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Original article

# A Research on the Development of a Sustainable *In Vitro* Propagation Method in Saffron (*Crocus sativus* L.)

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**Abstract**

Saffron (*Crocus sativus* L.) is the most expensive spice in the world. This plant species is propagated vegetatively by the formation of daughter corms from the mother plant. Unfortunately, many factors prevent the efficient propagation of saffron through this traditional practice. For example, the low production rate of daughter corms and the risk of fungal contamination affect the quality of daughter corms. Therefore, the traditional propagation method cannot meet the demand for planting material. The use of biotechnological tools, especially *in vitro* culture techniques, can be of great benefit in propagating saffron. Therefore, in recent years, various regeneration systems have been established for saffron through somatic embryogenesis and organogenesis using different explant types, media components, plant growth regulators (PGRs) and culture conditions. *In vitro* culture methods allow obtaining large amounts of propagation material for the saffron plant in a short time. This research was conducted to reveal the effects of different nutrient medium and hormone combinations on the *in vitro* propagation of saffron and to establish a sustainable *in vitro* micropropagation protocol. As a result of the research, it was revealed that the use of relatively high amounts of BAP in addition to maintaining a high cytokinin/auxin ratio in basic media (MS or DKW) is necessary for a sustainable saffron *in vitro* micropropagation protocol.

**Keywords:** *In vitro* Propagation, Sustainable Protocol, Media Composition, Saffron.

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## INTRODUCTION

Saffron (*Crocus sativus* L.) is the most expensive spice in the world. This plant species belongs to the family Iridaceae, which comprises approximately 80 species primarily distributed in the Mediterranean region and Southwest Asia (Karaoğlu et al., 2007). Saffron is a sterile triploid (2n = 3x = 24) geophyte with an underground corm (Hajyzadeh et al., 2020). It holds significant commercial value in international markets due to its color (crocin), taste (picrocrocin), and aroma (safranal) (Cardone et al., 2020). In fact, saffron is quite popular due to its colour (crocin), taste (picrocrocin), and aroma (safranal) (Cardone et al., 2020).

Saffron is vegetatively propagated through the formation of daughter corms from the mother corm. As its flowers are sterile, saffron cannot produce viable seeds. A single corm survives only one season and can produce certain numbers of daughter corms under natural conditions (Devi et al., 2011). Saffron propagation requires manual labour, as corms need to be removed and replanted. Challenges such as pathogen invasion, biotic and abiotic stresses, and poor crop management, combined with the low rate of corm propagation, limit the availability of saffron planting material (Menia et al., 2018). Due to the limitations of natural propagation methods, such as seasonal dependency and specific environmental requirements, developing efficient and sustainable strategies for large-scale production of selected saffron cultivars has become essential. The application of *in vitro* culture techniques has proven highly beneficial for the rapid and large-scale propagation of saffron (Small, 2016). *In vitro* micropropagation allows the production of uniform plants under controlled laboratory conditions throughout the year.

Research on the *in vitro* propagation of saffron began in the early 1980s. Ding et al. (1981) were the first to successfully culture saffron *in vitro* using Murashige and Skoog (MS; Murashige & Skoog, 1962) medium supplemented with indole-3-acetic acid (IAA) and/or 2,4-dichlorophenoxyacetic acid (2,4-D). Since then, numerous studies have been published on the direct and indirect organogenesis of saffron.

Different explants, including corms (Halim et al., 2018), apical and lateral buds (Mir et al., 2014), shoots (Zeybek et al., 2012), leaves (Zaffar et al., 2014), flower buds (Namin et al., 2010), stigmas (Karaoğlu et al., 2007), styles (Moradi et al., 2018), and ovaries (Mir et al., 2010), have been used for the *in vitro* regeneration of saffron. Among these, corms and apical/lateral buds are the most commonly used explants for organogenesis. For multiple shoot induction, corms have emerged as the most prominent explant.

