Crops and Products*,* 46. p. 97–104. https://doi.org/10.1016/j.indcrop.2013.01.013.

Salwee Y. et Nehvi F.A. (2014). *In vitro* microcorm formation in saffron (*Crocus sativus* L.). Journal of Cell and Tissue Research, 14 (2). p. 4463–4470.

Salwee Y., Nehvi F.A. et Shafiq A.W. (2013). Tissue culture as an alternative for commercial corm production in saffron: A heritage crop of Kashmir. African Journal of Biotechnology, 12(25). p. 3940-3946.

Salwee Y., Wani S.A. et Lone, A.A. (2010). Tissue culture: A tool for corm production in saffron. In: Souvenir and abstracts National seminar on technological innovations in saffron, November 25-26. pp. 19

Sarhan A.Z., Habib A.M., Azza M., Refaat A.M. et Awadalla S.S.A. (2013). Comparative effects of BAP and NAA on explant development of micropropagated saffron (*Crocus sativus* L.) corms. Journal of Applied Sciences Research, 9. 5141-5145.

Sarma K.S., Maesato K., Hara T. et Sonoda, Y. (1990). *In vitro* production of stigma-like structures from stigma explants of *Crocus sativus* L. Journal of Experimental Botany, 41. p.745–748. https://doi.org/10.1093/jxb/41.6.745

Sharma K.D. et Piqueras A. (2010). Saffron (*Crocus sativus* L.) tissue culture : micropropagation and secondary metabolite production. Functional Plant Science and Biotechnology*,* 4 (2). p. 15-24.

Simona L., Cerasela P., Florina F., Lazar A., Giancarla V., Danci M. et Maria B. (2013). *In vitro* regeneration of *Crocus sativus* L. Journal of Horticulture, Forestry and Biotechnology*,* 17. p. 244- 247.

Soukrat S., Benlhabib O., Alfaiz C. et Lage M. (2017). Boosting saffron (*Crocus sativus* L.) micro-propagation through *in vitro* corm production. Acta Horticulturae*,* 1184. p. 87-95. https://doi.org/10.17660/ActaHortic.2017.1184.13

Tahiri A., Mazri M.A., Karra Y., Ait Aabd N., Bouharroud R. et Mimouni A. (2022). Propagation of saffron (*Crocus sativus* L.) through tissue culture: a review. *The* Journal of Horticultural Science and Biotechnology, 98(1). p. 10-30. DOI:10.1080/14620316.2022.2078233

Teixeira da Silva T.A., Kulus D., Xinhua Z., Zeng S., Guohua M. et Piqueras A. (2016). Disinfection of explants for saffron (*Crocus sativus* L.) tissue culture. Environmental and Experimental Biology,14. p.183-198. http://doi.org/10.22364/eeb.14.25.

Vahedi M., Kalantari S. et Salami S.A. (2014). Factors affecting callus induction and organogenesis in saffron (*Crocus sativus* L.). Plant Tissue Culture and Biotechnology, 24. p.1-9. DOI: 10.3329/ptcb.v24i1.19184

Yildirim E. (2007). Development of *in vitro* micropropagation techniques for Saffron (*Crocus sativus* L.). Master of Science in Department of Biology, Middle East Technical University.