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Somatic Embryogenesis of Tuberose (*Agave amica* L.) was Improved by Milk as a Potential Biostimulant in Plant Tissue Culture

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ABSTRACT

An efficient *in vitro* method was established for indirect somatic emb ryogenesis of tuberose from pedicel-derived calluses on MS medium. The effects of 2,4-D, NAA, BAP, and ABA on callus induction, callus growth, embryogenic callus formation, embryo maturation, and plant let regeneration were evaluated. A combination of 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA resulted in the highest percentage of callus induction (100%) and callus quality. The highest average of callus growth was achieved at 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP. Embryogenic calluses were induced on the media containing 1 mg L^{-1} 2,4-D after 90 days of subculturing. The highest number of matured somatic embryos perpetri dish (81) and callus greening were observed on the medium containing 1 mg L^{-1} ABA + 45 g L^{-1} maltose. Further germination of embryos was observed on MS medium containing 10 to 15 ml L^{-1} goat colostrum and embryos with leaves regenerated after 90 days. In addition to the induction of osmotic pressure, colostrum is a rich source of organic nitrogen and calcium which play key roles in somatic embryogenesis. The whole plantlets were achieved after 60 days of subculturing on the media containing IBA (0.75 mg L^{-1}) with a survival rate of 88%. In conclusion, the present study provides a suitable somatic embryogenesis system that may serve in micropropa -gation, genetic transformation, and ploidy manipulation of tuberose.

Introduction

The *genus Agave* L. (Asparagaceae) is endemic to the Americas and comprises more than 200 species that are of great economic importance (Castañeda-Nava et al., 2019). One of the popular ornamental and aromatic plants in this genus is tuberose (*Agave amica* L.), formerly named "*Polianthes tuberosa*", which is endemic to Mexico. Tuberose is cultivated for use in manufacturing as a source of fragrant essences in cosmetic and perfumery products. Moreover, due to a long vase life and intense perfume, the long spikes of tuberose flowers with sweet fragrance are used in floral arrangements such as floral bouquets and table decorations (Barba-Gonzalez et al., 2012).

The flower color of all known cultivars of *A. amica* is white and thus flower color manipulation is the main breeding program in this species. Due to the limited range of flower colors among tuberose species, conventional cross-breeding has not been able to introduce new flower colors *to A. amica*. In such a situation, molecular breeding

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techniques and genetic engineering should be used for the successful introduction of foreign genes and subsequent new genetic traits, such as new colors, into germplasms (Azria and Bhalla, 2011). Setting up a protocol for successful *in vitro* plant regeneration is a critical prerequisite for the application of biotechnological approaches in molecular breeding. Moreover, successful *in vitro* plant regeneration in horticulture is a key step for micropropagation. In a case of relevant research, *in vitro*-produced leaf blades were used for the genetic transformation of *A. salmiana*. Transformed plantlets were regenerated via indirect somatic embryogenesis on the embryogenic calluses co-cultivated with *Agrobacterium tumefaciens* (Flores-Benítez et al. 2007). Also, *A. tequilana*, the most widely cultivated species of agave in México, persists under severe environmental stress. Thus, researchers have focused on the genetic improvement and micropropagation of this species (Rodríguez-Garay, 2016).

A thorough literature review reveals that some methods have been used to perform tissue culture and *in vitro* plant regeneration of tuberose. Micropropagation through direct (Gajbhiye et al., 2011; Naz et al., 2012) and indirect (Nalousi et al., 2019) *in vitro* shoot organogenesis has been reported. Abdullah (2012) reported somatic embryogenesis in *P. tuberosa*. Callus induction and plant regeneration in tuberose were examined by Nuzhat et al. (2000) and Jala et al. (2014). Several types of explants have been used in this regard including bulb (Naz et al., 2012; Nalousi et al., 2019), leaf (Abdullah, 2012), stem (Abdullah, 2012; Gajbhiye et al., 2011; Surendranath et al., 2016), flower buds (Abdullah, 2012; Surendranath et al., 2016), and axillary buds (Jala et al., 2014; Surendranath et al., 2016).

