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WITHANIA COAGULANS (STOCKS) DUNAL: BIOTECHNOLOGICAL ACHIEVEMENTS AND PERSPECTIVES

A review

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ABSTRACT

Withania coagulans (Stocks) Dunal is an important medicinal plant of the Solanaceae. Biotechnological studies on this plant started in 2009 and are still in a nascent phase of development. Even so, some important advances have been made, particularly in the field of tissue culture, which is an important means for its large-scale propagation and *in vitro* conservation. This review focuses on methods for surface sterilization, culture initiation, multiplication, rooting and acclimatization of *W. coagulans.*

Key words: adventitious shoot regeneration, *in vitro* rooting, micropropagation, tissue culture, RAPD, vegetable rennet, *Withania coagulans*

INTRODUCTION

Withania coagulans (Stocks) Dunal (Solanaceae), commonly known in English as Indian cheese maker, Indian rennet or vegetable rennet and with trade names panir (or paneer) doda, dodi, bandh and dhodi, is a medicinal herb whose biotechnological attributes have not been extensively explored, unlike better known *W. somnifera*. *W. coagulans* is a seed propagated, berry bearing bush that has ample medicinal properties conferred by the presence of many biologically active compounds including alkaloids, steroids, phenolic compounds, tannins, saponins, carbohydrates, proteins, amino acids, organic acids and withanolides (steroidal lactones). It is known that these bioactive compounds possess antihyperglycaemic, antihyperlipidemic, hypolipidemic, antiinflammatory, antimutagenic and anticancer/ anticarcinogenic, hepatoprotective, immunomodulatory, antifungal, antibacterial, antihelminthic, hypocholesterolemic, free radical scavenging, wound healing, cardiovascular, immunosuppressive and diuretic activities (reviewed in Gupta 2012; Jain et al. 2012; Gupta & Keshari 2013; and references therein). Excessive collection of *W. coagulans* from wild resources has marked this plant species as threatened and endangered (Rawat 2008), although it is not listed on the IUCN Red List www.iucnredlist.org). The association between the roots of *W. coagulans* and arbuscular mycorrhizal fungi may confer upon the plant an ability to survive harsh climatic conditions such as drought (Panwar & Tarafdar 2006).

The use of tissue culture and biotechnology would allow the mass production of medicinally important compounds such as the withanolides from rare germplasm. According to Mishra et al. (2013), the difference between *W. somnifera* and *W. coagulans* is that withanolides are produced primarily in the roots of the former and primarily in the aerial parts of the latter. For sampling natural populations in order to identify superior genotypes or to assemble diverse genotypes for future breeding programmes, a suitable sampling procedure is required (described by Gilani et al. 2009). In this study, seven populations of *W. coagulans* from diverse districts in Pakistan

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were analysed using PBA (P450 based analogue) markers, identifying low interpopulation but high intrapopulation diversity. The diversity can further be mined by nondestructive sampling of tissues and propagated by *in vitro* techniques as summarized in this minireview.

