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Clonal propagation of *Trichocentrum stramineum* (Orchidaceae), a threatened species endemic to Mexico

Propagación clonal de *Trichocentrum stramineum* (Orchidaceae), una especie amenazada endémica de México

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Abstract
Background: Cloning techniques are applied to an endangered orchid species in order to reproduce individual plants and to preserve their genetic characteristics.
Objectives: To establish a new protocol for clonal propagation of a threatened orchid of horticultural importance.
Study species: *Trichocentrum stramineum*, a threatened orchid endemic to Mexico.
Study site and duration: Totutla, Veracruz, Mexico. All experiments were designed and carried out at the Botanical Garden-UNAM and the IIAF-UMSNH over a course of six years.
Methods: Seeds were germinated in a modified KC basal medium; protocorms and apical bud explants were obtained from the resulting *in vitro* plants and cultivated with or without plant growth regulators (PGRs). Both experimental groups were subcultured in order to evaluate the number of protocorm-like bodies (PLBs) and buds per explant.
Results: On average, protocorms generated 51.2 and 54.1 PLBs in the absence or presence of 1 mg l^{-1} 6-benzyladenine (BA), respectively, while 13.1 and up to 23.7 PLBs and / or shoots were observed on the apical bud explants in the absence or presence of 1 mg l^{-1} kinetin, respectively. In both cases, responses were direct, without the formation of an intervening callus. Approximately 200 PLBs were subcultured and developed into whole plants within 14 weeks. These were acclimatized to greenhouse conditions with a 90 % survival rate after 12 weeks. After 44 weeks, flowering was observed (3 %) individuals measuring at least 12 cm in height.
Conclusions: The developed protocol proved to hold great potential for commercial propagation and conservation programs.
Keywords: Apical bud, Flowering, PLB, Protocorm.

Resumen
Antecedentes: Las técnicas de clonación se utilizan para reproducir orquídeas en peligro de extinción, manteniendo las características genéticas de cada individuo a conservar.
Objetivos: Establecer un nuevo protocolo para la propagación clonal de una planta con importancia hortícola.
Especie de estudio: *Trichocentrum stramineum*, orquídea endémica y amenazada de México
Sitio y años de estudio: Totutla, Veracruz**.** El estudio duró 6 años, desarrollado en el Jardín Botánico UNAM y el IIAF, UMSNH.
Métodos: Las semillas germinaron en un medio basal KC modificado; se tomaron los protocormos y explantes de yemas apicales de las plantas *in vitro* resultantes y se cultivaron con o sin reguladores del crecimiento vegetal (PGR). Ambos se subcultivaron, evaluando el número de cuerpos similares a protocormos (PLB) y brotes unidos a cada explante.
Resultados: Los protocormos generaron en promedio 51.2 y 54.1 PLB en ausencia o presencia de 1 mg l^{-1} benciladenina respectivamente, mientras que se observaron 13.1 y hasta 23.7 PLB y / o brotes en los explantes de yema apical en ausencia o presencia de 1 mg l^{-1} de cinetina, en ese orden, ambas respuestas fueron directas sin presencia de callo. Aproximadamente 200 PLBs fueron subcultivados y desarrollados en plantas enteras en 14 semanas. Estas se aclimataron a condiciones de invernadero con una tasa de supervivencia del 90 % después de 12 semanas. Después de 44 semanas, se observó floración (3 %) individuos que medían al menos 12 cm de altura.
Conclusiones: El protocolo desarrollado demuestra su potencial en programas comerciales de propagación y conservación.
Palabras clave: Floración, PLB, Protocormo, Yema apical.

