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In Vitro Floral Emergence and Improved Formation of Saffron Daughter Corms

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Abstract: In vitro cormogenesis is a potential tool for improving saffron production under controlled conditions. In this study, the effects of explant type, culture type, and medium supplements on saffron daughter corm formation in vitro were assessed. Saffron flowers emerged 30 days after culture, and the sizes of in-vitro- and ex-vitro-produced flowers and stigmas were similar. In vitro daughter corm formation and the saffron life cycle was completed after 10 and 14 weeks of culture, respectively. Using in vitro intact corms was more effective for corm production than using apical buds. Compared with apical bud explants, mother corm explants produced more corms with a higher fresh weight and diameter. Compared with solid culture, liquid cultures using bioreactors provided corms with a higher fresh weight and diameter, regardless of explant type. An ebb and flow system provided the highest cormlet fresh weight and diameter but the fewest cormlets, whereas an immersion system provided more cormlets with a smaller size. Saffron apical buds cultured with salicylic acid at 75 mg L^{-1} or glutamine at 600 mg L^{-1} exhibited the highest cormlet diameter and fresh weight. These findings will improve the process of in vitro cormogenesis and the production of saffron under controlled conditions.

Keywords: *Crocus sativus*; glutamine; Iridaceae; jasmonic acid; liquid culture; salicylic acid

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1. Introduction

Saffron (*Crocus sativus*; Iridaceae) is a sterile autotriploid and stemless monocotyledonous geophyte plant [1,2]. Therefore, obtaining daughter corms from mother corms is the only available method of saffron propagation [3]. Within the life cycle of saffron, flowering occurs during autumn (October–November), and the vegetative stage, including the formation of replacement corms at the base of the shoots, occurs throughout winter. At the beginning of the dry season (April–May), the leaves senesce and wither, and the corms go into dormancy. The transition from the vegetative stage to the reproductive stage can occur shortly afterwards in the apex of the buds of underground corms [4]. Saffron is a valuable spice obtained from the stigmas of *C. sativus*, and the value of saffron is enhanced by its potential use in biomedicine [5]. Thus, the demand for saffron is expected to increase in the coming years owing to its nutraceutical and medicinal properties.

In vitro culture technologies could facilitate the sustainable indoor production of saffron through the growth of pathogen-free stock corms. Saffron microcorm production under in vitro conditions is a promising technique with respect to the rate of multiplication and the number of cormlets produced. However, few studies have investigated the production of complete plants with roots and/or corms [6–9]. Saffron flowers grown in vitro could also serve as a source of saffron spice; however, the induced in vitro flowering of saffron plants has not been reported previously.

In in vitro propagation, liquid culture/bioreactor systems enable automation and reduce the costs of plantlet production [10]. Liquid culture systems provide uniform

culture conditions in which the nutrient medium is renewed without changing the container. Moreover, the container can be cleaned with ease after the culture period. Plant tissues and organs from various plant species exhibit higher levels of growth performance in a liquid medium than in solid or semi-solid media. Liquid culture/bioreactor systems and their characteristic culture conditions have several advantages over solid cultures, including the convenient handling of cultures and enhanced plant growth [11]. Although bioreactor culturing has been used to grow many plant species, to the best of our knowledge, the use of bioreactors to produce saffron microcorms has not been reported previously. The main disadvantages of saffron tissue culture are the low frequency and the small size of cormlets induced from in-vitro-derived shoots [12–14].

The aim of the present study was to compare in vitro flower induction and the formation of daughter corms in gel and liquid cultures. Additionally, different concentrations of glutamine (as a nitrogen source), salicylic acid, and jasmonic acid were tested with the aim of improving the formation of saffron daughter corms. Overall, this study demonstrates that saffron flowering can be induced in vitro. Moreover, compared with solid cultures, liquid cultures/bioreactors improved daughter corm diameter and fresh weight. Thus, the production of saffron under controlled in vitro conditions is a viable option.