A micropropagation protocol typically consists of explant collection, surface sterilization, establishment of aseptic cultures, shoot induction and multiplication, rooting, acclimatization, screening of genetic fidelity and performance of micropropagated plants in field conditions. Nodal explants from field-grown plants or from *in vitro* seedlings are the most common explants for the tissue culture of *W. coagulans* (Table 1). There are only two alternative regeneration protocols for *W. coagulans*. One of them described adventitious shoot regeneration from leaf segments of field-grown plants (Jain et al. 2011), while the other described the use of leaves from axillary shoots emerging from nodes and shoot tips of *in vitro*-raised shoots (Mishra et al. 2013). There is also only one report on callus-mediated regeneration from leaves and internode segments of *in vitro* seedlings of *W. coagulans* (Valizadeh & Valizadeh 2009). There are no reports that consider direct or callus-mediated somatic embryogenesis in *W. coagulans*. The literature (Table 1) indicates that Murashige and Skoog's (MS, Murashige & Skoog 1962) medium has been the best choice for culture initiation, multiplication and rooting. No studies found any other basal media to be important. Most studies employed N^6-benzyladenine (BA) alone or in combination with kinetin (Kn) for shoot induction and multiplication (Table 1). Only one report is available in which a cytokinin, meta topoline (mT), was found to be the most effective for axillary shoot multiplication from nodal explants (Joshi 2014). Joshi (2014) studied the effects of 0.5, 1.0, 1.50, 2.0 and 2.50 $mg \cdot l^{-1}$ of BA, Kn, thidiazuron (TDZ), zeatin (Zea) and mT in combination with 50 $mg \cdot l^{-1}$ adenine sulphate (AdS) and 0.1 $mg \cdot l^{-1}$ α-naphthaleneacetic acid (NAA). The best shoot multiplication index (79.17%) was achieved with MS medium supplemented with 2.50 $mg \cdot l^{-1}$ TDZ, 50 $mg \cdot l^{-1}$ AdS and 0.1 $mg \cdot l^{-1}$ NAA but all regenerated axillary shoots were hyperhydric (Fig. 1A). On the other hand, when the MS medium was supplemented with 2.50 $mg \cdot l^{-1}$ mT, 50 $mg \cdot l^{-1}$ AdS and 0.1 $mg \cdot l^{-1}$ NAA, the multiplication index of healthy shoots was 75% (Fig. 1B). Indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) was the most frequently used as auxins for inducing *in vitro* rooting, usually in the presence of half-strength MS medium (Table 1). Jain et al. (2009, 2011) reported that the addition of phloroglucinol (PG) and choline chloride (CC) to the basal medium enhanced the rooting of *in vitro* cultured *W. coagulans* shoots.

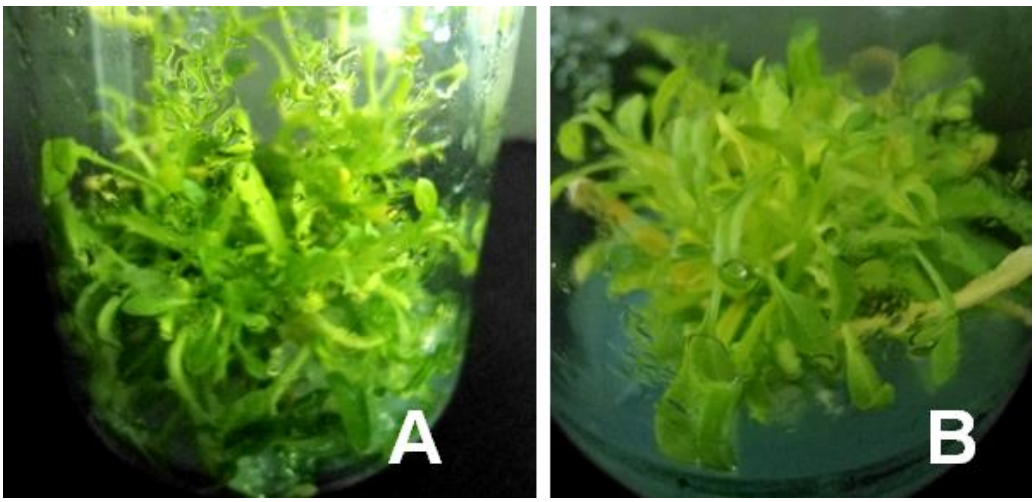

Fig. 1. Tissue culture of *Withania coagulans* (Stocks) Dunal (45-day-old cultures; unpublished results). A. Nodal explants cultured on MS medium supplemented with 2.5 $mg \cdot l^{-1}$ TDZ, 50 $mg \cdot l^{-1}$ AdS and 0.1 $mg \cdot l^{-1}$ NAA (showing hyperhydric shoots). B. Nodal explants cultured on MS medium supplemented with 2.5 $mg \cdot l^{-1}$ mT, 50 $mg \cdot l^{-1}$ AdS and 0.1 $mg \cdot l^{-1}$ NAA (showing healthy and uniform shoots).