With 23,314 species, almost half of them being endemic, Mexico ranks fourth in biodiversity of vascular plants by country (Villaseñor 2016). The Orchidaceae family is one of the four best represented families in the country and comprehends 1,254 reported species, 35 % of which are endemic (Soto-Arenas *et al*. 2007a, b). Unfortunately, most of them are at risk of extinction due to land use change and collecting of wild plants, a fact that is even more alarming in the case of species with restricted distribution and low population density. *Trichocentrum stramineum* (Lindl.) Chase & Williams, syn *Lophiaris straminea*: *Oreja de burro* (donkey ear), is a threatened endemic species (SEMARNAT 2010, Remolina 2015) and listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2017). Its distribution is limited to the tropical deciduous, oak, and pine-oak forests of the Mexican states of Veracruz and Puebla (Vovides *et al*. 1997, Espejo-Serna *et al*. 2005). The factors that put it at risk of extinction are the same that affect most orchids (Espejo-Serna *et al*. 2005); *e.g.,* the introduction of exotic timber species has caused many problems in this regard (Valdiviezo & Castillo 2011). Especially bamboos (*Bambusa* spp., *Gradua* spp. and *Phyllostachis* spp.) have shown to contribute significantly to the displacement of native species that occur in the distribution area of *T. stramineum*, due to the former's ability to spread rapidly and widely for a number of reasons: Bamboos can propagate themselves by asexual reproduction and, with their C4 carbon fixation pathway and dense stem growth, tend to be well-adapted to cloud forests. In some areas, the expansion of these species has gone out of control, invading the surrounding ravines at an ever higher rate. Although the plant is difficult to grow by traditional methods (Remolina 2015), *T. stramineum* is a species of horticultural interest (Espejo-Serna *et al*. 2005).

When cloning techniques are used to reproduce plants for commercial purposes, it is important that the selected genotype can be massively propagated in a short period of time without compromising the desired characteristics (George 1993, Arditti 2008). Cloning also occurs naturally in wild plants and is sometimes more efficient than sexual propagation, *e.g.*, favorable genotypes that contribute descendants by both sexual and asexual reproduction may perpetuate their genes within a population for generations (Gentry 1982, Nobel 1985, 1992). In the case of populations facing imminent extinction, represented by a few unique genotypes only, repeated small scale clonal multiplication can be the last resort to avoid vanishing of the former (Mistretta 1994, Martínez-Palacios *et al*. 2016). An efficient cloning method distinguishes itself by simple and short-term cultivation processes that promote direct morphogenic responses. It is also essential to keep plant growth regulator (PGR) concentrations low or not to add them at all to the culture media, in order to decrease the probability of irregular cell divisions or mutations (and thus genetic variation) during cultivation (George 1993, Martínez-Palacios *et al*. 2003, 2016). Plant tissue culture (PTC) is a widely used technique for mass propagation and can make use of regeneration pathways that either maintain the genetic stability of crops or promote genetic instability (George 1993); *e.g.*, the addition of PGRs can be avoided by cultivating plant tissues and organs in liquid media under continuous agitation (Mehrotra *et al*. 2007), which frequently results in the disruption of apical dominance and the subsequent induction and proliferation of numerous axillary buds. Furthermore, this technique provides appropriate levels of aeration and nutrients to the explants (Park *et al*. 2002, Mehrotra *et al*. 2007).

Genetic changes can occur in *in vitro* cultures at the cellular and / or molecular level, in the latter case including specific DNA mutations (Radić *et al*. 2005). Various factors, such as the genotype and age, as well as the culture and explant type, influence the incidence of somaclonal variation. Changes concerning chromosome number and structure are often related to ploidy levels (Evans 1989, Radić *et al*. 2005).

A crop's specific propensity for somaclonal variation can be indicated by the effects that different types and concentrations of PGRs have on the morphogenic response or dedifferentiation of the respective *in vitro* cultures, particularly when taking into account the number of subcultures necessary to induce these phenomena (Bairu *et al*. 2006, 2011, Vujović *et al*. 2010, Miguel & Marum 2011). For instance, high concentrations of the cytokinin benzyl amino purine (BAP) have been reported to induce somaclonal variation in plant tissue cultures established from several species belonging to the genera *Fragaria* (Biswas *et al*. 2009), *Musa* (Bidabadi *et al*. 2010), and *Saccharum* (Singh *et al*. 2008).

Environmental stress, such as high temperatures, can cause *in vitro* grown plant cells to attack their own plastids with antibiotic-like compounds or to undergo chromosome doubling. Doubled haploidy, for instance, may result in the formation of albino plants in the case of several important cereal crops (Torp & Andersen 2009).

