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Disinfection of explants for saffron (*Crocus sativus*) tissue culture

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Abstract

The choice of explant and its preparation are most likely one of the most important biotic factors that determine the success of a tissue culture protocol when the explant is derived from *ex vitro* organs. This review assesses the disinfection protocols available for saffron (*Crocus sativus* L.; Iridaceae) explants in an attempt to ascertain the most suitable set of parameters that could ensure successful tissue culture in subsequent treatments. From a methodological perspective, two explant types are most commonly adopted in saffron biotechnology, namely the use of fresh field-grown shoots, or dormant corms. The latter is more recommended to minimize contamination and to allow the use of more aggressive disinfection treatments.

Key words: corms, *Crocus sativus*, hydrogen peroxide, mercury II chloride, saffron, sodium hypochlorite, sterile distilled water, tissue culture.
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; $AgNO_3$, silver nitrate; BA, 6-benzyladenine; $Ca(ClO)_2$, calcium chloride; ELISA, enzyme-linked immunosorbent assay; GA_3, gibberellic acid; H_2O_2, hydrogen peroxide; $HgCl_2$, mercury II chloride; HWT, hot water treatment; IAA, indole-3-acetic acid; KIN, kinetin; NAA, α-naphthaleneacetic acid; NaOCl, sodium hypochlorite; PCR, polymerase chain reaction; PPM™, plant preservative mixture™; SDW, sterile distilled water.

Brief historical and cultural background

Saffron (*Crocus sativus* L.) stigmas are likely to be the most expensive spice on earth given the fine-scale processing required to harvest the stigmas and the tiny amount of crocin, safranin and picrocrocin that can be found therein (Melnyk et al. 2010). The agronomic and biotechnological aspects of saffron and its tissue culture have been recently reviewed (Ahrazem et al. 2015; Gantait, Vahedi 2015) and thus only a brief introductory overview is provided here. The induction of embryogenic callus (see choice of callus and notes in Teixeira da Silva 2012b) from non-embryogenic callus was first reported by George et al. (1992) in which the explant used was the meristematic region of corms. In that study, the non-embryogenic callus that developed on medium with 2,4-dichlorophenoxyacetic acid (2,4-D) was transferred to medium containing indole-3-acetic acid (IAA), kinetin (KIN) and ascorbic acid, resulting in the development of somatic embryos. This was followed by the induction of embryogenic callus from bulblet explants in the presence of 6-benzyladenine (BA; see choice of BA and notes in Teixeira da Silva 2012a) and α-naphthaleneacetic acid (NAA) (Ahuja et al. 1994). Saffron embryogenic callus has a generally nodular appearance and takes about 6 weeks to form from corm tissue cultures (Blazquez et al. 2009). At the nodular stage, callus contains proembryonic structures or proglobular embryos that develop into globular embryos after 3 weeks in culture, into monopolar embryos (containing a meristem and cotyledon) after 7 weeks in culture and into bipolar embryos (consisting of an apical meristem with a cotyledon at one end and a minicorm at the opposite end) after 10 weeks in culture (Blazquez et al. 2009). Somatic embryogenesis in saffron is not very efficient and plantlet conversion from somatic embryos is low. These problems must be resolved before somatic embryogenesis can become a viable method for the mass propagation of saffron. Direct shoot regeneration without an intervening callus phase is an alternative to somatic embryogenesis and has the advantage of greater genetic uniformity compared to an indirect organogenic route from callus cultures or somatic embryos, and may take less time to generate direct shoots than callus (Blazquez et al.

2009). In saffron, shoots have been generated directly from apical and lateral buds, small corms and ovaries (Plessner et al. 1990; Bhagyalakshami 1999; Blazquez et al. 2004a; Sharma et al. 2008; Diaz-Vivancos et al. 2011).

Micropropagation through direct organogenesis is the best technique available so far to generate a large number of genetically uniform clones. Direct shoot induction followed by microcorm formation holds promise for commercialization especially if genetically improved saffron corms (Agayev et al. 2009) are to be multiplied in a short amount of time. There are three concerns that need to be addressed to make this technology viable for saffron micropropagation: (i) effective establishment of an *in vitro* regeneration system; (ii) sustained multiplication of shoots from tissue culture-derived explants; (iii) field evaluation of microcorms derived from *in vitro* culture. As a first step, the disinfection of the explant is essential for the successful establishment of an *in vitro* culture, as has been elaborated in detail for *Anthurium* (Teixeira da Silva et al. 2015) and *Dendrobium* (Teixeira da Silva et al. 2016). Even though there are a substantial number of studies on saffron *in vitro* regeneration, the effect of disinfection on explant contamination and regeneration ability is rarely described in detail. This review looks at some theoretical aspects related to tissue disinfection in preparation for plant tissue culture, and then examines how this has been achieved in saffron to achieve a successful culture.

Theory of disinfection for the establishment of plant tissue cultures

Importance of and problems related to the disinfection of underground organs

Even though micropropagation allows for the production of numerous plants of high quality in a relatively short time, the greatest problem in this technique is contamination (Altan et al. 2010). A great variety of microrganisms (filamentous fungi, yeasts, bacteria), micro-arthropods (mites, thrips and their vectors), as well as viruses and viroids have been identified as contaminants in plant tissue cultures (Altan et al. 2010). Therefore, the asepsis of both tissues and other materials is one of the main conditions that needs to be met when establishing and maintaining plant *in vitro* cultures. Sterilization of equipment is not a problem in a modern laboratory that uses novel microwave-based autoclaves or pulsed-light systems that allow for the rapid (a few-minutes-long) and efficient disinfection of tools and media. The disinfection of biological material, however, is much more difficult and requires greater consideration. In plants, in theory, only the shoot apical meristem is free from any endophytes and viruses (Nesi et al. 2009). This is because apical meristems are an actively diving group of cells without a well-established vascular system but their plasmodesmata are too small to allow the cell-to-cell movement of endophytes; in addition, virus replication (i.e., the viral inactivating system) cannot cope with rapid meristem cell division, and a low water concentration and a high osmotic pressure, or high auxin levels might also limit endophytes (Elmi, West 1995; Everett 2006; Alam et al. 2013). Other tissues, however, host various microorganisms and require proper treatment prior to tissue culture initiation. Some organs are easier to disinfect than others (for example, seeds, due to the presence of their protective testa, which allows for longer or more intense treatments) or are naturally less contaminated – such as the generative organs covered by flower petals – while others, especially those that are located closer to the soil, are more heavily colonized by microorganisms. The disinfection of initial explants is a major problem in the micropropagation of geophytes (Bach, Sochacki 2013), which include important and highly profitable ornamental, cosmetic and medicinal species useful also in food production. *Tulipa* L., *Lilium* L., *Narcissus* L., *Gladiolus* L., *Iris* L. and *Hyacinthus* L. lead the cut flower sector worldwide (Çığ, Basdogan 2015). In such plants, underground organs such as modified stems, including the corms of *Crocus* L. spp., stem tubers of *Zantedeschia* Spreng. spp., the rhizomes of *Iris*, the pseudobulbs of orchids or the caudex of *Adenium* Roem. & Schult., are the most effective or even the sole source of axillary buds (Podwyszyńska 2012). The latter are considered to be valuable explants due to their high multiplication rate and genetic stability (Ngezayaho, Liu 2014). Rhizomes also have a greater direct regeneration potential compared to other explants that are easier to obtain, such as leaves (Ma, Gang 2006). In *Lilium*, *Narcissus* and *Tulipa*, the modified leaf blades born in bulbs (i.e., an underground stem with fleshy, scale-like leaf blades surrounding the apical bud) are the most important source of material for micropropagation since even though they do not contain meristematic tissue, they are able to regenerate adventitious true-to-type bulbs (Jerzy, Krzymińska 2006; Yadav et al. 2013). The level of contamination in corms and other underground organs can reach as high as 95 to 100% (Yasmin et al. 2013). Marinescu et al. (2013) compared the disinfection efficiency of various *Iris aphylla* L. explant types. As for rhizomes, the percent of contamination was reported to be 86%, but only 20% for leaf explants. However, even though a review on the micropropagation of geophytes was published (Ascough et al. 2009), it did not – surprisingly – focus on any aspect related to the elimination of contamination. Therefore, studies on the disinfection of underground tissues and organs, as for saffron, are extremely important.