Table 1. Micropropagation of *Withania coagulans* (chronological order)

Genotype	Sterilisation procedure and explant	Culture medium, PGRs, additives, subcultures*	Culture conditions**	Experimental outcome, maximum productivity, acclimatisation and variation	Reference
Wild plants from Ajmer district (India)	Nodal segments: 5% Teepol → 70% EtOH 30 s → 0.1% $HgCl_2$ 3 min → SDW	MS + 0.5 mg·l^{-1} BA + 0.5 mg·l^{-1} Kin + 0.5 mg·l^{-1} PG (SIM, SMM). MS + 10 mg·l^{-1} CC + 0.5 mg·l^{-1} PG 7 d → MS + 0.25 mg·l^{-1} IBA + 0.5 mg·l^{-1} phenylacetic acid + 2 mg·l^{-1} CC (RIM) using 2-3 cm long shoots. pII 5.8. 3% sucrose. Agar conc. NR.	16-h PP. CWFT 25 µmol·m^{-2}·s^{-1} 25 ± 1 °C	83% of nodes from mother plants formed shoots (18.6/explant). Shoots and shoot tips from *in vitro* shoots formed 23.4 and 24.6 buds/explant, respectively. 80% of shoots formed roots. About 75% survival in soil + manure (1:1). No variation between in vitro clones and mother plants claimed using RAPD.	Jain et al. 2009
Wild plants from Sistan and Baluchestan Province (SE Iran)	Seeds washed in tap water overnight → 70% EtOH and 3% NaOCl 30 min → several washes SDW	½ MS + 30 mg·l^{-1} sucrose (SG). Sterile leaf section (size NR) → *Agrobacterium tumefaciens* C58 Cl1 (pRiA4) duration of treatment (NR) → MS + carbenicillin 500 mg·l^{-1} → PGR-free MS medium as liquid medium suspension culture at 100 rpm. pH and carbon source (NR).	For SG: seeds kept in dark for first 2 d then under 16-h PP. 28-36 mmol·m^{-2}·s^{-1} For RI: Cultures kept in darkness 25 °C	> 90% of leaf explants formed roots within 3-6 w. Two types of roots obtained: callus-like and hairy roots. Withanolide A (0.891 µg·g^{-1}) and withaferin (37 ng·g^{-1}) from hairy root cultures and withanolide A (1.905 µg·g^{-1}) and withaferin (47 ng·g^{-1}) from callus-like root cultures (all dry weight values).	Mirjalili et al. 2009, 2011
Saravan (Iran)	Seeds from mature fruits: RTW 30 min → 70% alcohol 30 s → 2% NaOCl 10 min → SDW 16 h. Leaves and internodes from 1-mo-old seedlings used as explants	MS (SG). MS + 2-4 mg·l^{-1} 2,4-D or 0.5-1 mg·l^{-1} BA/Kin (CIM: leaves). MS + 2-4 mg·l^{-1} 2,4-D or 0.25-0.5 mg·l^{-1} BA (CIM: internodes). MS + 2 mg·l^{-1} BA + 0.5 mg·l^{-1} IBA (SIM). ½ MS + 2 mg·l^{-1} IBA (RIM). pH 5.8. 2% sucrose. 0.8% agar.	16-h PP. CWFT. 40 µmol·m^{-2}·s^{-1} 25 ± 2 °C	100% of leaves (42% in internodes) formed callus in 14-16 d. Shoots obtained only from internode callus, but only in max. 33% of explants. 100% of shoots rooted *in vitro*. 75% survival after acclimatisation in soil + sand (2 : 1).	Valizadeh & Valizadeh 2009
NR (local market)	Seeds: conc. H_2SO_4 2 min, roots of 3-w-old seedlings used as explants	MS (agarised) (SG). MS (liquid) + 0.25 mg·l^{-1} IBA (RIM; 30 ml·100 ml^{-1} Erlenmeyer flask; 80 rpm). 3% sucrose. pII and agar conc. NR.	Continuous light. No other details provided. 25 °C	50% germination. 3-w old root cultures produced 11.65 µg·g^{-1} Withaferin A with 88% recovery success, assessed by RP-HPLC.	Abouzid et al. 2010
Same as Jain et al. 2009	Leaf (0.8-2 cm) → RTW 15 min → 20% Extrans® (liquid detergent) 5 min → 0.1% $HgCl_2$ 3 min → 4-5X SDW	MS + 22.2 µM BA + 2.3 µM Kn (SIM). SMM same as Jain et al. (2009). Pulse treatment ½ MS + 71.6 µM CC + 3.9 µM PG for 7 d → ½ MS + 1.2 µM IBA + 3.6 µM phenylacetic acid + 14.3 µM CC (RIM). pH 5.8. 3% sucrose. 0.9% agar.	16-h PP. CWFT. 25 µmol·m^{-2}·s^{-1}. 26 ± 1 °C	80% of leaf explants showed direct shoot formation with 17 shoots/leaf segment. Pulse-treated shoots were successfully rooted (data NR). Acclimatisation same as Jain et al. (2009). No variation reported compared with mother plants using RAPD.	Jain et al. 2011

