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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

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ABSTRACT

Saffron is a triploid sterile crop with a low vegetative propagation rate, which prevents the widespread use of selected genotypes. Despite efficient *in vitro* propagation *via* direct organogenesis being very interesting, high contamination levels and low propagation rates preclude the commercial use of this technique. In order to obtain a source of explants for the *in vitro* multiplication of saffron that allows low contamination levels and efficient propagation, daughter corms were produced by storing non-planted mother corms at low temperatures (1–3 °C) for 9 months. This method avoids field cultivation and yields more corms than field production (8 *vs.* 3 corms per mother corm). Corms produced under cold storage conditions were *in vitro* sprouted on media with a high level of cytokinins (5 mg l^{-1} 6-benzylaminopurine or 1 mg l^{-1} thidiazuron) and 0.5 mg l^{-1} naphthalene acetic acid, and sprouted buds were cultivated on the same media. After 8 weeks of culture, multiple shoot primordia emerged from the base of the sprouted buds with no callus formation, and up to 400 shoot primordia were produced from one initial mother corm. These shoot primordia can be elongated and 90% produce corms. The high multiplication rate, lack of contamination, and the fact that multiplication occurs through direct organogenesis make the method suitable for the propagation of selected genotypes. Otherwise shoot clusters can be maintained for 6–7 months. However for longer maintenance periods, shoot proliferation capacity by direct organogenesis diminishes, and the emergence of somaclonal variation cannot be ruled out.

1. Introduction

Saffron is produced from the long scarlet styles of *Crocus sativus* L. and is highly valued for flavouring and colouring foods. It also has many medicinal properties and has been used in traditional medicine of many cultures (Abdullaev, 1993; Ríos et al., 1996; Fernández, 2004). In recent decades, saffron-cultivated areas in traditional saffron-producing European countries have drastically reduced (Fernández, 2004; Gresta et al., 2009). The intensive manual labour required for saffron harvesting, and processing and increasing labour costs, have made saffron production unprofitable. Recently, however, there have been indications of revival in saffron production in Europe and other developed countries, and considerable efforts have been made to improve saffron production technology (Molina et al., 2005). Preference for natural dyes instead of synthetic chemicals has stimulated the food industry to pay more attention to this spice (Kafi et al., 2006). Growing interest has also been shown in the potential medical applications of saffron, particularly those based on its cytotoxic, anticarcinogenic and antitumour properties (Abdullaev, 2002; Abdullaev and Espinosa-Aguirre, 2004; Fernández, 2004; Magesh et al., 2006; Chryssanthi et al., 2009; Dalezis et al., 2009).

One important factor that limits the areas in which saffron is cultivated is the difficulty in obtaining high quality propagation material with guaranteed levels of purity, homogeneity and health (European Saffron White Book, 2006).

Saffron is a triploid plant that is conventionally propagated by corms. It is a slow-growing species for which 3–4 daughter corms per mother corm are obtained each season under natural field conditions. This scenario limits the availability of material and the widespread use of improved genotypes obtained in the future. It would take 9–10 years to produce the corms required to sow one hectare (50 corms m^{-2}) from an initial corm.

Conversely, tissue culture methods offer great potential for large-scale multiplication. This fact has led research to be conducted into the *in vitro* propagation of saffron *via* embryogenesis

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and organogenesis. Induction of somatic embryogenesis from calluses of diverse origins has been described (George et al., 1992; Ahuja et al., 1994; Blazquez et al., 2004, 2009; Raja et al., 2007; Karamian and Ranjbar, 2010). Saffron propagation through direct and indirect organogenesis has also been studied (Sharifi et al., 2010; Majourhat et al., 2007; Díaz-Vivancos et al., 2011; Devi et al., 2011; Zeybek et al., 2012). The effects of temperature, growth regulators and season on callus, bud and root induction, and corm development, have been assayed to optimise protocols.

A high multiplication rate (39.5 shoots per callus from one corm explant) has been described based on shoot induction from calluses on thidiazuron (TDZ)-containing media (Sharifi et al., 2010). However, propagation *via* direct organogenesis is a more interesting method for the clonal propagation and *ex situ* conservation of saffron genetic resources because it avoids somaclonal variation.

Direct organogenesis from sprouted saffron buds has been recently published (Díaz-Vivancos et al., 2011; Devi et al., 2011; Zeybek et al., 2012) and multiplication rates ranging from 1.25 to 5 buds per explant have been reported. In addition, shoot multiplication from initial shoot primordia clusters has been described by Devi et al. (2011). However, it remains unclear whether shoot regeneration can be achieved *via* direct or indirect organogenesis in the late shoot multiplication phase from original clumps, which lasts 160 days. In most works on saffron, the use of 1.5–6 mg l^{-1} 6-benzylaminopurine (BAP) for direct organogenesis has been described as optimal. However, Díaz-Vivancos et al. (2011) described a similar multiplication rate with both BAP and TDZ (1–5 mg l^{-1}). Although some works have added auxins to the media to improve multiple shoot formation (Sharma et al., 2008; Zeybek et al., 2012), these hormones do not seem essential (Agüero and Tizio, 1994; Devi et al., 2011; Díaz-Vivancos et al., 2011).