Factors affecting disinfection efficiency

There are numerous factors that can influence the efficiency of disinfection, including the conditions of cultivation and physiological state of the stock plant, the size, age and type of the explant, the type of disinfectant and its concentration, time and temperature of exposure (Teixeira da Silva et al. 2015). Finally these factors will not only affect the asepsis of

the explant, but also its survival and regeneration potential, which are also prerequisites for an efficient transformation system and other applications of *in vitro* cultures. The rate of *Zingiber officinale* Rosc rhizome bud sprouting and differentiation increased from 4 to 50% when compared to other methods after two treatments (50 °C water and exposure to plant preservative mixture – PPMTM) were introduced into the disinfection procedure (Ma, Gang 2006). Langens et al. (1997) observed that hot water (40 °C) decreased contamination of *Lilium* explants, but water at 45 °C decreased their regeneration capacity.

Mother plants, which are the source or donors of explants, should be grown under protected conditions such as a glasshouse, or growth chambers, to minimize the population of epiphytes and pathogens (Leifert, Waites 1994, cited by Reed, Tanprasert 1995). Underground organs can be pre-incubated in perforated plastic bags containing a mixture of loose, moist soil. In *Lilium philippinensis* Baker, however, this resulted in a low level of aseptic culture (11 to 33%), probably due to high moisture, which is a vehicle for the spread of bacteria (Zamora, Gruezo 1999). Reducing relative humidity during the pregrowth period can improve the outcome of infection. Seabrook and Farrell (1993) found that irrigating donor plants with filtered water, rather than city water, decreased bacterial contamination. They observed that watering greenhouse-grown *Solanum tuberosum* L. stock plants with filtered city water decreased contamination of explant cultures by 30 to 50%. Installing an ultraviolet light water-disinfecting equipment at the glasshouse inlet point can successfully reduce contamination (Seabrook, Farrell 1993).

Various types of disinfectants can be used, including chemotherapeutics (fungicides, antibiotics) hydrogen peroxide (H_2O_2), bromine water, formaldehyde (CH_2O), silver nitrate ($AgNO_3$), mercury II chloride ($HgCl_2$), sodium hypochlorite (NaOCl) or calcium hypochlorite ($Ca(ClO)_2$). Chlorine dioxide (ClO_2), which was shown to be effective for gerbera, has not yet been tested in geophytes (Cardoso, Teixeira da Silva 2012). For bulbs, corms and rhizomes, $HgCl_2$ (mainly) and/or NaOCl are the most commonly utilized (e.g., in *Muscari* species; Teixeira da Silva, Dobránszki 2016). $HgCl_2$ is generally believed to be a stronger disinfectant than NaOCl (Marinescu et al. 2013). Unfortunately, heavy metals such as mercury are known for their immunotoxic and neurotoxic properties and are environmental pollutants (Marinescu et al. 2013). Therefore, it is recommended to use disinfection methods without $HgCl_2$ (Yildiz 2012). Hypochlorites have been known for at least two centuries, but the main problem with their application is their instability and sensitivity to light (Connell 2006). $Ca(OCl)_2$ is very effective, more stable than NaOCl, but is poorly soluble in water (Boyette et al. 1993). Chloramine, used with tulips (Podwyszyńska, Marasek 2003), is less efficient but much more persistent (WHO 2000). $AgNO_3$, sometimes applied to extend the vase life of ornamental plants, including geophytes (Doğan et al. 2013), can also be useful in disinfection (Yildiz 2012), since its exogenous application may increase the *in vitro* multiplication rate and induced floral initiation and development in shoot cultures, as was observed with *Cichorium intybus* L. (Bais et al. 2000). Due to the instability of $AgNO_3$ in the presence of chlorides and other compounds in tap water or in soil-clinging organs, its application alone has limited use (Newton et al. 1933). However, when 0.05% $AgNO_3$ was combined with (0.15%) potassium cyanide (KCN; 1:3 w/w), effective results with plant tissue culture were obtained (Newton et al. 1933). Silver, gold and copper nanocolloids also have antibacterial, antifungal and antiviral activities even at a low concentration and a short period of disinfection (5 to 10 mg L^{-1} 5 to 10 min) (Tymoszuk 2014). In addition they do not require additional washing of the explants with sterile distilled water (SDW) and cause no damage to tissue (Tymoszuk 2015). Even though these novel disinfectants may increase in popularity, their influence on genetic stability of biological material is still unknown and they have not yet been used with geophytes. It is also possible to add H_2O_2 as a chemical sterilizer into the culture medium (even without autoclaving) or to use it for washing explants (Curvetto et al. 2006). This is a non-phytotoxic solution due to the activity of plant peroxidases and catalases that act against H_2O_2 by transforming it into water and oxygen (Arora et al. 2002). This also provides a protection mechanism to preserve tissues from the harmful effects of peroxides produced by their own metabolism (Curvetto et al. 2006). An increase in H_2O_2 concentration from 0.005 to 0.020% reduced the contamination level of *Lilium longiflorum* 'Snow Queen' bulb scales from 52.5 to 40% after 18 weeks of culture (Curvetto et al. 2006). By increasing the H_2O_2 level in the culture medium, the number of explants with bulblets improved. Fungicides (e.g. 0.01% carbendizime and 0.1% mancozeb) and/or bacteriocides (e.g. 0.25% chloramphenicol) can also be applied prior to proper disinfection, as was done with *Chlorophytum borivilliens* Santapau & R.R. Fern tuberous roots (Sharan et al. 2010).