Saravan (Iran)	Nodal segments from 5-y-old plant during April-May 2008 → RTW → 70% EtOH 30 sec → 2% NaClO 10 min → several rinses with SDW → 250 $mg \cdot l^{-1}$ Cefotaxime for 5 min	MS + 2 $mg \cdot l^{-1}$ BA + 0.5 $mg \cdot l^{-1}$ IBA (SIM). ½ MS + 2 $mg \cdot l^{-1}$ IBA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	Same as Valizadeh & Valizadeh (2009)	100% contamination-free cultures. 100% axillary shoot multiplication with 7 shoots/node. 100% rooting with 35 roots/node. Rooted plants transplanted to sterile soil + sand (2 : 1). After 25 d, plants transferred to greenhouse and maintained under partial shade and irrigated daily with 75% plant survival.	Valizadeh & Valizadeh 2011
Three locations in Western Rajasthan (India)	Apical tips, nodal segments (3-5 air dried	MS + 8.88 µM BA + 0.57 µM IAA + additives like antioxidants (SIM). MS + 4.44 µM BA + 0.57 µM IAA + additives → MS + 1.11 µM BA + 0.57 µM IAA + additives (SMM). ½ MS + 29.5 µM IBA + 200 $mg \cdot l^{-1}$ activated charcoal (RIM). pH 5.8. 3% sucrose. 0.8% agar.	CWFT. First 3-4 d: 12-h PP. 20-25 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 28 ± 2 °C. 60-70% RH. Subsequent culture: as above, but 35-40 $\mu mol \cdot m^{-2} \cdot s^{-1}$	95.5% of explants formed shoots (4.1/explant). 67.3% of shoots rooted *in vitro*, or 73.8% if shoots were rooted *ex vitro* by pulsing with 2.46 mM IBA for 5 min. NOA was also an effective rooting agent. Pulsed shoots potted in sterile Soilrite®, watered with ¼ MS (26 ± 2 °C. 70-80% RH → 34 ± 2 °C. 40-50% RH). Final transfer to sandy soil + garden soil + farmyard manure (1 : 1 : 1) with > 90% survival.	Rathore et al. 2012
NR	Leaves of 3rd node from shoot tip of *in vitro* multiple shoots	MS + 10.0 µM BA + 8 µM IAA + 500 $mg \cdot l^{-1}$ Cef 1-5 d then 10-15 d (SIM). MS + 1.0 µM BA + 0.8 µM IAA + 50 $mg \cdot l^{-1}$ Kan (SEM). MS + 2.5 µM IBA 1 w → ½ MS 2 w (RIM). pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. 24 ± 1 °C	Integration of *npt*II and *gus*A genes shown with PCR following selection of Kan-resistant shoots buds on selection medium.	Mishra et al. 2013
Medicinal Plant Garden Anand Agriculture University, Gujarat, India	Nodes → RTW 10 min → Liquid detergent 10 min (conc. NR) → RTW 30 min → 0.1% $HgCl_2$ 1-2 min → 3-4X SDW	MS + 2 $mg \cdot l^{-1}$ BA (SIM). Rooting (NR). pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. CWFT. 2500-3000 lux. 25 ± 2 °C	100% of nodal explants produced 24 shoots/node. Rooting and acclimatisation not performed.	Nekkala 2013
12 ecotypes from wild populations in Sistan Baluchistan Province (Iran)	Seeds: RTW → dried at RT 1 d → 4 °C → 70% alcohol 30 s → 5% NaOCl 20 min → 4X SDW	Water or B_5 (SG). 0.7% agar. Carbon source and pH NR.	Darkness or light (16-h PP; Light source NR; 9684 lux). 25 °C. 65% RH	Germination response was strongly genotype-dependent. Germination percentages ranged from 5-63% in the light and from 67-98% in the dark, in the latter taking 6-10 days to achieve while in the former 8-13 days. Germination on filter paper higher than on B_5 or plain agar (70%, 57%, 52%, respectively).	Edalatifard et al. 2014