Somaclonal variation can also be induced by subculturing PTCs over prolonged periods of time, as has been demonstrated previously in pineapple (*Ananas comosus* (L.) Merr; da Silva *et al*. 2016). In other species like chestnut rose (*Rosa roxburghii* Tratt), however, Random Amplification of Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) analyses proved that *in vitro* propagated shoots are able to maintain genetic stability even after 25 subculture cycles (Wen & Deng 2005). This suggests that subculturing on itself does not cause somaclonal variation, but may do so in combination

with other factors, some of which have already been mentioned above.

In contrast, the use of explants obtained from apical meristems or immature plant tissues and organs facilitates the production of genetically stable regenerants (Williams & Maheswaran 1986, Balachandran *et al.* 1990, George 1993, Rani & Raina 2000, Martins *et al*. 2004), as the former are mainly composed of totipotential undifferentiated or meristematic cells, respectively (Williams & Maheswaran 1986, George 1993). Clonal multiplication has been successfully applied to ginger sprouts (Hosoki & Sagawa 1977, Balachandran *et al.* 1990, Rout *et al.* 2001), and RAPD, Simple Sequence Repetition (SSR), and / or Inter Simple Sequence Repetition (ISSR) analyses have shown the genetic stability of micropropagated poplar plants (Rani *et al*. 1995), somatic embryos of *Psidium guajava* L. (Rai *et al.* 2012) and different fir species (Isabel *et al.* 1996), as well as of *Saccharum officinarum* L. seedlings (Pandey *et al.* 2012) and *Prunus dulcis* (Mill.) DA Webb. shoots (Martins *et al.* 2004) produced by direct organogenesis or axillary branching, respectively.

The addition of organic extracts (coconut water and banana pulp, among others) to the culture medium favors the *in vitro* development of orchid plants (Ernst 1967, Morel 1974, Aktar *et al*. 2008). The use of activated charcoal can also have beneficial effects, because it retains toxic elements generated by tissue, organ, and whole plant cultures in a time-dependent manner (Morel 1974). With regard to the orchids, the development from seeds to flowering plants is a slow process that can take eight years of cultivation (Morel 1974). The phenological stages of this development have been described in the case of the *in vitro* asymbiotic germination of *T. stramineum*, and germination rates of up to 47.69 % have been reported (Flores-Escobar *et al*. 2008). Methods for the *in vitro* culture of this species in organic media have also been published, but the adaptation of the micropropagated plants to soil conditions and their subsequent development has not been covered yet (Flores-Escobar *et al*. 2008). Therefore, the objective of the present work was to establish a protocol for the clonal propagation of *T. stramineum*, a threatened species endemic to Mexico, by inducing the formation of multiple PLBs on protocorms, and of PLBs and shoots on apical bud explants, respectively, as well as to describe the propagated plants' development under *in vitro* and greenhouse conditions.

Materials and methods

Biological material. Trichocentrum stramineum seeds were obtained from three mature fruits taken from different plants in a tropical deciduous forest near the Conejos-Huatusco highway, locality of Mata Oscura, Municipality of Totutla, Veracruz, Mexico. Voucher specimens were deposited at the Herbarium of the Autonomous University of Tamaulipas (voucher number UAT MA2019). Apical buds were obtained from *in vitro*-grown plants.

Study design and conduction: All experiments were designed and carried out at the Botanical Garden-UNAM and the IIAF-UMSNH over a course of six years.

Culture medium. The basal medium was KC-E, a modified KC medium (Knudson 1946) enriched with micronutrients (in $mg \cdot l^{-1}$, 0.056 of H_3BO_3, 0.016 of MoO_3, 0.040 of $CuSO_4$, 0.331 of $ZnSO_4.7H_2O$, 0.083 of KI, 0.0025 of $CoCl_2.6H_2O$), vitamins of the B5 medium (Gamborg *et al.* 1968), 100 $mg \cdot l^{-1}$ of myo-inositol, 20 $g \cdot l^{-1}$ of sucrose, and 8 $g \cdot l^{-1}$ of agar-agar (SIGMA A-1296). After adjusting the medium's pH to 5.0, it was sterilized in an autoclave at a pressure of 1.5 $kg \cdot cm^{-2}$ for 15 minutes.