Various culture media, such as MS, Quorin and Lepoivre (QL), White, Linsmaier and Skoog (LS), Gamborg (B5), Nitsch and Nitsch (N6), Schenk and Hildebrandt (SH), and Woody Plant Medium (WPM), have been utilized for saffron micropropagation. However, the most widely used basal

formulation is the MS medium, supplemented with a carbon source, usually sucrose, amino acids, vitamins, and other additives (Gantait and Vahedi, 2015).

Plant growth regulators (PGRs) such as auxins (e.g., 2,4-D and 1-naphthaleneacetic acid [NAA]) and cytokinins (e.g., 6-benzylaminopurine [BAP], kinetin [KIN], and zeatin) are reported to be crucial for saffron micropropagation (Sharma & Piqueras, 2010). Studies have shown that combinations of auxins and cytokinins are more effective than the use of either alone (Gantait and Vahedi, 2015).

This study aimed to evaluate the effects of various basic media and PGRs combinations on the *in vitro* propagation of saffron collected from Turkey and to establish a sustainable *in vitro* micropropagation protocol in order to overcome the limitations of vegetative propagation.

## MATERIAL and METHOD

The study was conducted in the laboratory and greenhouse of the Güney Agripark R&D Center. Corms sourced from Safranbolu-Türkiye were used as plant material. Corm slices were employed as explants in tissue culture experiments. For the surface sterilization of explants, a sterilization protocol previously developed and proven effective in our laboratory was applied. According to protocol, surface sterilization was performed using 100% ethanol for 1 minute, followed by 30 minutes in 50% sodium hypochlorite (NaOCl, 12%). Sterilized explants were rinsed three times with distilled water.

The sterilized corm slices were cultured in MS and DKW media supplemented with various hormone combinations and sugar concentrations, as detailed in Table 1. Later on, these cultures were incubated in a climate-controlled chamber set at 23 ± 2°C with 3000 lux light intensity and a 16/8-hour light/dark photoperiod.

**Table 1.** Basic media, hormone combinations and sugar concentrations used in the research

| Basic Media | PGRs combinations | Sucrose concentrations |
|---|---|---|
| MS0 (Control) | - | - |
| MS | 0,5 mg $L^{-1}$ NAA + 0,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ NAA + 1,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ NAA + 1,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ NAA + 2,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ IAA + 0,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ IAA + 1,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ IAA + 1,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| MS | 0,5 mg $L^{-1}$ IAA + 2,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW0 (Control) | - | - |
| DKW | 0,5 mg $L^{-1}$ NAA + 0,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ NAA + 1,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ NAA + 1,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ NAA + 2,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ IAA + 0,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ IAA + 1,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ IAA + 1,5 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-1}$ sucrose |
| DKW | 0,5 mg $L^{-1}$ IAA + 2,0 mg $L^{-1}$ BAP | 30 g $L^{-1}$ sucrose and 40 g $L^{-}$ sucrose |

The number of applications was 9, the number of replications was 10, the number of explants per jar was 5, and each jar was counted as 1 (one) replication.

## RESULTS and DISCUSSION

The results obtained using the MS basal medium supplemented with 30 g $L^{-1}$ sucrose (shoot number, shoot length [cm], and number of daughter corms) are presented below in Table 2.

**Table 2.** Results obtained using MS basic medium and adding 30 g L-1 sugar to the medium (number of shoots, shoot length (cm), number of corms)

| Media Composition | Number of Shoots | Average Shoot Length (cm) | Number of corms (average) |
|---|---|---|---|
| MS0 | 1.0 | 2.0 | 1.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 0,5 mg $L^{-1}$ BAP | 2.0 | 2.0 | 1.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 1,0 mg $L^{-1}$ BAP | 2.0 | 2.0 | 1.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 1,5 mg $L^{-1}$ BAP | 2.0 | 2.0 | 2.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 2,0 mg $L^{-1}$ BAP | 3.0 | 3.0 | 3.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 0,5 mg $L^{-1}$ BAP | 2.0 | 2.0 | 1.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 1,0 mg $L^{-1}$ BAP | 2.0 | 2.0 | 1.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 1,5 mg $L^{-1}$ BAP | 2.0 | 2.0 | 2.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 2,0 mg $L^{-1}$ BAP | 3.0 | 2.0 | 2.0 |

The results obtained using the MS basal medium supplemented with 40 g $L^{-1}$ sucrose (shoot number, shoot length [cm], and number of daughter corms) are presented below in Table 3.