The concentration of the disinfection agent (0.1 to 6%) and period of exposure (typically ranging from between one minute and one hour) has to be optimized individually depending on the biological material. Obviously longer treatment with more concentrated disinfectants provides better asepsis. An increase in NaOCl concentration from 2 to 4% (30 min) resulted in a 13.4% greater share of pure cultures of *Allium aflatunense* B. Fedtsch. 'Purple Sensation' and 20.0% of *Allium karataviense* Regel 'Ivory Queen' (Kozak, Stelmaszczuk, 2013). Prolonged application of 1:500 carbendazim solution and 2% NaOCl (up to 30 min) provided more clean *Lilium* 'Eyeliner' bulblet cultures in comparison to 10 min (Liu et al. 2012). One should keep in mind though that the viability of tissues is negatively affected by disinfectants at high concentrations

(especially with increasing application period) resulting in the dehydration and yellowing of the explants (Pandey et al. 2009). Prolonging the treatment of *Lilium* bulb scales with 0.1% $HgCl_2$ in combination with 2% Bavistin from 2.5 to 10 min increased the disinfection efficiency from 29.3 to 96.0% (evaluated after one week), but the survival of biological material after 4 weeks decreased by 4% after 2.5 min disinfection and by more than 14% after 10-min treatment (Pandey et al. 2009). This indicates that contamination and survival levels of disinfected explants should not be evaluated at the same time; the issue of timing of sampling can strongly influence the reported results in plant tissue culture (Teixeira da Silva, Dobránszki 2013). Damage to explants during disinfection may have the opposite effect and lead to the spread of endogenous microorganisms, while longer disinfection with NaOCl may stimulate the subsequent regeneration potential of explants, probably as a response to prolonged stress, as observed with *Kalanchoe tubiflora* (Harvey) Hamet (Kulus 2014). Liu et al. (2012) reported that a 15-min treatment with 2% NaOCl induced over 10% greater bulblet formation in *Lilium* 'Eyelier' scales than a 10-min treatment. Besides the type, concentration and application period, temperature of the disinfectant is also important. Temperatures exceeding 10°C increase the disinfection activity of NaOCl, which penetrates more easily into tissues (Yildiz 2012). However, heating NaOCl solutions may cause unpredictable changes to the concentration of available Cl^-, depending upon the conditions (Frais et al., 2001). Also Altan et al. (2010) obtained a greater share (16.6%) of fungi-infected *Lilium candidium* L. scale bulbs cultured in MS medium and supplemented with 0.1 mg L^{-1} NAA + 0.01 mg L^{-1} BA in the dark relative to a 16-h photoperiod (10.4%).

Disinfection procedures

A proper disinfection procedure should preferably be cheap, efficient in terms of surface and endogenous microorganism elimination, simple enough to be performed even by an unexperienced tissue culture staff, but should also be friendly to the environment (Purohit et al. 2011). The surface disinfection of underground organs is usually carried out in four steps. (i) First, tissues are gently scrubbed under running tap water for about 10 min to a few hours to remove microorganisms ubiquitously covering them. The protective layer surrounding the shoot apical meristem, i.e., the tunics in saffron, can also be removed gently from the corms. Next, after optional scarification, the tissues are rinsed in running water with a drop of detergent (e.g. 0.5% Extran) and a surfactant (Tween-20) for approx. 15 min (with agitation) in order to remove the lipid layer from the plant material. Detergents and other surfactants facilitate mixing of dirt with water, alter the pH of the organ surface, which results in breaking of hydrogen bonds via which dirt is bound to the tissue surface or leads to decomposition of the material forming the dirt through oxidation. Moreover, they reduce the water hardiness, which allows for better wetting of the plant surface and easier dissolution of ionic compounds and by forming foam they increase the contact surface with dirt and grease (Schmiedel, von Rybinski 2006). (ii) Tissues are then pretreated with 70 to 96% ethanol for a few seconds to a few minutes (in order to remove air bubbles and provide better accessibility of the tissue to the disinfectant), a fungicide (dry or liquid) and/or a bactericide, sulfuric acid (1 to 3 min), or PPM™, a biocide. (iii) Explants are then transferred to a laminar air-flow chamber and treated with an appropriate disinfectant, usually a commercial bleach. (iv) Lastly, three to five rinses in sterile (double) distilled water (SDW) are applied. Sometimes explants are treated with hot water (40 to 50 °C for 1 to 4 h) followed by optional overnight drying before sterilizing them with commercial bleach. Applying a two-step procedure may also be beneficial as was observed with *Allium aflatunense* 'Purple Sensation' and *Allium karataviense* 'Ivory Queen' bulbs. When a one-step disinfection procedure was applied (4% NaOCl for 30 min), 26.7% pure cultures were obtained, but after applying a two-step protocol (2% NaOCl for 30 min followed by 1% NaOCl for 15 min) the success rate increased to 66.6 to 80.0%.

Winarto and Teixeira da Silva (2012a) observed an interaction between the disinfection method and regeneration medium on the percentage contamination, total browning and regeneration efficiency of *Rumohra adiantiformis* (G. Forst.) Ching rhizome cultures proving a strong correlation between those parameters. These authors also underlined the significance of the area of exposure to the sterilant and its effect on explant survival and level of disinfection. The sliced shoot tips area of rhizomes inoculated on a semi-solid regeneration medium were nearly 100% contaminated. Furthermore, it was observed that slicing the rhizomes with a tissue culture blade led to browning of the explants within 72 h from culture initiation and in the end caused explant necrosis. The highest share of clean cultures (50%), as well as, highest percentage of rhizome regeneration, was achieved after inoculating full rhizomes on a simple paper bridge containing liquid regeneration medium. Only when disinfection procedures are optimized (Winarto, Teixeira da Silva 2012a) can a successful *in vitro* regeneration protocol be established (Winarto, Teixeira da Silva 2012b). Better regeneration potential of the explants can be achieved after cutting the underground organs (as a result of mechanical stress), however, after their disinfection (not prior to it) (Kanchanapoom et al. 2011).

Elimination of endophytes

Surface contaminants (epiphytes) are relatively easy to eliminate. Endogenous contamination (mostly bacteria, but also fungi) are a much more complicated issue, and may require antibiotic therapy (Reed, Tanprasert 1995). The antibiotics used should be stable, soluble, unaffected

by pH or medium components, broadly active and cheap. Furthermore, they should have bactericidal (not only bacteriostatic) activity and not phytotoxic (Falkiner 1990). The most popular are gentamicin, rifampicin and streptomycin (they act on the prokaryotic 30 S or 50 S ribosome subunits) (Falkiner 1988). Antibiotics, however, often have a narrow target spectrum for bacteria, thus combinatorial use of antibiotics should be used in order to obtain a synergistic effect, i.e., control of microorganisms and reduction of plant damage (Altan et al. 2010). *Zephyranthes grandiflora* Lindl. bulb explants were treated with 0.2% Bavistin and 0.1% Pantomycin (streptomycin sulfate and tetracyclin tetrachloride) for 2, 3, 4 or 5 h under continuous shaking on a magnetic stirrer, prior to final disinfection with 0.1% $HgCl_2$ for 30 s. It was observed that 4 h pretreatment resulted in the highest share (67%) of pure living cultures after 30 days (Gangopadhyay et al. 2010). The antimicrobial effect of an antibiotic can be improved by increasing the pH in the culture medium to a more neutral level (approx. 7.0) (Falkiner 1988; Falagas et al. 1997).

Epiphytes and endophytes can be removed in two different stages. For *Lilium candidum* bulb scales, the most effective treatment against fungal contamination (over 95% of clean cultures) and to eliminate endophytes was achieved after surface disinfection with 96% ethanol (2 min), then 2.25% NaOCl with one drop of 0.1% Tween 80 (20 min) and four rinses in SDW while elimination of epiphytes was followed by treatment with Benomyl (100 mg L^{-1}) + Nystatin (100 mg L^{-1}) for 30 min (Altan et al. 2010).

The immersion of underground *Narcissus* bulbs in a liquid solution of disinfectant (silver nitrate-potassium cyanide solutions) *in vacuo* may also be successful in eliminating endophytes by reducing infection from 26.8 to 1% (Newton et al. 1933).