Asymbiotic seed germination. The seeds were disinfected in a solution of 7 % calcium hypochlorite for 30 min, and then rinsed thrice with sterile distilled water. Between 200 and 300 seeds were suspended in 0.5 ml of sterile distilled water and dispersed on the surface of 15 ml of solid KC-E medium in 100 × 15 mm Petri dishes. The number of germinated seeds was recorded twenty days later with a stereo microscope ZEISS Stemi 200-C. Increase in size (measured with a vernier scale), green coloring, rupture of the seed coat, and initiation of the protocorm stage were all considered signs of germination. The experimental group consisted of six repetitions, and the corresponding germination rates were indicated as average values. Leftover disinfected seeds were sown in 250 ml jars containing 40 ml of basal medium in order to facilitate their germination. All cultures were incubated at 26 ± 1 °C and exposed to a 16 h light / 8h darkness photoperiod and at photosynthetic photon flux density (PPFD) of 60 µmol m^{-2} s^{-1}, during 30 days.

Cultivation of protocorm and apical bud explants in liquid medium under orbital agitation at 100 rpm. Thirty days after germinating, protocorms with a morphology characteristic of development stage 2 (diameter of 1 mm; spherical shape; absent or incipient apical buds) were subcultivated in liquid KC-E medium, in the absence or presence of 1 $mg \cdot l^{-1}$ of 6-benzyladenine (BA). In either case, approximately 0.5 g of protocorms were transferred to 250 ml Erlenmeyer flasks containing 80 ml of medium. The cultures were maintained for eight weeks under the following conditions: continuous orbital shaking at 100 rpm; temperature of 27 ± 1 °C; 16 light/ 8h darkness photoperiod (PPFD 20 µmol m^{-2} s^{-1}) provided by 40 W fluorescent white light tubes.

Apical bud explants measuring between 1 and 3 mm in length were obtained from *in vitro*-grown plants, and cultivated in three different liquid KC-E media: One did not contain any PGRs, while the other two were enriched with 1 $mg \cdot l^{-1}$ of kinetin (Kin) or 2 $mg \cdot l^{-1}$ of Kin and 1 $mg \cdot l^{-1}$ of 2, 4-dichlorophenoxyacetic acid (2,4-D), respectively. Each repetition consisted of ten explants in 125 ml Erlenmeyer flasks filled with 25 ml of liquid medium and was maintained for 16 weeks under the conditions described for orbital shaking.

PLB and shoot formation in solid medium. Some of the protocorms developed under agitation in liquid KC-E media with and without BA were randomly selected for subculturing in solid KC-E medium without PGRs. Each protocorm was placed in a 125 ml jar containing 30 ml of medium, and each treatment consisted of 12 repetitions. The cultures were maintained at 27 ± 1 °C and a 16 h photoperiod (PPFD: 60 µmol m^{-2} s^{-1}) for eight weeks.

Subculture of PLBs and shoots derived from protocorm and/or apical bud explants, and their in vitro development into whole plants. Protocorms, PLBs, and shoots originated from asymbiotic germination or from protocorm and apical bud explants under orbital agitation, respectively, were subcultured individually in jars (13.2 × 7.6 cm) containing 80 ml solid KC-E medium enriched with 20 % coconut water and 1 $g \cdot l^{-1}$ of activated carbon, under the above mentioned conditions. Apical bud explants cultivated in liquid medium under orbital agitation were subcultured identically, with 20 repetitions per treatment. After 14 weeks, they had developed into whole plants with at least four leaves and three roots.

Acclimation of regenerated whole plants to greenhouse conditions. The whole plants originated from the protocorm-derived PLBs were extracted from the jars, washed under running water, and planted in circular trays (40 cm in diameter and 18 cm in height, with 10 mm perforations in the bottom to facilitate drainage) filled with a bottom layer of basalt rock (2 to 3 cm in diameter) and a top layer (4 cm in height) of moss substrate (*Thuidium* sp. and *Hypnum* sp.). The trays were watered between 2 and 3 times a day for 12 weeks, and then once a day for the remaining time. The propagation process of *T. stramineum* is described in Figure 1.