Somatic embryogenesis is a process with several advantages over regeneration by organogenesis, such as improvements in the *in vitro* multiplication rate of ornamental geophytes and the probable single-cell origin, thus avoiding chimeras in the regenerated plants (Skirvin et al., 1993). Beside micropropagation purposes, callus induction and indirect somatic embryogenesis are very crucial for the production of plant material after plant transformation via agrobacterium-mediation or gene gun (Yuan et al., 2013). On the other hand, some disadvantages have been reported for somatic embryogenesis. For example, success in the production of somatic embryos is a very genotype-dependent process. Moreover, the germination rate of somatic embryos is low (Rout et al., 2006). Thus, enhancing the conversion of somatic embryos to plantlets is a crucial step in the tissue-culture of many plant species. Somatic embryogenesis in the genus *Agave* was firstly reported on *A. victoria-reginae* using leaf explants (Rodríguez-Garay et al. 1996). Direct somatic embryos were induced on MS medium containing 2,4-D.

In cases where a defined medium fails to fulfill growth requirements, a natural complex with undefined composition may be used to support the growth and regeneration of *in vitro* cultured plant tissues (Bansal and Gokhale, 2012). These additives may contain different concentrations of amino acids, peptides, carbohydrates, vitamins, growth promoters, and minerals (Molnár et al., 2011). Casein hydrolysate, coconut water, yeast extract, malt extract, potato extract, banana homogenate, tomato juice, and immature maize seed extract are among the most applied natural amendments in plant tissue culture media. Nambiar et al. (2012) studied the proliferation rate of protocorm-like bodies of a *Dendrobium* hybrid in the presence of homogenates of banana, tomato, and coconut water. With a four-fold increase in fresh weight, compared to the initial weight in four weeks, coconut water was found to be the best organic supplement. Milk could also be an animal-based biostimulant for better plant growth. It is rich in essential proteins and other compounds that are good for plant growth (Dawood et al., 2019). However, to the best of our knowledge, there is no scientific report on the application of milk in plant tissue culture.

To achieve molecular breeding and micropropagation in tuberose, a reliable and efficient plantlet regeneration system needs to be established first. Therefore, the present work reports a protocol for callus induction and indirect somatic embryogenesis of tuberose. This protocol may serve as the first step in the establishment of flower color manipulation of tuberose via genetic engineering. Moreover, the pathway introduced here can be used for micropropagation of this valuable ornamental plant.

Material and Methods

Culture media and explant preparation

Murashige and Skoog (MS 1962) basal media with 30 g L^{-1} sucrose and 6.0 g L^{-1} agar were used for all treatments. Based on the developmental stage, different combinations and concentrations of plant growth regulators (PGRs) were added. To complete embryo germination, goat colostrum was added to culture media as a novel organic additive.

Well-developed long spikes of donor plants were harvested and used as explant sources. Florets

were detached from spikes and washed under running tap water. Detached florets were surface-disinfected by immersion in 70% (v/v) ethanol for 30 s, followed by immersion in 10% sodium hypochlorite solution (using commercial bleach at 5.25% of active chloride) for 5 min and then rinsed three times with sterile distilled water. Pedicels of disinfected florets were cut and used as initial explants (Fig. 1A).