**Table 3.** Results obtained using MS basic medium and adding 40 g $L^{-1}$ sugar to the medium (number of shoots, shoot length (cm), number of corms)

| Media Composition | Average Shoot Length (cm) | Average Shoot Length (cm) | Number of corms (average) |
|---|---|---|---|
| MS0 | 2.0 | 2.0 | 1.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 0,5 mg $L^{-1}$ BAP | 2.0 | 3.0 | 2.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 1,0 mg $L^{-1}$ BAP | 3.0 | 3.0 | 3.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 1,5 mg $L^{-1}$ BAP | 3.0 | 4.0 | 4.0 |
| MS+ 0,5 mg $L^{-1}$ NAA + 2,0 mg $L^{-1}$ BAP | 4.0 | 5.0 | 5.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 0,5 mg $L^{-1}$ BAP | 2.0 | 3.0 | 2.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 1,0 mg $L^{-1}$ BAP | 3.0 | 4.0 | 3.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 1,5 mg $L^{-1}$ BAP | 5.0 | 4.0 | 4.0 |
| MS+ 0,5 mg $L^{-1}$ IAA + 2,0 mg $L^{-1}$ BAP | 5.0 | 6.0 | 6.0 |

On the other hand, the results obtained using the DKW basal medium supplemented with 30 g $L^{-1}$ sucrose (shoot number, shoot length [cm], and number of daughter corms) are presented below in Table 4.

**Table 4.** Results obtained using DKW basic medium and adding 30 g $L^{-1}$ sugar to the medium (number of shoots, shoot length (cm), number of corms)

| Media Composition | Average Shoot Length (cm) | Average Shoot Length (cm) | Number of corms (average) |
|---|---|---|---|
| DKW0 | 2.0 | 3.0 | 1.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 0,5 mg $L^{-1}$ BAP | 2.0 | 3.0 | 1.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 1,0 mg $L^{-1}$BAP | 2.0 | 3.0 | 3.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 1,5 mg $L^{-1}$ BAP | 2.0 | 3.0 | 4.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 2,0 mg $L^{-1}$ BAP | 5.0 | 4.0 | 5.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 0,5 mg $L^{-1}$ BAP | 5.0 | 3.0 | 5.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 1,0 mg $L^{-1}$ BAP | 4.0 | 3.0 | 4.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 1,5 mg $L^{-1}$ BAP | 5.0 | 4.0 | 4.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 2,0 mg $L^{-1}$ BAP | 6.0 | 6.0 | 7.0 |

The results obtained using the DKW medium supplemented with 40 g $L^{-1}$ sucrose (shoot number, shoot length [cm], and number of daughter corms) are presented below in Table 5.

**Table 5.** Results obtained using DKW basic medium and adding 40 g $L^{-1}$ sugar to the medium (number of shoots, shoot length (cm), number of corms)

| Media Composition | Average Shoot Length (cm) | Average Shoot Length (cm) | Number of corms (average) |
|---|---|---|---|
| DKW0 | 2.0 | 3.0 | 1.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 0,5 mg $L^{-1}$ BAP | 2.0 | 3.0 | 2.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 1,0 mg $L^{-1}$ BAP | 3.0 | 3.0 | 3.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 1,5 mg $L^{-1}$ BAP | 3.0 | 3.0 | 3.0 |
| DKW + 0,5 mg $L^{-1}$ NAA + 2,0 mg $L^{-1}$ BAP | 6.0 | 5.0 | 6.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 0,5 mg $L^{-1}$ BAP | 4.0 | 3.0 | 2.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 1,0 mg $L^{-1}$ BAP | 5.0 | 3.0 | 3.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 1,5 mg $L^{-1}$ BAP | 5.0 | 4.0 | 4.0 |
| DKW + 0,5 mg $L^{-1}$ IAA + 2,0 mg $L^{-1}$ BAP | 7.0 | 8.0 | 6.0 |

As observed from the results above, both MS and DKW basal media, combined with specific hormone doses and combinations, have demonstrated potential for successful *in vitro* propagation of saffron. The data clearly show that varying hormone doses and combinations in both media result in different numbers of shoots (Figure 1).