Statistical analysis. Statistical Analysis Software (SAS 1999) was used to evaluate the study results. Data was normalized with PROC STANDARD (z score; mean = 0, std = 1). Significant differences between treatments were determined by Student's t-test ($P = 0.95$) in the case of the induction of PLB formation on protocorms cultivated in liquid medium with and without BA, and by ANOVA ($R^2 = 0.150026$, $P = < 0.0001$*) in the case of the response of the apical buds.

Results

Seed germination. Notwithstanding that first indicators of asymbiotic seed germination were observed nine days after initiation of the cultures, the number of germinated seeds was only registered after 20 days. An average germination rate of 87.9 % was determined in solid KC-E medium (pH 5).

Diameter increase of protocorms and shoot induction in stem apex explants exposed to liquid medium under constant orbital agitation. After eight weeks of cultivation in liquid medium under orbital agitation, the protocorms obtained by asymbiotic seed germination showed diameter increases between 3 and 9 mm (relative to the initial 1 mm). Although the medium darkened over time, the protocorms presented a hard consistency, a light-yellow color, and a spherical shape (Figure 2B and C). Occasionally, either aborted shoots (Figure 2C above) or small protuberances (Figure 2C below) formed on the surface of the protocorms. The protuberances could develop into PLBs and originate new protocorms when separated from their respective explants.

While only a few apical bud explants developed PLBs, it was common to observe direct shoot formation on the axillary buds of the former during the 14 weeks of cultivation in liquid medium. Explants grown in basal KC-E medium produced an average of 13.1 shoots, whereas the addition of Kin resulted in a significantly higher number of shoots (up to 23.7 per explant; Table 1). When transferred to solid medium, the regenerated shoots continued to develop satisfactorily into whole plants.

PLB formation on protocorm explants and development of apical bud explant-derived shoots in solid medium. In all cases, direct PLB formation was observed on the surface of the protocorms. The number of PLBs per explant varied from 14 to 108, and similar average values were determined for protocorms previously cultivated in the absence or presence of BA (51.2 and 54.1 PLBs, respectively). The PLBs soon acquired an intense green color (Figure 2D) and could be separated from the protocorm explants with a spatula. Interestingly, it became evident that the PLBs had partly absorbed the tissue of the protocorm explants and that they were not connected with each other. They eventually developed into plantlets and shoots (Figure 2D-F).