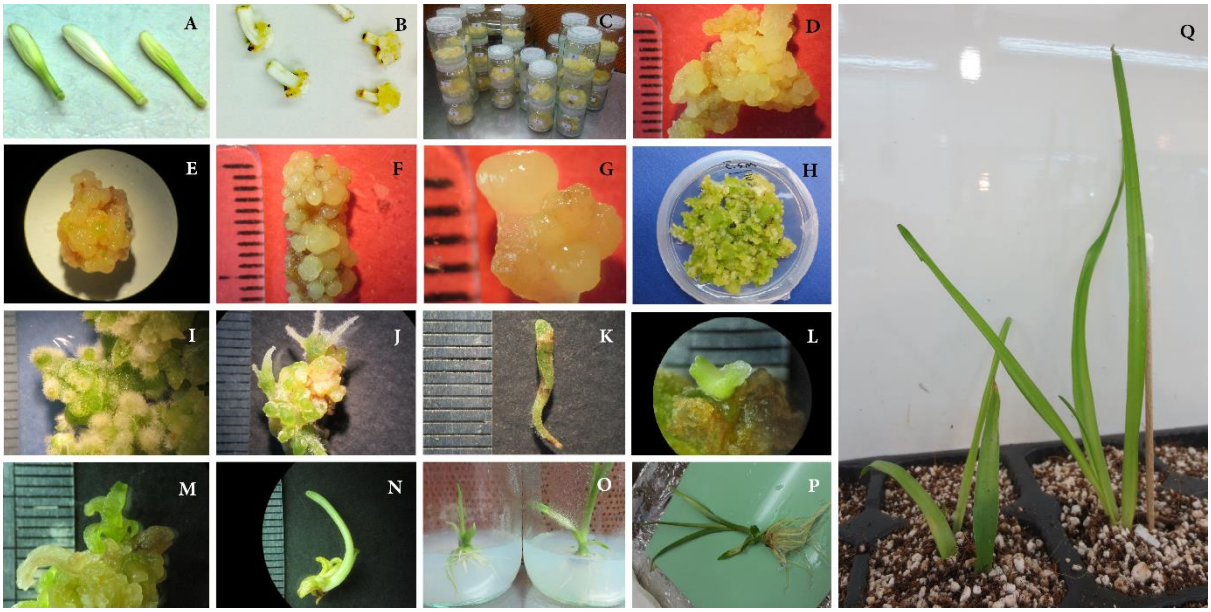

Fig. 1. Embryogenic callus induction and plantlet regeneration of tuberose (*Agave amica* L.). a) The portion of the pedicel used as the initial explant is shown in a circle. b) Swelling of the explants and callus initiation at the cut edges of the pedicels were observed on MS medium containing 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA after 10 and 35 days of cultures, respectively. c) Proliferation of creamy to yellowish and compact calluses after around 120 days of *in vitro* culture on MS medium containing 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP. d and e) Production of a nodular and friable callus after 90 days in the presence of 1 mg L^{-1} 2,4-D. f) Polyembryogenic masses with well-formed globular embryoids 60 days on media containing 1 mg L^{-1} 2,4-D. g) A single globular embryoid and its basal conjunction to callus. h-j) Callus greening (the formation of green dots which will germinate in the next stages), development of embryos, and formation of root/shoot axis on MS medium supplemented with 1 mg L^{-1} ABA and 45 g L^{-1} maltose. k) A somatic embryo with shoot and root apical meristems. l and m) An embryo being germinated with leaves regenerated on MS medium supplemented with 15 ml L^{-1} goat colostrum after 90 days of subculturing. n) Germinated somatic embryo with shoot and root obtained on the medium containing 15 ml L^{-1} goat colostrum. o) Simultaneously shoot/root elongation and bulb formation on MS medium supplemented with 0.75 mg L^{-1} IBA within 60 days of culture. p) A whole regenerated plantlet from somatic embryo ready for acclimatization. q) Acclimatized *in vitro* plantlets in plug trays.

Tuberose callus induction and growth

Cut pedicels were placed on 9 cm diameter petri dishes (4 per one) filled with 20 ml of different callus induction media and sealed with Parafilm®. The effects of different combinations and concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and benzylaminopurine (BAP) were evaluated for callus induction and growth. Selected treatments for callus induction and callus mass increment were 2,4-D (0 or 0.5 mg L-1) + NAA (0, 0.5, 1, or 1.5 mg L-1) + BAP (0 or 0.5 mg L-1) and 2,4-D (0.5, 1, or 1.5 mg L-1) + BA (0.5 or 1 mg L-1), respectively. The cultures were incubated in the dark at 27 ± 1°C. After 35 days of incubation, the percentage of callus induction (PCI), callus quality (CQ by visual scoring), or no reaction of explants (NR) were recorded.

For visual scoring, calluses were classified as follows:

1) Color (scale of 1 through 3): 1 = white to pale yellow callus / 3 = creamy to yellowish callus (Fig. 2A, B).
2) Freshness (scale of 1 through 3): 1 = dry and necrotic callus / 3 = translucent and fresh callus (Fig. 2C, D).
3) Friability (scale of 1 through 3): 1 = non-friable callus that must be divided by scalpel / 3 = friable callus that was easily spread by forceps (Fig. 2E, F).
4) Compactness (scale of 1 through 3): 1 = dispersed callus / 3 = compact callus

(Fig. 2G, H)
Grades 1, 2, and 3 represent calluses of the poor, moderate, and excellent quality, respectively.
For further growth, calluses with grade 3 were cultured on the solid MS media containing 0.5 or 1 mg L^{-1} 2,4-D + 0.5, 1, or 1.5 mg L^{-1} BAP. Cultures were incubated at 27 ± 1°C with a daily 16-h illumination regimen of 40 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by white fluorescent lights and transferred every four weeks. After 120 days of proliferation under light, callus growth (cm^2) was measured.