Endophytes (as well as epiphytes) can be eliminated by the use of hot-water treatment (HWT). Another advantage is the lack of chemical residues required. The technique was first developed in the 19th century in Denmark and has, ever since, been used on a large scale with bulbs, tubers and seeds (Langens-Gerrits et al. 1997). HWT reduced initial contamination in *Narcissus* and *Lilium* bulbs from 40–60 to 5% but the temperature used is species-dependent since various pathogens have different heat sensitivities, which also depend on the host plant. Thus, for *Narcissus*, 54 °C for 1 h was optimal, but for *Lilium* explants, regeneration decreased after HWT at 45 °C for 1 h (Langens-Gerrits et al. 1997).

Elimination of viruses

Microorganisms are not the only problem while establishing a micropropagation protocol. Bacteria and fungi can be removed during the initiation of *in vitro* cultures from standard disinfection of organs, but viruses [which are a serious threat with vegetatively propagated geophytes (Nesi et al. 2009), decreasing plant growth vigour] cannot. The detection of viral agents in micropropagated geophytes can be achieved by enzyme-linked immunosorbent assay (ELISA) or by more sensitive polymerase chain reaction (PCR) techniques (Dorrigiv et al. 2013; Nesi et al. 2013). Viruses can be eliminated by heat treatments (thermotherapy; e.g. 30 to 40 days at 35 °C) of *in vivo*- or *in vitro*-cultured plants and bulbs/bulblets and/or meristem isolation (Nesi et al. 2009).

Low-cost disinfection

Initiation of axenic cultures requires expensive equipment such as autoclaves and laminar air-flow hoods that are not affordable for hobbyists or even small producers. This limitation can be overcome by the use of easily-available substitute materials to disinfect cultures, either using fluent vapour or vapour under pressure which might be possible with a pressure cooker. This possibility allowed Curvetto et al. (2006) to obtain 60% clean cultures (80% in the 'traditional', i.e. autoclaved method) from *Lilium longiflorum* 'Snow Queen' bulb scales. In addition, greater bulblet biomass was achieved and these bulblets had a higher relative growth ratio than bulblets derived from the traditional disinfection method (Curvetto et al. 2006). (Re) distilled water can be substituted by boiled tap water for media preparation. The latter costs only one third of the former, while a pH value of 6.0 to 6.5 is suitable for most ornamentals (Liu, Liu 2010).

Another possibility is the use of carbohydrate-free medium, which has been successfully used with numerous ornamental plant species, such as: *Cymbidium grandiflorum* Griff. and *Zantedeschia aethiopica* (L.) Spreng. (Liu, Liu 2010). In such media the contamination rate is low and there are more new leaves than when cultured in sugar-containing medium. Photoautotrophic micropropagation significantly increased the chrysanthemum shoot mass relative to control plants, even when the density of plants was doubled (Teixeira da Silva 2014). Furthermore, such plantlets are easier to acclimatize since they are autotrophic, unlike the heterotrophic plantlets derived from sugar-containing media. Furthermore, bacteriostatic agents extracted from plants can be used to achieve asepsis by adding them directly to medium instead of autoclaving (Liu, Liu 2010). By applying phytobiotics (i.e., plant anti-microbial agents in which the combination of numerous compounds extracted from plants can kill endophytes in plants and that results in little harm to the cultured plant) instead of autoclaved medium, the contamination rate could be sustained at an acceptable level under 10% (Cui et al. 2004).

While developing a disinfection protocol, a researcher should remember that different genotypes may display variable sensitivity to sterilizing agents and sterilization period. Therefore, choosing different disinfection methods for different cultivars is necessary, as observed with lily bulbs (Lu et al. 2005), although with *Allium aflatunense*

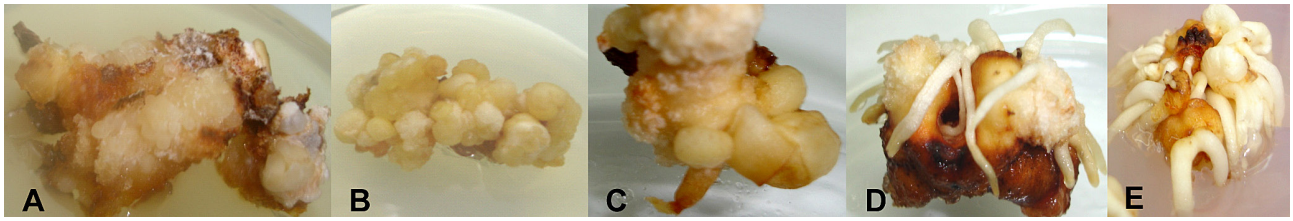

Fig. 1. *In vitro* response of corm slices of *Crocus sativus* L. formed after surface disinfection described in Table 1. (A) Callus formation on corm slices after 8 weeks. (B) Multiple cormlet production after 12 weeks. (C) Cormlet size (less notable) and callus formation after 12 weeks. (D) Cormlet (notable size) accompanied with multiple root regeneration and callus formation after 12 weeks. (E) Multiple root formation and increase in cormlet size. Figure reproduced from Quadri et al. (2010), with kind permission from Global Science Books (www.globalsciencebooks.info).

'Purple Sensation' and *Allium karataviense* 'Ivory Queen' bulbs the same procedure (out of five), i.e. when NaOCl containing 2.0% of Cl^- for 30 min and 1.0% Cl^- for 15 min, was used for sterilization, provided the best culture purity – 66.6 and 80.0%, respectively (Kozak, Stelmaszczuk 2013).

Disinfection of saffron tissue cultures

Saffron *in vitro* tissue cultures have been initiated from several organs (Table 1): leaves, shoots, corms (Fig. 1, 2), whole flower buds or parts of the inflorescence, including the stigma, style, ovary, anthers and petals. The disinfection protocol described for most studies, however, was rarely followed by any indication of the level of infection or explant mortality after the disinfection procedure. There are also several *in vitro* culture studies that appeared to have used corms or other tissues from field or greenhouse-grown plants for culture initiation, but which did not describe the disinfection procedure – those studies have not been cited in this review. Other studies, like Raja et al. (2007), describe surface disinfection loosely and imperfectly, not allowing for the procedure to be replicated: "healthy leaves… were sterilized after treating them with sodium hypochlorite for 8-10 minutes." Basing their experiment on earlier knowledge of tulip bulb forcing after cold storage, one important study of saffron determined that low temperature storage of corms (1 to 3 °C for 9 months) provided tissue that was not infected *in vitro* [i.e., 0% vs 50 to 100% contamination in field-grown corms (control)], and that resulted in greater organogenesis (i.e., a greater proportion of organ-regenerating explants) as a direct result of lower contamination, but also as a direct fine-scale tuning of medium conditions such as plant growth regulators (Renau-Morata et al. 2013).