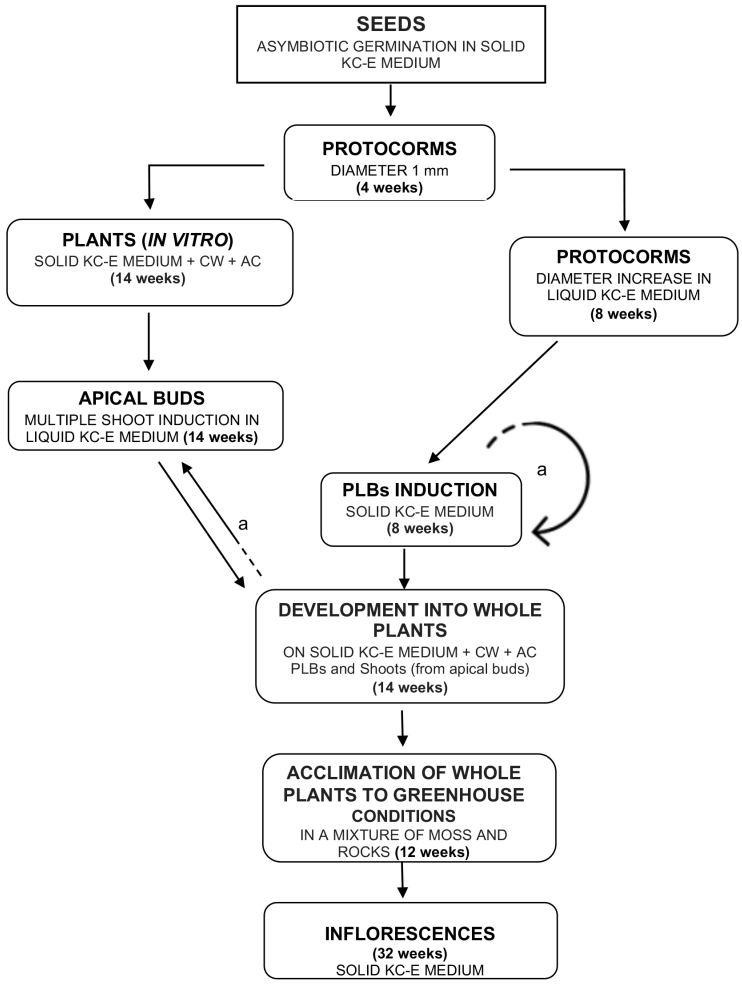

Figure 1. Workflow diagram for clonal propagation of *Trichocentrum stramineum*. [a]: *In vitro* potential for mass propagation of horticultural interest. CW: 20 % coconut water; AC: activated carbon 1 $g \cdot l^{-1}$; PGR: plant growth regulator.

Since the protocorm explants previously cultivated in BA-containing liquid medium did not show statistically significant differences (P = 0.7195) with reference to diameter increase and PLB formation in comparison to the control group, this PGR was not added to the solid basal KC-E medium.

In vitro development of protocorms, PLBs, and shoots into whole plants. The protocorms, as well as the regenerated PLBs and shoots originated whole plants following a normal development pattern, devoid of callus formation, hyperhydric symptoms, or further PLB generation. The roots and leaves of the *in vitro* whole plants did not present morphological abnormalities (Figure 2G). It should be noted that only the PGR-free experimental groups were monitored at this stage, and it can thus not be ruled out that the use of certain PGRs could induce somaclonal variation in any of the mentioned *in vitro* cultures and structures.

Acclimation of regenerated whole plants to greenhouse conditions. After 12 weeks of having been transplanted to non-sterile substrate under greenhouse conditions, 90 % of the regenerated whole plants were still alive. Morphological abnormalities were not observed at all, and sizes ranged from 4 to 12 cm in height (Figure 2H). The leaves were elliptic, thick, and reddish-green due to the presence of red pigments. They measured between 10 and 15 cm in length and between 2.7 and 3.5 cm in width. After 44 weeks in the greenhouse, 3 % of the plants that had grown to a height of at least 12 cm under *in vitro* conditions, developed inflorescences of 12 to 15 flowers whose sizes (diameter of 12 to 15 mm), shapes, and colors (off-white or ivory-colored sepals and petals with a yellow spot at the base; yellow red-spotted trilobed lips with a white middle part and red dots at its base) were similar to those characteristic of wild plants (Figure 2I). Flowering under greenhouse conditions in Mexico City took place in March.