Unclear	Seeds: Tween 20 → distilled water	½ MS + 100 mg·l^{-1} activated charcoal + 3% sucrose (SG). MS + 13.6-18.1 µM 2,4-D (CIM). MS + 2.1 µM BA + 2.32 µM Kin + 0.57 µM IAA (SIM). MS + 13.31 µM BA + 13.93 µM Kin + 7.13 µM IAA (SMM). MS + 24.6 µM IAA (RIM). pH 5.8. 3-4% sucrose. 0.5-0.8% agar.	SG: PP and light source NR. 1500-2000 lux. 25 ± 2 °C. CIM, SIM, SMM: 14-h PP. CWFT. 2000-2500 lux. 28 ± 2 °C. 60-70% RH	Epicotyledonary nodes (explants, 2.65 cm long) subcultured every 4-5 w, forming 3 shoots/explant in 98% of nodes in 4-5 w. Roots formed in 4-6 w. Hardening for 2 w at 30 ± 2 °C. 50% RH. NR Acclimatisation in vermiculite + sand + gravel soil (1 : 1 : 1) after treatment with carbendazim (0.1%, 5 min) and a rinse with distilled water (grown under same conditions as *in vitro* for 2 w). % survival NR.	Jat et al. 2014
Same as Nekkala (2013)	Node → RTW 30 min → 10% liquid detergent 10 min → RTW duration (NR) → 0.1% $HgCl_2$ 1-2 min → 3-4X SDW	MS + 2.5 mg·l^{-1} meta-topolin + 50 mg·l^{-1} adenine sulphate + 0.1 mg·l^{-1} NAA or MS + 2.5 mg·l^{-1} TDZ + 50 mg·l^{-1} adenine sulphate + 0.1 mg·l^{-1} NAA (SIM). Subcultured on MS + 0.5 mg·l^{-1} BA + 50 mg·l^{-1} adenine sulphate (SMM). ½ MS + 2 mg·l^{-1} IAA (RIM). pH 5.8. 3% sucrose. 0.8% agar.	16-h PP. light source and intensity (NR). 25 ± 2 °C	75% nodal explants show axillary shoot multiplication on SIM1 and 79% axillary shoot multiplication observed in SIM2 but all shoots were hyperhydric in SIM2. During second subculture 18.63 shoots/node with 4.84 cm shoot length. 58% shoots were rooted with 2.43 roots/shoot. Acclimatisation NR.	Joshi 2014
AUFWc008 and AUFWc025	Seeds → RTW 10 min → 2X distilled water→ soaked in distilled water overnight → 70% EtOH 3 min → SDW → 0.1% $HgCl_2$ 30 sec → 3X SDW. Nodes from *in vitro* germinated seedlings.	MS or ½ MS + 2% sucrose (SG). MS + 4.44 µM BA + 2.32 µM Kn (SIM)	Walizadeh & Walizadeh (2009)	5.66 shoots/node AUFWc008 and 5.33 shoots/node AUFWc025 within 20d. Rooting of *in vitro* raised shoots and acclimatisation (NR).	Thamarai 2014

2,4-D, 2,4-dichlorophenoxyacetic acid; B_5 medium, or Gamborg medium (Gamborg et al. 1968); BA, N^6-benzyladenine, is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva (2012a); CC, choline chloride; cef, cefotaxime; CIM, callus induction medium; CWFT, cold white fluorescent tubes; d, day(s); EtOH, ethyl alcohol (ethanol); IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kan, kanamycin; Kin, kinetin (6-furfuryl aminopurine); mo, month; MS, Murashige and Skoog (1962) medium; NaOCl, sodium hypochlorite; NOA, β-naphthoxyacetic acid; NR, not reported in the study; PCR, polymerase chain reaction; PG, phloroglucinol; PP, photoperiod; RAPD, random amplified polymorphic DNA; RH, relative humidity; RIM, root induction medium; RP-HPLC, reverse-phase high-performance liquid chromatography; rpm, revolutions per minute; RT, room temperature; RTW, running tap water; SDW, sterilised (by autoclaving) distilled water; SEM, shoot elongation medium; SG, seed germination; SIM, shoot induction medium; w, week(s);
* Even though calli was used in the original, the term callus has been used here based on recommendation of Teixeira da Silva (2012b).
** The original light intensity reported in each study has been represented since the conversion of lux to µmol·m^{-2}·s^{-1} is different for different illumination (main ones represented): for fluorescent lamps, 1 µmol·m^{-2}·s^{-1} = 80 lux; the sun, 1 µmol·m^{-2}·s^{-1} = 55.6 lux; high voltage sodium lamp, 1 µmol·m^{-2}·s^{-1} = 71.4 lux (Thimijan & Heins 1983).