Surprisingly, only a few studies examined the disinfection procedure in detail. Karaoğlu et al. (2007) tested seven disinfection procedures and made some important, but incomplete, observations. They noted that a one year quarantine period (details not provided) was insufficient to eliminate endogenous contaminants and that any treatment with high temperature or sulphuric acid was damaging to explants (floral and corm segments) and did not eliminate latent endogenous contaminants that reappeared after 3 to 5 weeks in culture. The precise nature of these contaminants was not described, i.e., bacterial fungal or viral, and the authors noted that only a single treatment could eliminate contaminants, but only resulted in the formation of a

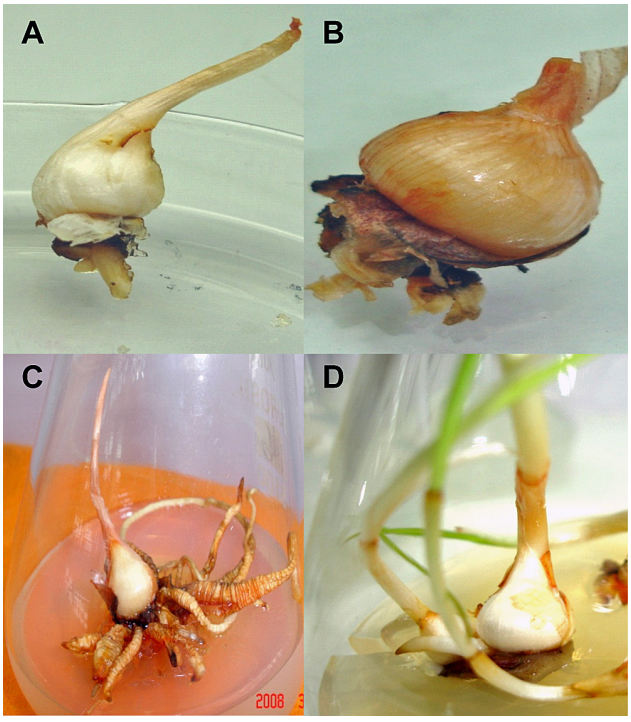

Fig. 2. *In vitro* response of active vegetative buds of *Crocus sativus* L. after the surface disinfection of corms using the following procedure: wash in running tap water with 0.5% Cedepol, a detergent, and a few drops of Tween-20, transfer to 70% ethanol for 1 min then to 0.1% $HgCl_2$ for 10 min completed with five rinses in sterile distilled water. (A) Increase in corm size on 2 µM BA + 2 µM NAA + 40 to 60 g L^{-1} table sugar after 12 weeks. (B) Increase in corm size on 2 µM BA + 2 µM NAA + 40 g L^{-1} sucrose after 12 weeks. (C) Increase in corm size and multiple thick root formation on 8.8 µM IBA + 40 g L^{-1} table sugar after 12 weeks. (D) Increase in corm size and germination on 2 µM BA + 2 µM NAA + 2.5 g L^{-1} KCl + 40 g L^{-1} table sugar + 30 g L^{-1} sucrose after 12 weeks. Figure reproduced from Quadri et al. (2010), with kind permission from Global Science Books (www.globalsciencebooks.info).

Table 1. Disinfection of *ex vitro*–derived tissues for saffron tissue culture (chronological listing). Ø, diameter; AA, ascorbic acid; $AgNO_3$, silver nitrate; d, day(s); DDW, double distilled water; DW, distilled water; EtOH, ethyl alcohol (ethanol); GA_3, gibberellic acid; H_2O_2, hydrogen peroxide; $HgCl_2$, mercury chloride; H_2SO_4, sulphuric acid; mo, month(s); NaOCl, sodium hypochlorite (values presented are assumed to represent the % of active chlorine); NR, not reported in the study; PPM™, Plant Preservation Mixture (Plant Cell Technology); RTW, running tap water; SDW, sterilized (by autoclaving) distilled water; SDDW, sterilized (by autoclaving) double distilled water; SW, sterile water; TW, tap water; w, week(s). * Duplicate publication. ** Authors contacted for clarification, but none was provided

Explant(s) used, explant disinfection, size and source	Reference (country source of material)
Corms → TW → 70% EtOH 10–20 s → 0.1% $HgCl_2$ 15 min → 3–4× SW. Subcultures NR.	Ding et al. 1979, 1981 (China)
Corms → TW → NaOCl or 0.1% $HgCl_2$ 30 min → SW. Subcultures NR.	Cheng, Huang 1980 (China)
Corms → 0.1% $HgCl_2$ 3 min → DW (10 min). Subcultured every 2 mo.	Homes et al. 1987 (Belgium)
1–2 cm Ø corms → RTW → 1% $HgCl_2$ 3–4 min → 75% alcohol + Tween-80 30 min → 3× SW. Subcultured every 30 d.	Ilahi et al. 1987 (Pakistan)
Flower buds → 0.1% NaOCl 5 min → 1% NaOCl 5 min → 70% EtOH 2–3 min → 3× SW. Subcultured every 60 d.	Koyama et al. 1987; Namera et al. 1987 (Japan)
Flower buds → 1% NaOCl 10 min → 3× SW. Subcultures NR.	Sano, Himeno 1987 (Japan)
Corms → TW → 0.1% $HgCl_2$ 8 min → 10% NaOCl 8 min. Rinses and subcultures NR.	Gui et al. 1988 (China)
Descaled corms → RTW 2 h → excised flower buds 3–4 cm long in 7% Domestos 30 min → 5× SW. Subcultured every 30 d.	Fakhrai, Evans 1990 (UK)
Corms → RTW 30 min → dip in 70% EtOH → 0.25% $HgCl_2$ 30 min → 3× SW. Subcultures NR.	Plessner et al. 1990 (India)
Flower buds → 70% EtOH 1 min → 2.5% NaOCl 8 min → 3× SW. Subcultured every 2 mo.	Sarma et al. 1990, 1991 (Japan)
Sprouted corms with green leaves → 0.15% $HgCl_2$ 4 min → SW. Subcultures NR.	George et al. 1992 (India)
Corms → 75% EtOH 3× → 0.1% $HgCl_2$ 10 min → 3× SW. Subcultures NR.	Liu et al. 1992 (China)
Corms → 0.1% $HgCl_2$ 2 min → SW. Subcultured every 14 d.	Dhar, Sapru 1993 (India)
Corms → TW → 0.1% $HgCl_2$ 10 min → 3× SDW. Subcultured every 4 w.	Ahuja et al. 1994 (India)
Corms → 70% EtOH 1 min → 0.1% $HgCl_2$ 15 min → SW. Subcultures NR.	Guang, Shi 1995 (China)
Corms → TW 40 min → 70% EtOH 10 s → 0.1% $HgCl_2$ 15 min → 4–5× SW. Subcultures NR.	Liu et al. 1995 (China)
Corms → TW → 70% EtOH 1–2 min → 0.1% $HgCl_2$ 7–10 min → 4–5× SW. Subcultures NR.	Yang et al. 1996 (China)
Floral buds, style, stigma, anthers, ovaries, and corms treated at 4 °C for 21 d → soapy water → 70% EtOH 30 s → 0.1% $HgCl_2$ 8–10 min → 3× SW. Subcultures NR.	Jia et al. 1996 (China)
Descaled corms → RTW → excised flower buds in 70% EtOH 5 min → 2.5% NaOCl 15 min → 3× SDW. Subcultured every 30 d.	Ebrahimzadeh et al. 1996 (Iran)
Corms with developed leaves and ensheathed flowers just prior to flowering → EtOH 5 min → 10% Domestos 30 min → 3× SDW. Subcultured every 3 mo.	Castellar, Iborra 1997 (Spain)
Ovaries (2.5–5 cm Ø) of 8 developmental stages → 0.15% $HgCl_2$ 4 min → several rinses in SDW. Subcultures NR.	Bhagyalakshmi 1999 (India)
Corms → RTW 1–2 h → DW → 80% EtOH 25 s → 3× SDW → 0.8% NaOCl 20 min with sonication (Hz NR) → 3× SDW. Subcultured every 5 w.	Escribano et al. 1999; Piqueras et al. 1999; Blázquez et al. 2004a, 2004b (Spain)
Corms with floral buds → RTW → floral buds excised → soapy water 10 min → DW → 70% EtOH 1 min → 5.25% NaOCl + 3–4 drops Tween–80/500 mL 15 min → 4× SDDW. Subcultured every 3 w.	Loskutov et al. 1999 (USA)
Descaled corms → TW → 0.1% $HgCl_2$ 10 min → 3× SDW. Subcultured every 30 d.	Ebrahimzadeh et al. 2000a (Iran)
5–10 cm long flower buds → RTW → 70% EtOH 5 min → 1% NaOCl 10 min → 3× SDW. Subcultured every 45 d.	Ebrahimzadeh et al. 2000b (Iran)
Corms → 75% EtOH 3× → 0.1% $HgCl_2$ 10 min → 3× SW. Subcultures NR.	Wang, Chen 2000 (China)
Corms → TW → 70% EtOH 10 min → 0.1% $HgCl_2$ 10 min → 4–5× SW. Subcultures NR.	Zhao et al. 2001a (China)
Corms → TW 40 min → 70% EtOH 8 min → 0.2% $HgCl_2$ 8 min → 4–5× SW. Apical buds → TW → 70% EtOH 10 min → 0.1% $HgCl_2$ 10 min → 4–5× SW. Subcultures NR.	Zhao et al. 2001b (China)
Corms → TW → 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 4× SW. Subcultured every 1–2 mo.	He et al. 2002 (China)