2. Materials and Methods

2.1. Surface Disinfection and the Establishment of Aseptic Culture

Crocus sativus corms (3.2–3.5 cm diameters; Figure 1a,b) were obtained from Bloembollenbedrijf J.C.Koot (Vennewatersweg, The Netherlands) during two successive seasons in 2020 and 2021 and kept at room temperature. The corms were descaled, and injured or infected corms were discarded. The corms were washed three times using sterile distilled water containing Tween-20 and then rinsed with a fungicide solution (2 mL L^{-1} Ortiva; Syngentia, Switzerland) containing 200 g L^{-1} of azoxystrobin and 125 g L^{-1} of difenoconazole^{-1} for 15 min. The corms were then submerged in 10% (v/v) commercial bleach (5.2% sodium hypochlorite) for 10 min for disinfection and transferred to a 0.1% (w/v) mercuric chloride solution for 5 min. Subsequently, the corms were rinsed three more times using a sterile solution containing 2 g L^{-1} of ascorbic acid and 1 g L^{-1} of citric acid. They were then cultured in Magenta GA-7 culture vessels (77 mm × 77 mm × 97 mm; Sigma Chemical Co., St. Louis, MO, USA) containing semi-solid Murashige–Skoog (MS) medium [15] without plant growth regulators and incubated in the dark for 1 week. The cultures were checked regularly, and the percentage of corm contamination was recorded.

2.2. Saffron Flowering in In Vitro Culture and Hydroponic Culture

In vitro aseptic corms were grown in MS medium supplemented with 3% sucrose and solidified with 0.8% agar–agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C and 1.2 kg cm^{-2} pressure for 20 min. The environmental conditions of the growth chamber were adjusted to 15 °C ± 1 °C and 70 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) for a 16 h photoperiod using cool white fluorescent lamps. The flowering characteristics, i.e., the number of flowers per plant, stigma length, and stigma fresh and dry weights, under in vitro culture were compared with those under an aerated, volcanic-rock-based hydroponic system as described by Dewir and Alsadon [16]. The corms were obtained from the same source (Bloembollenbedrijf J.C.Koot) and were of the same size grade (3.2–3.5 cm diameters).

2.3. Effects of Explant Type and Solid/Liquid Culture on Saffron Daughter Corm Formation

Two types of liquid culture systems, temporary immersion (ebb and flow) and continuous immersion (with a net), were tested to select a suitable method for saffron cormlet production in liquid media and then compared with solid culture. Intact corms or apical buds (15 explants per bioreactor) were transferred to a 3 L balloon-type bubble bioreactor with 1.2 L of MS liquid medium supplemented with 30 g L^{-1} of sucrose and 0.5 mg L^{-1} of NAA.

The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C and 1.2 kg cm^{-2} pressure for 30 min. In the immersion-type bioreactor, a supporting net was used to hold the plant material to avoid the complete submersion of explants in the liquid medium. The volume of air input was adjusted to 0.2 vvm (air volume/culture volume min^{-1}). The ebb and flow system (PLT Scientific SDN BHD, Puchong, Selangor D.E., Malaysia) was programmed to immerse the plantlets in medium four times per day for 60 min per immersion. All the bioreactors and culture vessels were maintained at 15 °C ± 1 °C and 70 µmol m^{-2} s^{-1} PPFD for a 16 h photoperiod using cool white fluorescent lamps. There were 15 explants in each treatment, and the diameter, fresh weight, and number of daughter corms were recorded after 14 weeks of culture from 9 randomly selected explants.

2.4. Effects of Salicylic Acid, Glutamine, and Jasmonic Acid on Saffron Daughter Corm Formation In Vitro

Saffron apical buds were grown in MS medium supplemented with 3% sucrose and the following additives: salicylic acid (75 or 150 mg L^{-1}), glutamine (600 or 1200 mg L^{-1}), and jasmonic acid (1 or 2 mg L^{-1}). The explants grown in MS medium without additives were used as controls. Thus, the effects of nine different treatments on daughter corm size were assessed. All media were solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C and 1.2 kg cm^{-2} pressure for 20 min. The environmental conditions in the growth chamber were adjusted to 15 °C ± 1 °C and 70 µmol m^{-2} s^{-1} PPFD for a 16 h photoperiod using cool white fluorescent lamps. There were 15 explants in each treatment and the diameter and fresh weight of daughter corms were recorded after 14 weeks of culture from 9 randomly selected explants.

2.5. Experimental Design and Data Analysis

The experiments were conducted using a completely randomized design with three replicates per treatment. The effects of the treatments were assessed using Tukey's range test in SAS (version 6.12; SAS Institute, Inc., Cary, NC, USA).