The *in vitro* induction of saffron shoots is influenced by various factors, with the most critical being the type of explant, plant growth regulators (PGRs), and incubation temperature (Vahedi et al., 2014). Successful regeneration of saffron shoots has been observed from various types of explants,

including apical and lateral buds, corms, and ovaries. The first study on shoot induction using corms as explants was reported by Homes et al. in 1987. In this study, as supported by the literature, successful shoot induction from corms has also been achieved.

**Figure 1.** Saffron plantlets developing *in vitro* in different media

As evident from the results, varying doses of NAA, IAA, and BAP combined with MS and DKW basal media produced different numbers of shoots, consistent with findings from previous studies. Previous research has highlighted the importance of a high cytokinin-to-auxin ratio for initiating and enhancing shoot regeneration. Salwee et al. (2010) demonstrated that high concentrations of zeatin or BAP combined with low concentrations of NAA or IAA significantly promote shoot proliferation. Specifically, high BAP concentrations have been reported as crucial for inducing shoot regeneration.

The data presented in the tables clearly indicate that media with a high cytokinin-to-auxin ratio, particularly with elevated BAP concentrations, resulted in better shoot numbers and lengths, aligning with findings in the literature. Regarding the type of plant growth regulators (PGRs), the addition of IBA, BAP, and NAA positively impacted shoot induction and subsequent corm formation (Sharma et al., 2008). Piqueras et al. (1999) also emphasized that using BAP alone or in combination with other PGRs effectively stimulates microcorm formation in saffron during *in vitro* propagation. Similarly, in this study, the combination of BAP with NAA or IAA positively influenced both shoot formation and corm development.

One of the critical factors influencing shoot regeneration from saffron explants is the sucrose concentration in the culture medium. Sucrose concentration plays a significant role, as higher sucrose levels have been reported to promote more widespread and successful shoot induction (Mazri et al., 2016). In this study, the best results for shoot and corm formation were obtained with a medium prepared using 40 g $L^{-1}$ sucrose in both basal media (MS and DKW) and a high cytokinin-to-auxin ratio, specifically the 0.5 mg $L^{-1}$ IAA + 2.0 mg $L^{-1}$ BAP combination. These findings align with previous studies, further supporting the role of sucrose concentration and hormone balance in optimizing in vitro propagation conditions for saffron.

During the *in vitro* development of saffron shoots, it was observed that the shoots tend to swell at their bases, eventually forming small corms, referred to as "microcorms" (Sharma et al., 2011). Several studies have reported the formation of microcorms from shoots. Subsequent research has confirmed that all explant types used in saffron micropropagation, including corms, ovaries, buds, or floral parts, possess the capability to form corms, regardless of the explant type (Raja et al., 2007). These findings are consistent with the results obtained in this present study, further validating the potential of various explants for successful microcorm induction in saffron propagation.

**Conclusion**

In conclusion, the findings of this study clearly demonstrate that in vitro micropropagation is a highly effective and rapid method for the large-scale production of disease-free saffron corms. This method addresses the limitations of traditional propagation methods, particularly the low yield of corms, by leveraging *in vitro* techniques. The study highlights the necessity of using basal media such as MS or DKW, supplemented with a high cytokinin-to-auxin ratio and relatively high concentrations of BAP, to establish a sustainable *in vitro* micropropagation protocol for saffron. These findings provide a valuable contribution to the development of efficient and scalable propagation strategies for this economically and ecologically significant species.

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