(continued)

Table 1. *continued*

Explant(s) used, explant disinfection, size and source	Reference (country source of material)
Corms → TW → 70% EtOH 30 s → NaOCl 15 min → 5× SW. Subcultures NR.	Chen et al. 2003a, 2003b (China)
Floral buds → soapy water → DW → 70% EtOH 1 min → 0.1% $HgCl_2$ 8 min → 5× SDW. Subcultures NR.	Zeng et al. 2003 (China)
Shoots, leaves, flowers → TW 1–2 h → DW → 70% EtOH 20 s → NaOCl 20 min → 5× SDW. Subcultures NR.	Chen et al. 2004 (China)
Cormlets → TW → 0.15% $HgCl_2$ 10 min → 3× SW. Subcultures NR.	Karamian 2004 (Iran)
Corms → 70% EtOH time NR → 0.1% $HgCl_2$ 10 min → 5× SW. Subcultured every 21 d.	Sharma et al. 2005 (India)
Corms → TW → 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 0.05% HgCl2 5 min → 3–4× SW. Subcultures NR.	Zhao et al. 2005a, 2005b (China)
Descaled corms → RTW 30 min → dip in 70% EtOH → 2% $HgCl_2$ 30 min → 3× SDW → 1% AA 10 min. Subcultured every 1 mo.	Darvishi et al. 2006a, 2006b* (Iran)
Shoots derived from sprouting corms at 25 °C in greenhouse → RTW 30 min → 70% EtOH 15 s → 0.1% $HgCl_2$ 8 min → 4–5× SDW. Subcultures NR.	Wang et al. 2006 (China)
Corms stored at 4 °C for 40 d → 0.5–1.0 mg/l GA_3 → TW 40 min → 75% EtOH 1 min → 0.1% $HgCl_2$ 15 min → 5× SW → 0.1% $HgCl_2$ 5 min → 6× SW. Subcultures NR.	Zhang et al. 2007 (China)
Styles and perianths excised from closed flower buds → 70% EtOH 1 min → 0.1% $HgCl_2$ 8 min → 3× SDW. Subcultures NR.	Jun et al. 2007 (China)
Descaled corms → one of 6 surface disinfection treatments: 1) H_2SO_4 1 min → 80% bleach (ACE) 20 min; 2) H_2SO_4 2.5 min → 80% bleach (ACE) 20 min; 3) H_2SO_4 1 min → 0.7% $AgNO_3$ 10 min; 4) H_2SO_4 2.5 min → 0.7% $AgNO_3$ 10 min; 5) 3% dry or liquid fungicide (manufacturer NR); 6) 1, 2, 3, 4, 5 or 6% PPMTM 1 h; 7) hot water (40, 42.5, 45 and 47.5 °C) (period NR) → 50% bleach (Axion) 20 min. In all 7 treatments, surface disinfected corms → 5× SDW. Subcultures NR.	Karaoğlu et al. 2007 (Turkey)
Descaled corms → TW 30 min → 70% EtOH 1 min → 20% NaOCl 15 min → 3× SDW → 0.15% $HgCl_2$ 7 min → 3× SDW. Subcultures NR.	Rajabpoor et al. 2007 (Iran)
Descaled corms → 70% EtOH 45 sec → 0.2% $HgCl_2$ 20 min → 3× SDW 15 min. Subcultured every 4 w.	Sheibani et al. 2007a, 2007b (Iran)
Corms stored at 4°C → TW → 70% EtOH 10 sec → 0.1% $HgCl_2$ 10 min → 5× SDW. Subcultured every 1 mo.	Sharma et al. 2008 (India)
Dormant corms (age and size NR) → RTW 2 h → dip in DW → 80% EtOH 30 sec → 3× SDW → 0.8% NaOCl 20 min with sonication (Hz NR) → 3× SDW. Explants 10 mm^3 from corm center. Subcultured every 6 w.	Blázquez et al. 2009 (Spain)
Flower buds → RTW 30 min → 0.5% benzalconium chloride 15 min → 70% EtOH 2 min → 1% NaOCl + few drops Tween-80 20 min → 3× SDW. Subcultured every 4 w.	Namin et al. 2009, 2010 (Iran)
Corms → TW → 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 4–5× SW. Subcultures NR.	Wang et al. 2009 (China)
Corms → TW overnight → 70% EtOH 1 min → 0.1% $HgCl_2$ 6–15 min → 4× SW. Subcultures NR.	Yuan et al. 2009 (China)
Ovaries from flower buds → RTW → 70% EtOH 3 min → NaOCl (% NR) 10 min → 4× SDW. Subcultured every 4 w.	Mir et al. 2010 (India)
Corms → RTW + 0.5% Cedepol (detergent) + Tween–20 (time NR) → DDW → 70% EtOH 1 min → 0.1% $HgCl_2$ 10 min → 5× DW. Subcultures NR.	Quadri et al. 2010 (India)
Flower buds → RTW 30 min → dishwashing liquid → 1% benzalconium chloride 10 min → TW → 70% EtOH 2 min → 1% NaOCl 15 min → 3× SDW. Subcultured every 7 d.	Sharifi et al. 2010a, 2010b, 2012 (Iran)
Corms → RTW → 70% EtOH 2 min → 0.1% $HgCl_2$ 5 min → 20% bleach + Tween-20 10 min → 3× SDW. Subcultures NR.	Vatankhah et al. 2010, 2014 (Iran)
Descaled corms → RTW 1 h → brushed with Tween–20 → 0.1% streptomycin sulphate + 0.1% Bavistin 30 min → DW → 70% EtOH 30–45 s → 0.1% $HgCl_2$ 10–12 min → 5–6× SDW. Subcultures NR.	Devi et al. 2011, 2014 (India)

(continued)