3. Results and Discussion

3.1. In Vitro Establishment of Saffron Aseptic Culture and Flower Emergence

Preliminary trials were conducted in October 2020 to test the surface disinfection of saffron corms using sodium hypochlorite, mercuric chloride, and hydrogen peroxide for different durations and at various concentrations following the protocols described in previous studies [9,17–19]. Despite careful cleaning during corm preparation (Figure 1a–c), pathogen contamination was a serious challenge during the establishment of aseptic saffron culture (Figure 1d,e). Only 10% of the corms survived fungal and bacterial contamination, and we were unable to establish sufficient aseptic corms in this season. In the following season, we surface-disinfected saffron corms in July 2021 using the protocol described in the Materials and Methods Section, and a high percentage of aseptic corms (70%) was obtained. Teixeira da Silva et al. [20] reviewed various saffron corm disinfection methods, highlighting the numerous factors that can influence the efficiency of disinfection: cultivation conditions; the physiological state of the stock plant; the size, age, and type of the explant; the type and concentration of disinfectant; and the time and temperature of exposure. In the present study, establishing the aseptic saffron culture after the corm harvest in July, rather than in later months, increased the efficiency of corm disinfection. Bud sprouts during corm storage can increase microorganism abundance within the saffron corm, making it difficult to eradicate contaminants.

The growth of the main apical bud and the emergence of roots occurred within 1 week (Figure 1f), and all lateral buds grew within 3 weeks of culture (Figure 1g). Saffron flower buds appeared 30 days after culture (Figure 1h,i). Interestingly, the sizes of the flowers and stigmas produced in vitro and via hydroponics were similar (Figure 1j,k; Table 1). Additionally, only large saffron corms (≥2.8 cm in diameter) produced flowers in vitro; small corms did not produce flowers.

Table 1. Comparison of stigma and flower size of saffron produced under in vitro culture and via a hydroponic system (Dewir and Alsadon [16]).

	Number of Flowers/Plant	Stigma Length (mm)	Stigma Fresh Weight (mg)	Stigma Dry Weight (mg)
In vitro	1.80	41.32	42.68	5.29
Hydroponic	1.90 ^{NS}	42.46 ^{NS}	43.81 ^{NS}	5.68 ^{NS}

^{NS} = non-significant according to *t*-test.