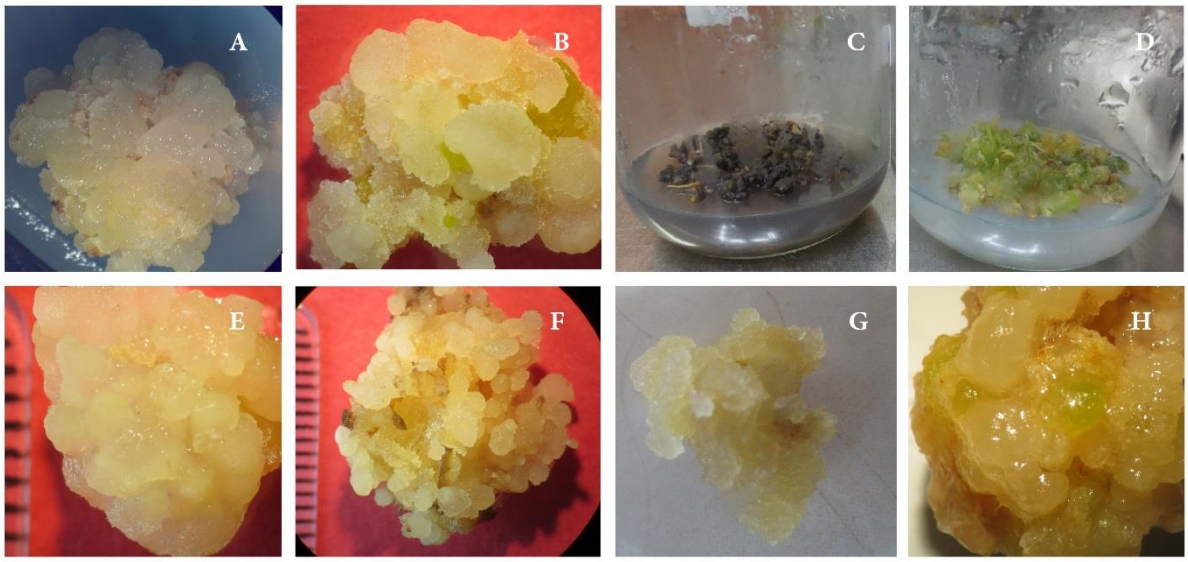

Fig. 2. The rating of callus quality with relative visual scores based on color (a and b), freshness (c and d), friability (e and f), and compactness (g and h) of callus tissue. Scores 1 and 3 represent calluses of poor and excellent quality, respectively. A) White to pale yellow callus (score 1). B) Creamy to yellowish callus (score 3). C) Dry and necrotic callus (score 1). D) Translucent and fresh callus (score 3). E) Non-friable callus that must be divided by scalpel (score 1). F) Friable callus (score 3). G) Dispersed, watery and smooth callus (score 1). H) Compact callus (score 3).

Stabilization of embryogenic callus

Proliferated calluses were subcultured on MS media containing 0, 0.5, 1, or 1.5 mg L^{-1} 2,4-D to acquire embryogenesis potential. After 90 days (3 subcultures), nodular and friable calluses were obtained in some cultures. In the next stage, 1 mg L^{-1} 2,4-D was used to produce polyembryogenic masses.