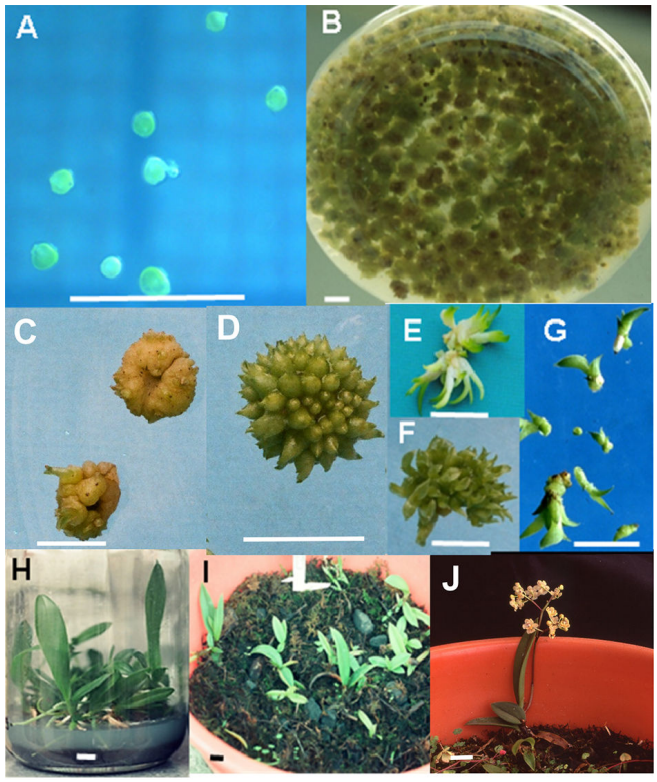

Figure 2. Clonal propagation of *Trichocentrum stramineum* in liquid culture medium under orbital agitation. A) Protocorms in development stage 2, 30 days after initiating the cultures; B) Protocorm explants after eight weeks in liquid medium under orbital agitation at 100 rpm; C) Protocorm with first signs of PLB formation, after eight weeks in liquid medium; D) PLBs formed on protocorm cultivated for eight weeks on solid medium; E) Regenerated shoots obtained from the apical bud explants cultivated in liquid medium; F) Regenerated shoots obtained from the protocorm explants cultivated on solid medium for eight weeks; G) PLBs and plantlets separated from their respective explants; H) *in vitro* whole plants derived from regenerated PLBs, after 12 weeks of cultivation on solid medium; I) Whole plants acclimated to greenhouse conditions, 32 weeks after initiating the *in vitro* cultures; J) Flowering plant after 44 weeks in the greenhouse. Scale bar = 10 mm

Discussion

Seed germination. In this work, the asymbiotic germination rate of *T. stramineum* seeds exposed to solid KC-E medium proved to be significantly higher than the reported 47.69 % of seeds that germinated on MS medium according to a previous study (Flores-Escobar *et al.* 2008). This difference may be due to the particular high concentrations of salts in the latter medium (Bell *et al.* 2009), which can exert inhibitory effects on seed germination (Rodríguez *et al.* 2014), alter *in vitro* development, or even induce tissue necrosis (George 1993, Cassells & Curry 2001). With respect to the pH of the medium, the optimum values for germination of orchid seeds range from 4.8 to 5.2, with an extended interval of 3.6 to 7.6 (Arditti & Ernst 1984).

The relatively low concentrations of mineral salts and inorganic nitrogen contained in KC (pH 5.0) and VW media (pH 5.2) promoted germination in species belonging to the *Paphiopedilum* genus, an effect not observed in the case of quarter- or half-strength MS medium (pH 5.8; Long *et al.* 2010). Johnson & Kane (2007) also reported higher germination frequencies in *Vanda* hybrid seeds when exposed to KC medium. This stimulating effect on the germination of orchid species may be associated with the medium's higher calcium concentration (4.23 mM) in comparison to the VW (1.94 mM) and MS media (quarter-strength: 0.75 mM; half-strength: 1.5 mM). Furthermore, in contrast to most other plant groups, many orchids grow on fairly acid soils.

Diameter increase of protocorms and shoot induction in stem apex explants exposed to liquid medium under constant orbital agitation. The protocorms increased considerably in diameter, reaching values of up to ten times their initial size. In this regard, it should be pointed out that diameter increases of plant tissues grown in liquid media under orbital agitation are often associated with the disruption of apical dominance (Mehrotra *et al.* 2007), and that the effective use of this technique is in agreement with other studies that have applied similar culture conditions to different types of explants and orchid species, such as leaf segments of *Phalaenopsis* spp. (Park *et al.* 2002) and nodal segments of *Dendrobium candidum* (Cui *et al.* 2015).

Table 1. Effect of PGRs on shoot induction in *T. stramineum* apical bud explants cultivated in liquid media under orbital agitation.

Treatment	*n*	Shoots/explant (average)	Tukey	*e.e.*
0 (control)	13	13.1	b	2.0
1 mg·l^{-1} (Kin)	16	23.7	a	1.8
2-1 mg·l^{-1} (Kin-2,4-D)	16	18.2	ab	1.8

Kin: Kinetin; 2,4-D: 2,4-dichlorophenoxyacetic acid; $P = 0.001$

As high concentrations of cytokinins like BA can interfere with cell division and may induce somaclonal variation in some species (Bairu *et al*. 2006), the effect that this PGR might have on the induction of PLBs in protocorm cultures was not evaluated. Interestingly, in *in vitro* cultures of *Turbinicarpus valdezianus* (Möeller) Glass & R.A. Foster (Martínez-Palacios *et al*. 2016), the above mentioned side effects did occur when the same species was exposed to BA (Dávila-Figueroa *et al*. 2005). Whenever use of these compounds is essential for propagation purposes, their concentrations should therefore be kept as low as possible, and multiplication periods be reduced (Martínez-Palacios *et al*. 2003, 2016).