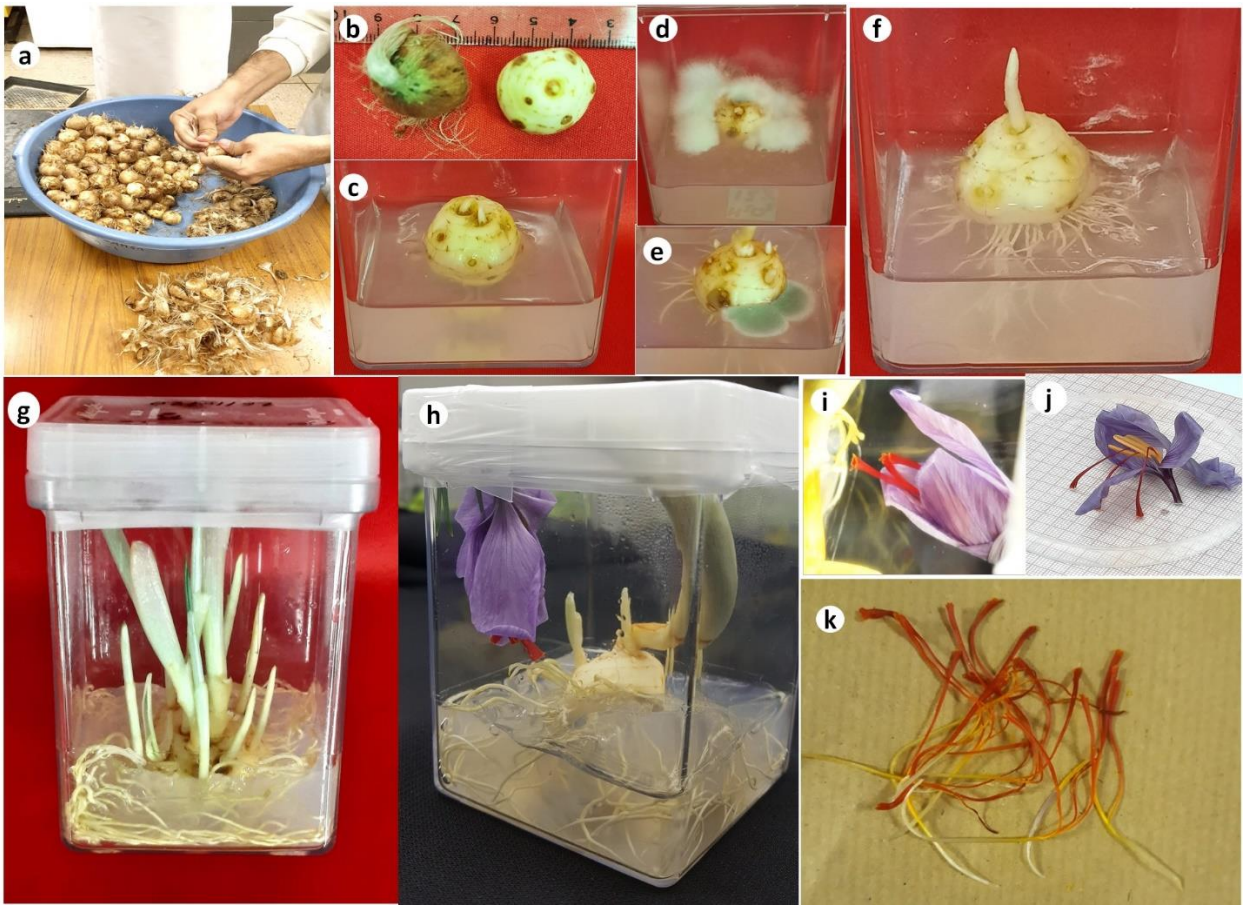

Figure 1. Surface disinfection and the establishment of in vitro saffron aseptic culture: (**a**–**c**) Cleaning, surface disinfection, and culture of corms on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose; (**d**,**e**) Fungal contamination of in-vitro-cultured corms 1 week after incubation; (**f**) Bud break and rooting of saffron corms 7 days after incubation at 16 °C; (**g**) Shoot emergence and growth of saffron buds 3 weeks after incubation; (**h**,**i**) In vitro flowering of saffron corms 30 days after incubation; (**j**) Normal size and structure of in-vitro-produced saffron flowers; and (**k**) Harvested stigma of in-vitro-produced saffron.

In vitro flowering systems are considered convenient tools for studying the transition to flowering. They can be used to study specific aspects of flowering, as well as the mechanisms underlying the reproductive process [21]. Both developmental cues and environmental signals control the timing of flowering, and genetic studies have revealed the complexity of the mechanisms underlying flowering [22]. A combination of environmental, developmental, hormonal, and genetic factors determines the eventual transition to flowering [23]. However, saffron corms have no cold requirement to break dormancy or to complete flower formation, which is often the case in geophytes [24,25].

Under field conditions, floral initiation usually occurs during summer, coinciding with the revival of meristematic activity in the apical bud. Thus, corms can be cultivated

directly in the field to force flowering, or they can be stored for up to 60 days at 2 °C to extend and facilitate harvesting [26,27]. Temperature, among several environmental parameters, plays a pivotal role in saffron flower induction [4,28,29]. Therefore, saffron corms are incubated at a moderately high temperature (23 °C–27 °C) to induce flowering before they are exposed to a moderately low temperature (17 °C) for floral emergence. In the present study, floral emergence occurred in vitro 30 days after the incubation of the saffron corms at 15 °C ± 1 °C. These in-vitro-produced saffron stigma can be utilized as a source of biochemicals and pharmaceuticals. They also can be explored as initial explants for callus formation and cell cultures. In-vitro-formed flowers are generally undersized compared with ex-vitro-formed flowers, as reported previously for *Phyllanthus niruri* [30] and *Spathiphyllum cannifolium* [31]. However, some in-vitro-grown plant species form normal-size flowers, e.g., *Chamomilla recutita* [32] and *Euphorbia milii* [33]. Comparing the in vitro and hydroponic flowering of saffron, the flowers formed in vitro had a similar size compared with those formed ex vitro. This may have been due to the early initiation of the saffron floral buds and the available nutrient supply from the mother corm supporting floral bud emergence and flower development.