Table 1. *continued*

Explant(s) used, explant disinfection, size and source	Reference (country source of material)
Shoots derived from sprouting corms → RTW 2 h → DW → 80% EtOH 30 s → 6× SDW → 0.8% NaOCl 20 min with sonication (Hz NR) → 3× SDW. Subcultures NR.	Diaz-Vivancos et al. 2011 (Spain)
Corms stored at 4°C for 48 h → TW 0.5– 1 h → 2× SW → 70% EtOH 30 s → 0.1% $HgCl_2$ 8 min → 4–5× SW. Subcultures NR.	Wang et al. 2011 (China)
Corms → RTW + 0.5% Extran (detergent) + Tween-20 (time NR) → DDW → 70% EtOH 1 min → 0.5% HgCl2 6 min → 5× DW. Subcultures NR.	Parray et al. 2012 (India)
Leaves (age / developmental stage NR) → RTW 20 min → 5.25% NaOCl + Tween-80 10 min → 3× DW. Subcultures NR.	Sharafzadeh 2012 (Iran)
Corms → RTW 30 min → tunics removed → 70% EtOH 30 sec → 0.15% $HgCl_2$ 20 min → 3× DW. Subcultured every 3 w.	Zeybek et al. 2012 (Turkey)
Corms → immersed in water with dishwashing liquid for 5 min → TW 30 min → 0.05% $HgCl_2$ 30 min → 2× SW → 0.02% $HgCl_2$ 2 min → 4× SW for 10 min. Subcultures NR.	Wang, Xiao 2012 (China)
10–45 mm Ø corms used in 5 disinfection procedures: 1) 70% EtOH 5 min → 50% NaOCl 15 min; 2) 5% Tween-20 in DW 60 min → 70% EtOH 20 min → 50% NaOCl 5 min → 7% H_2O_2 10 min; 3) 5% Tween-20 in DW 75 min → 70% EtOH 20 min → 7% H_2O_2 20 min; 4) 5% Tween-20 in DW 90 min → 70% EtOH 15 min → 7% H_2O_2 15 min; 5) 5% Tween–20 in 10% EtOH 10 min → 0.15% H_2O_2 10 min. 1) to 5) → 3× SDW. Explants 5–10 mm^3 with apical and axillary buds. Subcultured every 45 d.	Cavusoglu et al. 2013 (Turkey)
Corms → TW → 70% EtOH 2 min → 5% NaOCl + 0.1% Tween-20 10 min → corms cut into segments → 70% EtOH 10 sec → 0.1% $HgCl_2$ 3 min → 4× SDW. Subcultures NR.	Lapadatescu et al. 2013 (Romania)
Corms (low temperature storage and controls) with tunics removed → 70% EtOH 30 sec → 3× SW → 0.8% NaOCl 20 min → sonication (time and Hz NR) → 3× SDW. Subcultures NR.	Renau-Morata et al. 2013 (Spain)
Corms → 70% EtOH 1 min → 35% NaOCl 7 min → 35% nano–silver (precise specifications NR) 10 min → several washes in SDW. Subcultures NR.	Shahabzadeh et al. 2013 (Iran)
Corms → RTW 10 min → 0.1% carbendizime + 0.2% mancozeb (fungicides) + Tween-20 10 min → 50% NaOCl 10 min → 1.6% $HgCl_2$ 5 min → SDW. Subcultures NR.	Yasmin et al. 2013; Yasmin, Nehvi 2014a, 2014b (India)
Apical buds from flower buds → RTW → 70% EtOH 3–4 min → 0.1% $HgCl_2$ 10 min → 5× SDW. Subcultured every 4 w.	Mir et al. 2014 (India)
Corms → TW 1 h → 70% EtOH 30 s → 0.1% $HgCl_2$ 15 min → 4× SW. Subcultures NR.	Peng, Hu 2014 (China)
Corms → RTW 30 min → detergent → 1% benzalconium chloride 10 min → TW → 70% EtOH 4 min → 1% NaOCl 15 min → 3× SDW. Subcultured every 1 mo.	Vahedi et al. 2014, 2015 (Iran)
Corms → 5% NaOCl 8 min (shaking) → 80% EtOH 1 min → 37% nanosilver** 17 min (shaking) → 3× DW. Subcultured every 1 mo.	Abbas, Elahe 2015 (Iran)
Corms → RTW → 70% EtOH 3 min → NaOCl 10 min → 4× SDW. Subcultured every 3 w.	Mir et al. 2015 (India)
Corms → TW → 75% EtOH 60 s → 3–4× SW → 0.1% $HgCl_2$ 12–15 min → 3–4× SW. Subcultures NR.	Yang et al. 2015 (China)

limited number of viable explants (corms). Cavusoglu et al. (2013) compared five disinfection procedures and assessed the percentage explant (corm segments) contamination after 30 days, finding the percentage of contaminated cultures to be 0, 17, 60, 83 and 93% for methods 1 to 5 in Table 1, respectively. Yasmin et al. (2013) found that just over 30% of control cultures (i.e., corms not undergoing any surface disinfection) were uncontaminated, but that the level of contamination was reduced as the level of $HgCl_2$ increased, peaking at 94% contaminant-free culture when 1.6% $HgCl_2$ was used (time period not described). In all cases, fungicides were also simultaneously applied and concentrations of $HgCl_2$ exceeding 1.6% or high levels of sodium hypochlorite surprisingly (as the opposite would be expected) decreased the percentage of contaminant-free cultures, and also explant survival to 46.43% (Yasmin and Nehvi 2014b). Abbas and Elahe (2015) claimed to use 37% nanosilver, but what this percentage means, or its commercial source, were not indicated, nor was the final explant contamination level quantified.

Since saffron leaves are basal without a general stem, and since the growth point (shoot apical meristem) is located at the base of the leaves where they are connected to the corm, it is difficult to isolate shoot tips. In the Chinese literature,

it was shown that the survival of shoot tips less than 0.5 mm in length – essential for the development of virus-free material – was low, but that disinfection of large shoot tips (> 0.5 mm) was better than for shoot tips less than 0.5 mm. The survival rate of explants increased to 85% when shoot tips longer than 1 mm were cultured after disinfection (Zhu et al. 2009). Chen et al. (2006) used a two-step disinfection method to obtain uncontaminated shoots from the corms of *C. sativus*. In the first step, shoots induced from callus tissue according to the method of Chen et al. (2003a) were subcultured on MS supplemented with 0.25 mg L^{-1} 2,4-D, 2 mg L^{-1} BA and 200 mg L^{-1} casein hydrolysate and cultured at 35 °C for 25 days (10-h photoperiod). The new shoots were used to induce callus once again. Induced callus was transferred to MS containing 0.5 mg L^{-1} NAA and 0.25 mg L^{-1} BA and cultured at 35 °C for 40 days (continuous darkness), then shoots were induced from callus tissue on MS medium supplemented with 0.25 mg L^{-1} 2,4-D and 2 mg L^{-1} BA. Finally, uncontaminated shoots that were generated after 50 days of culture were tested by combining ELISA and RT-PCR. Zhu et al. (2009) obtained saffron plantlets free of CMV (*Cucumber mosaic virus*), TuMV (*Turnip mosaic virus*), TRV (*Tobacco rattle virus*) and IMV (*Iris mosaic virus*) free by two methods: (i) temperature treatment combined with shoot tip culture; (ii) chemical treatment combined with shoot tip culture, after corms were disinfected with 75% EtOH and 0.1% $HgCl_2$ for 12 min. In method 1, corms (age not indicated) with shoots were treated at 36 °C for 12 h then at 18 °C for 12 h, outer tunics were removed and 0.5 to 1.0 mm shoot tips were cultured, resulting in 26.7% survival. In method 2, 0.5 to 1.0 mm shoot tips stripped from corms 3 to 5 cm in diameter with leaves were added to MS medium supplemented with 5.0 mg/L BA, 3.0 mg L^{-1} NAA, and 5 to 10 mg L^{-1} ribavirin. The survival rate of shoot tips ranged from 25 (when 10 mg L^{-1} ribavirin was used) to 45% (when 5 mg L^{-1} ribavirin was used) (Zhu et al. 2009).