Development and germination of somatic embryos

After initiation of polyembryogenic masses in the presence of 2,4-D, calluses with plenty of embryoids were transferred to new petri dishes containing 20 ml of MS basal salts supplemented with 3 levels of ABA (0, 0.5, or 1 mg L^{-1}) and different concentrations of maltose (0, 30, 45, or 60 g L^{-1}) plus 30 g L^{-1} sucrose as the additional carbon source. To develop somatic embryos, the callus cultures were incubated at 27 ±1 °C in 16 h light: 8 h dark photoperiod. For visual scoring of greening, the calluses were classified into 3 scores, i.e. poor, moderate, and excellent.
Further conversion of embryos to plantlets was studied on the solid MS medium supplemented with 5 levels (0, 5, 10, 15, or 20 ml L^{-1}) of goat colostrum. Colostrum was added to culture media using a membrane filter with a pore-size of 0.45 microns. Physiochemical properties of goat colostrum used in this study are presented in Table 1.
In callus induction and somatic embryo maturation experiments, each treatment included 10 replicates and each replicate contained 4 explants/calluses per culture vessel.

Plantlet growth, hardening, and survival

Germinated bipolar embryos with well-formed shoots and roots were subcultured onto MS medium containing 0.75 mg L-1 IBA + 30 g L^{-1} sucrose to produce bulbs. For acclimatization, regenerates were washed from agar and planted into plug trays containing a 1:1 mixture of coco peat: perlite and maintained in the hardening room (at 26 ± 1°C with a daily 16-h illumination regimen of 40 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by white fluorescent lights). The hardened plants were then cultured in pots containing a 1:1 mixture of coco peat: perlite and

kept under greenhouse conditions (sunlight, 26–28 °C and 65–70% of relative humidity).

Table 1. Physiochemical properties of goat colostrum in this study.

pH	Fat (%)	Total protein (%)	Casein (%)	Water (%)	Total solids (%)
6.34	7.62	10.48	5.21	75.43	24.57

Statistical analysis

A completely randomized design was used for all the experiments. The data collected from the experiments were subjected to ANOVA (analysis of variance) using SPSS 16.0 software with Duncan's multiple range test at the 5% level of significance ($P \leq 0.05$).

The scheme representing implemented experiments including media composition, optimal treatments, culture conditions, and durations for each stage is shown in Table 2.

Table 2. Representation of different stages during somatic embryogenesis of *Agave amica* in this study.

No.	Stages	Description Media composition	Conditions and durations
1	*Culture establishment:* Florets were surface-sterilized.	Florets were immersed in 70% (v/v) ethanol for 30 s, followed by immersion in 10% sodium hypochlorite solution for 5 min and then rinsed three times.	All steps were performed under laminar air flow cabinet.
2	*Callus induction:* Cut pedicles were used as explants.	MS basal medium 2,4-D (0 or 0.5 mg L^{-1}) NAA (0, 0.5, 1, or 1.5 mg L^{-1}) BAP (0 or 0.5 mg L^{-1}) Optimal: 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA	27 ± 1°C Darkness 35 days
3	*Callus growth:* Calluses of the highest quality were selected for further proliferation.	MS basal medium 2,4-D (0.5 or 1 mg L^{-1}) BAP (0.5, 1 or 1.5 mg L^{-1}) Optimal: 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP	27 ± 1°C 16 h light 40 µmol m^{-2} s^{-1} PAR 120 days (4 subcultures)
4	*Stabilization of embryogenic callus:* Nodular and friable embryogenic calluses were initiated.	MS basal medium 2,4-D (0, 0.5 or 1 mg L^{-1}) Optimal: 1 mg L^{-1} 2,4-D	27 ± 1°C 16 h light 40 µmol m^{-2} s^{-1} PAR 90 days (3 subcultures)
5	*Initiation of polyembryogenic masses:* Embryogenic calluses were used.	MS basal medium 1 mg L^{-1} 2,4-D	27 ± 1°C 16 h light 40 µmol m-2 s-1 PAR 60 days (2 subcultures)
6	*Development of somatic embryos:* Calluses with a plenty of embryoids were transferred to new petri dishes.	MS basal medium ABA (0, 0.5, or 1 mg L^{-1}) Maltose (0, 30, 45, or 60 g L^{-1}) 30 g l^{-1} sucrose Optimal: 1 mg L^{-1} ABA + 45 g L^{-1} maltose	27 ±1 °C 16 h light 40 µmol m-2 s-1 PAR 60 days (2 subcultures)
7	*Germination of somatic embryos:* Further conversion of embryos and plantlet regeneration was obtained.	MS basal medium Goat colostrum (0, 5, 10, 15, or 20 ml L^{-1}) Optimal: 15 ml L^{-1}	27 ± 1°C 16 h light 40 µmol m^{-2} s^{-1} PAR 90 days (3 subcultures)
8	*Bulb and root formation:* Germinated bipolar embryos with well-formed shoots and roots were used.	MS basal medium 0.75 mg L^{-1} IBA 30 g L^{-1} sucrose	27 ±1 °C 16 h light 40 µmol m-2 s-1 PAR 60 days (2 subcultures)
9	*Acclimatization:* Regenerates were washed from agar and planted into plug trays	1:1 mixture of coco peat:perlite	26 ±1 °C 16 h light 40 µmol m-2 s-1 PAR 60 days (2 subcultures)
10	*Plantlets culture:* Survived plantlets were transplanted in pots and kept under greenhouse conditions.	1:1 mixture of coco peat:perlite	26–28 °C, Sunlight 65–70% RH