3.2. Effects of Explant Type and Solid/Liquid Culture on Saffron Daughter Corm Formation

The use of intact mother corms or apical buds as initial explants (Figure 2a,e) influenced saffron daughter corm formation. In vitro, daughter corms were formed successfully after 10 weeks of culture (Figure 2b–g). After 14 weeks of culture, the life cycle of saffron was completed in vitro; the leaves were dried, and the saffron daughter corms were harvested (Figure 2d,g). All corms and apical bud explants formed daughter corms. However, the use of intact corms was more effective for corm production than the use of apical buds. More corms were produced, and the average fresh weight and diameter of corms were higher in mother corm explants than in apical bud explants. The number of cormlets, cormlet fresh weight, and cormlet diameter were also influenced by the culture type (i.e., continuous immersion bioreactors, ebb and flow bioreactors, and solid culture) (Figure 2h,i and Figure 3). Intact corms produced a higher number of corms than were produced by apical bud explants. The average number of cormlets per intact corm was 2.0–4.5 cormlets, owing to the presence of several lateral buds in the mother corms, whereas the apical buds produced single cormlets. For both intact corms and apical buds, liquid cultures using bioreactors resulted in corms with a higher fresh weight and diameter than those grown via solid culture. The average fresh weight and diameter of cormlets was the highest under the ebb and flow system although a low number of cormlets was obtained. Conversely, a higher number of cormlets with a smaller size was obtained using the immersion system.

Liquid medium is known to promote nutrient uptake and encourage growth and proliferation in vitro. The bioreactors designed for plant cultures provide optimal aeration and mixing with oxygen, without subjecting the propagules to shear stress [11]. Several plant species exhibit better growth in liquid/bioreactor cultures than in solid cultures, e.g., *E. milli* [34,35], *S. cannifolium* [36], *Gentiana triflora* [37], *Lessertia frutescens* [38], *Kalopanax septemlobus* [39], and *Philodendron bipinnatifidum* [40]. Piqueras et al. [41] highlighted the improved development of the cormogenic nodules and microcorms of saffron grown in liquid cultures relative to solid cultures. In the present study, the high number of cormlets and fresh mass of saffron corms grown in a bioreactor system might be attributable to enhanced water and nutrient uptake, as well as aeration, i.e., conditions that favor better growth compared with those under a nonaerated, solid culture system. Compared with continuous immersion, the ebb and flow system produced fewer cormlets, but they were larger. The higher efficacy of the ebb and flow system, in terms of fostering optimum cormlet growth, is probably due to culture ventilation and the intermittent contact between the entire tissue surface and the liquid medium, two features that are not usually combined in other liquid culture procedures [42,43].

Figure 2. Effects of explant type (intact corms and apical buds) and culture system (solid culture, continuous immersion bioreactors, and ebb and flow bioreactors) on in-vitro-produced daughter saffron corm formation: (**a**) Established saffron aseptic culture; (**b**–**d**) Daughter corm formation following 10, 14, and 16 weeks, respectively, of solid culture using intact corm explants; (**e**) Bud break and shoot development of apical buds after 2 weeks of culture; (**f**,**g**) Daughter corm formation following 14 and 16 weeks, respectively, of solid culture using intact corm explants; (**h**) Layout of continuous immersion and ebb and flow bioreactors; (**i**–**k**) Daughter corm formation using intact corms in continuous immersion bioreactors, ebb and flow bioreactors, and solid culture, respectively, after 16 weeks of culture; (**l**) Daughter corm formation using apical buds in continuous immersion bioreactors, ebb and flow bioreactors, and solid culture, respectively, after 16 weeks of culture.