PLB formation on protocorm explants and development of apical bud explant-derived shoots in solid medium. In the case of orchids, direct shoot induction on stem apex explants has only been registered a few times (Rubluo *et al*. 1993, Martin & Madassery 2006), the vast majority of publications concerning the *in vitro* regeneration of these plants reports the direct or indirect formation of PLBs (Zhao *et al*. 2008, Khoddamzadeh *et al*. 2011, Niknejad *et al*. 2011). The direct induction of PLBs and shoots on protocorms and stem apex explants, respectively, implies morphogenetic responses that are among the most stable in PTC (George 1993, Rubluo *et al*. 1993, Martins *et al*. 2004, Bhattacharyya *et al*. 2014).

Furthermore, our results suggest that PLB formation was caused by disruption of apical dominance and subsequent diameter increase in the protocorm explants, both consequences of exposing the latter to orbital shaking in liquid medium. The low number of PLBs generated on apical buds may be due to the fact that the longest explants (3 mm) floated on the liquid medium without being able to rotate freely in all directions. In consequence, unlike the protocorm explants, they did not increase in diameter. However, the culture conditions allowed for the emergence of shoots from the axillary buds that are naturally present in stem apices.

In vitro development of protocorms, PLBs, and shoots into whole plants. In agreement with previous publications, the enrichment of the basal growth media with coconut water and activated carbon favored the *in vitro* development of the orchids, (Morel 1974, Martínez-Palacios 1991, Rubluo *et al*. 1993, Aktar *et al*. 2008). The fact that 20 % of the regenerated *T. stramineum* whole plants reached at least 12 cm in height (Figure 2G) may be attributed in part to this factor.

Acclimation of regenerated whole plants to greenhouse conditions. Despite the fact that micropropagated plantlets tend to be fragile and may not be sufficiently vigorous to survive the acclimatization shock resulting from transplanting them from *in vitro* to soil substrates (Jeon *et al*. 2005), the survival rate of *T. stramineum* plantlets was high. The first days after transplanting are crucial, and conditions should mimic those of the original *in vitro* cultures, providing dim light and high relative humidity. The plantlets can then gradually be subjected to higher stress levels in order to harden them off and prepare them for normal plant development under greenhouse or outdoor conditions (Rubluo *et al*. 1993, Jeon *et al*. 2005, Cha-um *et al*. 2010).

Considerations on mass propagation for commercial use. The protocol described in this study showed great potential for the mass propagation of *T. stramineum*. Assuming regeneration rates of 51.2 PLBs per protocorm and 13.1 shoots per apical bud explant after 18 weeks of cultivation in PGR-free liquid medium under orbital agitation, one protocorm could originate 134,218 PLBs in a period of 54 weeks (three cycles). For comparison, species of *Phalaenopsis* can be propagated at a rate of 18,000 PLB per 20 g of inoculum (approximately 1000 PLB sections) using 2 liters of medium and a cultivation time of eight weeks (Park *et al*. 2002). The solid KC-E medium enriched with coconut water and activated carbon proved to be appropriate for the development of the regenerated PLBs, shoots, and eventually whole plants. Particularly the occurrence of flower formation in less than a year suggests that commercial scale-up of our protocol could lead to the development of farming systems for *T. stramineum*, similar to those already existing for other orchid species, such as *Phalaenopsis* spp. (Tokuhara & Mii 1993, Wang 2000). Regarding morphology and flowering period, our results obtained from greenhouse-grown *T. stramineum* plants coincide with development patterns previously reported for natural populations of the species (Jiménez 1990).

Our protocol minimizes loss of genetic stability of the regenerated plants by inducing direct responses without the use of PGRs and limiting cultivation periods and cycles to a minimum (George 1993, Pandey *et al.* 2012). It could therefore also be applied to preservation programs, *e.g.*, selected genotypes of threatened or genetically erode *T. stramineum* populations may be propagated following the methods described herein, thus ensuring normal plant development and avoiding somaclonal variation. A good example for the establishment of a cloning system for conservation purposes of small populations has been given by Mistretta (1994): Several individuals could be clonally propagated from a wild population of *Cercocarpus traskiae* Eastw., represented by only seven unique genotypes, and were successfully reintroduced to their native habitat.

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