Cormlet development at the base of formed shoots has been accomplished in different media (Agüero and Tizio, 1994; Zeybek et al., 2012; Devi et al., 2011). Although some works describe the growth performance of *in vitro*-produced corms under greenhouse conditions (Devi et al., 2011), there are no detailed studies available on somaclonal variation induced by *in vitro* saffron cultures.

The major problem with these multiplication methods is the possibility of high contamination levels when using field-grown corms as the source of explants. Some works clearly state this problem (Piqueras and Fernández, 2004). In other works, this may be deduced from the sterilisation protocols in which streptomycin sulphate, Bavistin (fungicide) and mercuric chloride are employed for disinfection purposes (Devi et al., 2011; Zeybek et al., 2012). If the high incidence of *Fusarium* in saffron fields is considered, endogen contamination by this fungus can partially explain this problem. Another reason is the difficulty to sterilise buds from field corms.

Earlier studies about the physiology of bulbs (Le Nard and Cohat, 1968; Le Nard and De Hertogh, 1993) have shown the possibility of bulbing from buds of tulip bulbs by low-temperature storage (2 °C). Bulbs placed at low temperatures before flower differentiation can lead to apical bulbing, which prevents flower differentiation. Simultaneously, low temperatures induce the bulbing of axillary buds. In these works, two processes have been distinguished in tulip bulb production: (1) an inductive process requiring low temperatures (2–3 °C); (2) the manifestation of bulbing, which occurs faster at 10 °C than at 2–3 °C for those bulbs constantly stored at these temperatures. The successful implementation of the protocol described for tulip bulbs on non-planted saffron corms allows corm production by avoiding field cultivation. It also proves to be a very suitable starting material as a source of explants for *in vitro* culture by significantly reducing contamination levels.

These aspects are addressed herein in relation to the following objectives: (1) to produce saffron corms by storing non-planted corms at low temperatures to avoid field cultivation; (2) to study the efficiency of these materials as a source of explants for *in vitro* multiplication through direct organogenesis by following the described optimal protocols; (3) to test the possibility of the long-term maintenance of shoot multiplication from original clumps through direct organogenesis; (4) to characterise both *ex vitro* growth of *in vitro*-produced corms and variation in the ploidy levels of these materials.

2. Materials and methods

2.1. Corm production by low-temperature storage of non-planted corms

Saffron corms (*C. sativus* L.) of 10–30 mm in diameter obtained from a local farmer in Albacete (Spain), were stored at 1–3 °C from the end of July to April–May. At the end of this period, lateral buds development was checked. When lateral buds developed corms, the number and size of these corms were recorded, and the correlation between the initial weight of the mother and daughter corm productions was analysed for 60 mother corms. With another batch of corms, the storage temperature was raised to 10 °C from November onwards in order to test if this temperature was more favourable than 1–3 °C for the growth processes after corm induction. Daughter corm production (size and number) was also measured and compared with that of the corms stored at 1–3 °C. Twenty corms, 20–30 mm in diameter, were measured in each treatment.

2.2. In vitro bud sprouting and axillary bud formation from corms produced at low temperatures

Small corms produced at low temperatures (1–3 °C) were detached from their tunics and thoroughly washed under running tap water, dipped in 70% (v/v) of ethanol for 30 s and rinsed three times in sterile water. Afterwards, corms were surface-sterilised for 20 min in a 0.8% (w/v) solution of sodium hypochlorite, sonicated and then rinsed three times with sterile distilled water.

After sterilisation, corms were cultured in a basal medium composed of half-strength MS mineral media (Murashige and Skoog, 1962) supplemented with Skoog's vitamins and amino acids (Skoog, 1944), 3% (w/v) sucrose, 600 mg l^{-1} polyvinylpyrrolidone (PVP 10, Sigma) and 7 mg l^{-1} agar (Technical agar no. 3, Oxoid). Different plant growth regulators were tested to promote corm sprouting. After remaining in the dark at 17 °C for 10 days, cultures were maintained under a 16/8 h photoperiod with photon flux density of 140 μmol m^{-2} s^{-1} provided by Philips TLD lamps. After sprouting, sprouted buds per corm were counted and single shoots were cultured in the same medium to test axillary bud formation. The effect of TDZ at 1 mg l^{-1} was compared with BAP at 5 mg l^{-1}. All the media with cytokinins were also supplemented with 0.5 mg l^{-1} of naphthalene acetic acid (NAA). After 6–8 weeks, the number of shoots primordia per initial explant was counted.

In order to measure bud sprouting from the corms produced at low temperatures, 15–20 corms per treatment were tested. In order to study axillary bud formation, 20–30 sprouted buds from different corms (approx. 10 corms) per treatment were tested.

The propagation rate of the corms produced at low temperatures was compared with that of the sprouting buds of the field corms in the media containing TDZ 1 mg l^{-1} and NAA at 0.5 mg l^{-1}. Thirty small segments of the field-grown corms, each with one sprouting bud, were sterilised as described and were then *in vitro* cultivated.

2.3. Maintenance of proliferating meristematic clusters

The shoot primordia clusters (approx. 10–12 shoot primordia per cluster; a total of 194 shoots) from the media containing TDZ (1 mg l^{-1}) and NAA (0.5 mg l^{-1}) were cultured in the basal media