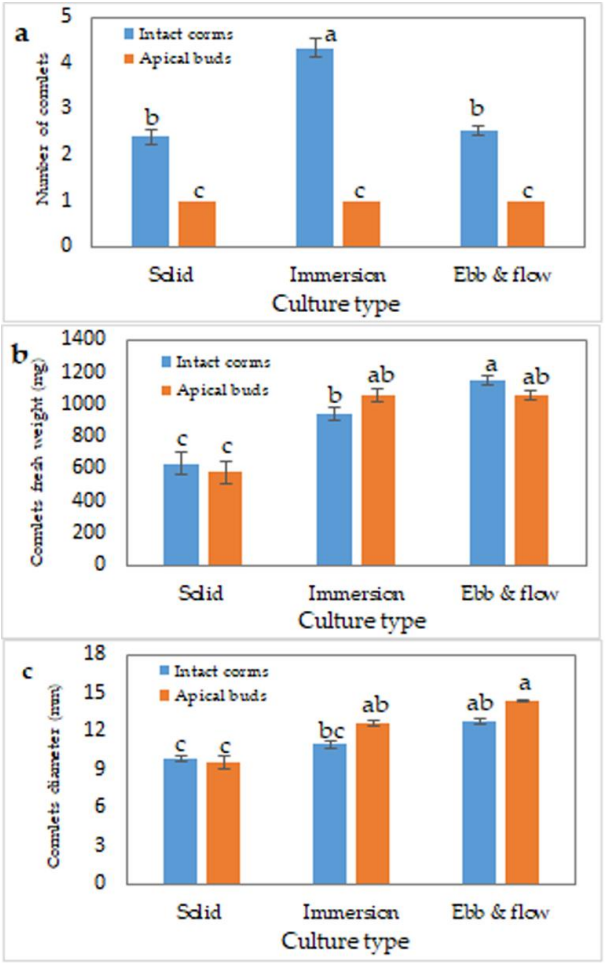

Figure 3. Effects of explant type (intact corms and apical buds) and culture type (solid culture, continuous immersion bioreactors, and ebb and flow bioreactors) on number of cormlets (**a**), cormlet fresh weight (**b**), and cormlet diameter (**c**) of saffron. Different letters show significant differences at $p \leq 0.05$.

3.3. Effects of Salicylic Acid, Glutamine, and Jasmonic Acid on Saffron Daughter Corm Formation In Vitro

The apical buds of saffron cultured on MS medium supplemented with different additives, i.e., salicylic acid, glutamine, and jasmonic acid, showed variation in cormlet diameter and fresh weight (Table 2; Figure 4). With the exception of 2 mg L^{-1} of jasmonic acid, all other treatments increased cormlet diameter and fresh weight relative to the control treatment, with salicylic acid at 75 mg L^{-1} and glutamine at 600 mg L^{-1} providing the highest and second highest increases, respectively, increasing the corm diameter by around 0.4 cm and nearly doubling the fresh weight compared with the control treatment. Salicylic acid is a phytohormone that plays important roles in many aspects of plant life, including seed germination, physiological and biochemical processes, flowering, and fruit yield [44,45]. Under field conditions, salicylic acid is effective for improving plant and bulb growth. In a plastic house experiment, soaking *Iris hollandica* bulbs in salicylic acid (200 mg L^{-1} for 4 h) improved plant vegetative growth [46]. In another study, the exogenous application of salicylic acid (250 mg L^{-1} at 30, 45, and 60 days after transplanting) increased not only the vegetative growth, but also the bulb weight, diameter, and yield of onion (*Allium cepa* 'ALR') [47]. In a study on saffron, corm dipping in a solution of salicylic acid (2 mM for 6 h) improved vegetative and reproductive characteristics, as well as yield quality [48]. Similarly, the application of salicylic acid (1–2 mM) to saffron, either during corm priming or foliar application, improved the vegetative growth and physiological and biochemical characteristics [49]. Under in vitro conditions, the exogenous application of salicylic acid is

useful for the growth and development of plants. Salicylic acid has been reported to enhance microtuber formation [50] and increase starch percentage [51] in potato plants (*Solanum tuberosum*) propagated in vitro. Glutamine is an amino acid and nitrogen source that supports several metabolic processes. The addition of amino acids provides a readily available primary source of nitrogen in tissue culture systems, and uptake occurs more rapidly than the uptake of inorganic nitrogen in the same medium [52]. Glutamine has been reported to have positive effects in plant tissue cultures [53,54]. Jasmonic acid is an endogenous plant-growth regulating substance. In the present study, jasmonic acid at 1 mg L^{-1} increased corm size compared with corm size in the control, but 2 mg L^{-1} of jasmonic acid did not produce positive effects. Exogenous jasmonic acid is considered to play an important role in bulb formation, as shown in studies on garlic [55] and onion [56]. In addition, exogenous jasmonic acid supported the microtuberization of three food yam (*Dioscorea*) species [57] and promoted the enlargement of in-vitro-grown bulbs in shoot cultures of *Narcissus* plants [58] and the daughter bulb development of *Tulipa gesneriana* [59]. However, the positive or negative effects of jasmonic acid are species- and concentration-dependent.

Table 2. Effects of salicylic acid, glutamine, and jasmonic acid treatments on saffron daughter corm diameter and fresh weight after 14 weeks of culture.