In order to produce withanolide A in root cultures as an alternative method to the destructive harvesting of roots, Abouzid et al. (2010) established liquid root cultures based on IBA-supplemented MS medium using seedling-derived roots as explants. One proposal for future experiments can be the use of exogenously applied PG in order to increase root production (Teixeira da Silva et al. 2013).

There are very limited studies that have employed molecular techniques to assess any aspect of *W. coagulans* research. For example, Jain et al. (2009, 2011) used random amplified polymorphic DNA to assess deviations from true-to-type plantlets derived from *in vitro* culture and mother plants. Kushwaha et al. (2013) characterized a tropinone reductase in *W. coagulans* using leaves from *in vitro* plantlets according to protocols established by Jain et al. (2009, 2011) and Mishra et al. (2013). The study by Mishra et al. (2013) was the first study on an enzyme involved in the secondary metabolism in this medicinal plant. Mirjalili et al. (2011) capitalized upon the ability of hairy root cultures, induced by *Agrobacterium rhizogenes*-mediated transformation, to increase the production of triterpenoids (phytosterols and withanolides) through the overexpression of the squalene synthase gene. Mishra et al. (2013) established the first *Agrobacterium tumefaciens*-mediated genetic transformation protocol for *W. coagulans* in which leaves of *in vitro* plantlets were used to introduce marker (*gus*A) and selector (*npt*II, *hpt*II) genes.

Synthetic seed technology, which involves the encapsulation of *in vitro* or *in vivo* generated explants in an alginate bead, is an efficient system that supports the multiplication, storage and exchange of germplasm having traits of choice that are difficult to propagate via traditional approaches (Sharma et al., 2013; Gantait et al., 2015). Rathore & Kheni (2015) recently reported the alginate encapsulation of microcuttings (shoot tip and nodal segments) with 3.0% sodium alginate and 100 mM calcium chloride. Microshoot encapsulates were regenerated (96%) on 0.75% agar-gelled MS medium containing 1.11 µM BAP and 0.57 µM IAA. Pulse-treatment of the base of microcuttings with 2.46 mM IBA for 2 min was essential for conversion of beads into plantlets, and more than 95% of the encapsulated microcuttings produced shoots and roots within 30 d.

Conclusion and future perspectives

The tissue culture and biotechnology of *W. coagulans*, a valuable medicinal plant, remain unexplored relative to the more famous *W. somnifera*. The focus of future studies to improve the research objectives of this plant species is: induction of somatic embryogenesis and use of somatic embryos in bioreactors, the use of somatic embryos and other propagules, increased production of withanolide or withaferin, the most important biologically active compounds of this plant; the induction of organs using a wide range of plant growth regulators (e.g. Teixeira da Silva 2014; Teixeira da Silva et al. 2014) and testing the use of different gelling agents, abiotic growth conditions, additives and even biotic interactions (e.g. arbuscular mycorrhizal fungi) in order to improve *in vitro* productivity and secondary metabolite production. For the better understanding of developmental regulatory events, investigation of thin cell layers could be useful (Teixeira da Silva 2014; Teixeira da Silva et al. 2014) while *in vitro* breeding could advance much more quickly if flowers could be artificially induced *in vitro* (Teixeira da Silva 2014; Teixeira da Silva et al. 2014, 2015), allowing for the reproductive tissue to be available on demand throughout the year. The biotechnology of this plant must be accompanied with an increased environmental regulation that suppresses illegal gathering and destructive exploitation of this plant species from nature, while also seeking ways to introduce genetically variable *in vitro*-derived clones back into wild, protected environments.

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