Most studies in the Chinese saffron literature have followed a fairly standard procedure for the disinfection of tissues for *in vitro* shoot or callus induction, broadly including a wash with tap water, surface disinfection with 70% EtOH, treatment with 0.1% $HgCl_2$ and a final rinse with sterile water (Table 1). The number of times explants were rinsed with tap water varied, although most studies did not report on the precise period while a few studies described it as 0.5 h to overnight (Liu et al. 1995; Yuan et al. 2009; Wang et al. 2011). Explant disinfection with 0.1% $HgCl_2$ for 8 to 15 min was a general trend, with the exception of three studies that employed NaOCl for 15 to 20 min (Chen et al. 2003a; 2003b; 2004a; 2004b). Chen et al. (2003a; 2003b; 2004a; 2004b) reported disinfection methods for different saffron explants that were not exactly the same. The disinfection procedure for shoots, leaves and flower explants involved a rinse in tap water for 1 to 2 h, a wash with distilled water, a dip in 70% ethanol for 20 s, treatment for 20 min in 1% NaOCl, and then five rinses with SDW. In contrast, the disinfection procedure for corms was two washes with distilled water, a dip in 70% ethanol for 30 s, transfer to 2% NaOCl for 15 min, and then five rinses with SDW. In two studies, corms were stored at 4 °C for 48 h and 40 days, respectively, prior to rinsing with tap water, but the exact reason for the cold treatment was not explained (Zhang et al. 2007; Wang et al. 2011), although Zhang et al. (2007) first treated explants with 0.5 to 1.0 mg L^{-1} gibberellic acid (GA_3) prior to cold storage. In geophytes in general and specifically in saffron, a cold period of vernalization is required for the adequate induction of flowering and further development of shoots. From a physiological point of view, cold treatments can modulate endogenous levels of hormones implicated (gibberellins) in the retardation of new meristem development and sprouting (Plessner et al. 1989).

In order to achieve better results, a two-step procedure is often used in saffron tissue disinfection procedures. During the first step, entire organs are surface disinfected. At first, redundant parts (leaves and/or roots) are removed, organs are washed extensively with water containing a few drops of Teepol (0.1%, v/v) and sterilized by washing in 70 to 90% ethanol (5 min) and then for 30 min in 3-5% Ca(ClO)2 with three or more rinses in SDW. During the second step, the outer tissues (epidermal layers) of the remaining organ are removed and the inner tissues are cut into pieces (approx. 3 cm thick). The fragments are washed with SDW and treated with $Ca(ClO)_2$ for 30 min, with three or more rinses in SDW (Ndong et al. 2006). It is also possible to use two sterilizing agents, sequentially (usually NaOCl followed by $HgCl_2$). For example, the combination of systemic fungicides with bleach at 50% (10 min) and a dip in 1.6% $HgCl_2$ for 5 min provided 94% clean viable saffron corm cultures (Yasmin et al. 2013). Sivanesan et al. (2014) was able to obtain 100% aseptic cultures of *Crocus vernus* (L.) Hill corms after applying 2.0% (v/v) NaOCl for 10 min followed by three washes with SDW, treatment with 0.01% (w/v) $HgCl_2$ for 15 min, while Cavusoglu et al. (2013) obtained the same results after combining 50% NaOCl (15 min) and 7% H_2O_2 (15 min).

It is curious to note that Zaffar et al. (2014) cultured corm directly onto tissue culture medium without describing any surface disinfection procedure, which is highly unlikely. Indeed, photographic evidence provided by these authors indicates that stigma-like structures formed in 4-month old cultures that were apparently free of contamination, although the authors did not specifically address the issue of contamination. Similarly, Chichiriccò and Grilli Caiola (1987) excised ovaries aseptically and plated them directly *in vitro* without describing any surface disinfection protocol or accompanying medium infection. Although Majourhat et al. (2007) claimed that less than 30% of apical and axillary shoots were contaminated *in vitro,* presumably less than corm tissue, the actual disinfection procedure was

not described.

This mini-review focuses on a segment of biotechnology that is rarely discussed in detail, mainly because it pertains to negative results. However, there is great value in negative results, but whose publication and discussion are limited primarily by a negative stigma (Teixeira da Silva 2015). Thus the objective of this review was to draw upon broad tissue culture principles for the establishment of *in vitro* cultures and to seek evidence and results from within the saffron literature, including negative elements, which will make future efforts to establish *in vitro* cultures by saffron researchers easier.

Insight to successful establishment of saffron tissue cultures from phytopathology

Many pathogenic and non-pathogenic microbiota have been described for saffron. Viral infections like those caused by TuMV are known (Ahrazem et al. 2010), but from a tissue disinfection perspective, fungal infections are the most important. Species/strains of many fungal/oomycete genera have been described and a significant proportion of them are endophytic, which might cause less efficient surface disinfection of explants used for tissue culture. Species of *Fusarium* are the most important pathogens, causing corm rot disease (Ahrazem et al. 2010; Husaini et al. 2010). During saffron flowering, if the crop is infected by *Fusarium oxysporum* f. sp. *gladioli* and *solani*, *Fusarium pallidoroseum, Fusarium equiseti*, *Mucor* sp. and *Penicillium* sp., then yellowing, wilting of shoots, basal stem and corm rot and major yield losses can occur (Di Primo et al. 2002; Kalha, Gupta 2009). *Fusarium* isolates can be characterized by pathogenicity and vegetative compatibility tests but can be eliminated from saffron corms with a simple surface-disinfection with 1% NaOCl (Di Primo et al. 2002). Other genera that have been detected are *Aspergillus, Beauveria, Penicillium, Phytophtora, Rhizoctonia* and *Uromyces* (*Uromyces croci* is known for the colonization of saffron organs) (Ahrazem et al. 2010; Husaini et al. 2010). Pathogenic bacteria affecting saffron yield are not common. However, there are some reports showing saffron diseases of bacterial origin. Fiori et al. (2011) proved that *Burkholderia gladioli* and other species belonging to this genus were responsible for shoot, leaf and corm rot in saffron cultivated in Sardinia, Italy. On the other hand, Ambardar and Vakhlu (2013) detected rhizosphere bacterial communities with *Bacillus, Brevibacterium* and *Pseudomonas* promoting saffron growth, one possible reason being that most of these bacteria produced IAA.

Conclusions

At present, saffron is propagated vegetatively by using annually renewed corms. Fungal infestation of corms is a bottleneck limiting the availability of sufficient quality planting material (Yasmin et al. 2013). As for geophytes, disinfection of underground organs to establish axenic cultures is very difficult. Most epiphytic microorganisms develop during the first stage of tissue culture, but endophytic microorganisms may remain cryptic and not be detected until a more advanced culture phase (Curvetto et al. 2006). The literature pertaining to saffron and other geophytes has considerable errors, including the lack of details related to disinfection protocols or the efficiency of protocols. In the future, traditional disinfectants such as NaOCl or $HgCl_2$ might be replaced with more efficient and user-friendly nanocolloids (Ag, Au, Cu). Thus far developed shoots or dormant corms have been used, but to obtain a balance between eliminating infection and having tissue that is receptive *in vitro*, future experiments could consider employing sprouting corms for culture initiation and multiplication.

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