Treatments	Concentration (mg L^{-1})	Average Daughter Corm Diameter (mm)	Average Daughter Corm Fresh Weight (g)
Control	0	9.93 d	0.94 c
Salicylic acid	75	14.33 a	1.99 a
	150	11.40 c	1.28 bc
Glutamine	600	13.89 ab	1.89 a
	1200	11.83 abc	1.73 ab
Jasmonic acid	1	12.49 abc	1.24 bc
	2	9.58 d	0.82 c

Different letters within a set of values denote significant differences at $p \leq 0.05$ according to Tukey's test.

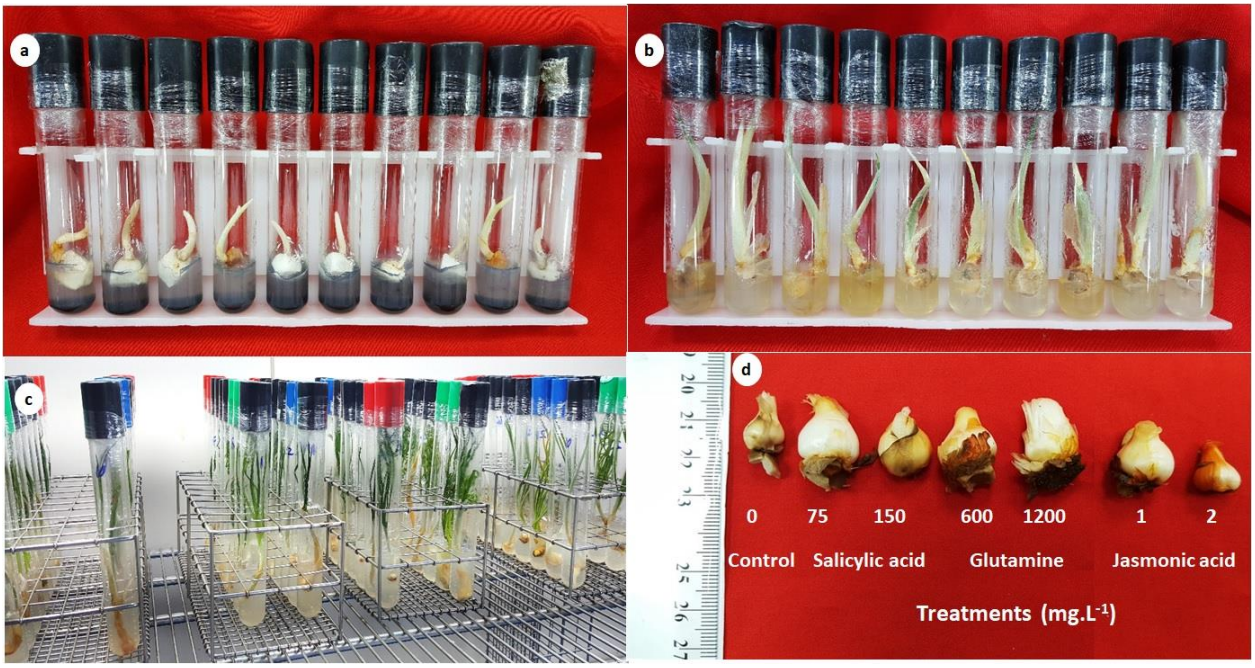

Figure 4. Influence of salicylic acid, glutamine, and jasmonic acid on saffron daughter corm formation using apical bud explants: (**a**) Surface disinfection and bud break of saffron apical buds cultured on MS medium supplemented with 3% sucrose and 1 g L^{-1} of activated charcoal 2 weeks after incubat-

ion; (**b**) Shoot development of saffron apical buds and growth 6 weeks after incubation; (**c**,**d**) Daughter corm formation 12 and 16 weeks, respectively, after treatment with salicylic acid (75 and 150 mg L^{-1}), glutamine (600 and 1200 mg L^{-1}), and jasmonic acid (1 and 2 mg L^{-1}).

4. Conclusions

The present results indicate that saffron flowering can be induced in vitro, and the harvested stigma of these flowers could be used as a source of spice or pharmaceuticals. Compared with solid culture, liquid cultures/bioreactors improved daughter corm diameter and fresh weight. Moreover, salicylic acid at 75 mg L^{-1} and glutamine at 600 mg L^{-1} increased corm diameter and fresh weight. These findings will help improve in vitro cormogenesis toward the production of saffron in a controlled environment. However, further investigations on optimal growth conditions, e.g., medium composition, light intensity and quality, and incubation temperature, are required to optimize in vitro cormogenesis.

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