Results

Tuberose callus induction and mass increment

Tuberose pedicels (Fig. 1A) were found to be more amenable explants to callus induction compared to leaf explants. Leaf-induced calluses turned brown and produced necrotic tissues. Inclusion of 2,4-D and NAA in the medium promoted callus production on pedicel explants. Swelling of the explants which is an early symptom of callus induction started after 10 days of culture. By day 35, callus initiation at the cut edges of the pedicels was observed (Fig. 1B). There were significant differences among calluses induced on the media supplemented with different PGRs concerning the percentage of callus induction (PCI) and callus quality (CQ). Combination of 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} NAA resulted in the highest PCI (100%) and CQ (Table 3). The exclusion of 2,4-D dramatically affected callogenesis so that no callus was produced. The inclusion of BAP had a negative effect on callus production as the percentage of callus formation was reduced by almost half.

To increase callus mass, the callus pieces were transferred onto the new MS medium and began to multiply and proliferate rapidly. The combinations of 2,4-D + BAP significantly increased callus mass and the highest rate of callus growth was achieved at 1 mg L^{-1} 2,4-D + 1 mg L^{-1} BAP (Fig. 3). The callus mass diameter doubled in two subcultures and a 5-fold increase was observed around 120 days (4 subcultures) of culture (Fig. 1C).

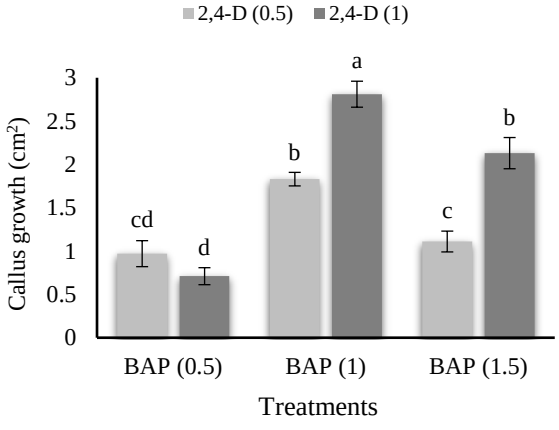

Fig. 3. Interaction effect of different concentrations of 2,4-D and BAP on callus growth of *Agave amica*. Error bars indicate the SE (n=40) and different letters on bars are significantly different at P ≤ 0.05 (Duncan's multiple range test).

Induction of somatic embryos

To induce embryogenic calluses, the pieces of proliferated calluses were transferred onto a new MS medium supplemented with different concentrations of 2,4-D. We found that only the calluses on MS medium containing 1 mg L^{-1} 2,4-D were able to produce nodular, friable, and semi-compact embryogenic calluses after 90 days (Fig. 1D, E). To induce polyembryogenic masses, embryogenic calluses were subcultured on the same media. After 60 days, polyembryogenic clusters with well-formed globular embryoids were observed (Fig. 1F, G).

Development of somatic embryos

For further growth and development of somatic embryos, calluses were subcultured on MS media supplemented with various concentrations of ABA and maltose under the light. Mean separation analysis showed that ABA and maltose had significant impacts on the embryoid development. Callus greening and formation of root-shoot axis are good signs of embryoids development. Using maltose and incubation of cultures under light, green dots were observed on the surface of the embryogenic calluses during the first week of culture. After 4 weeks, the entire surface of the calluses became green. In the next subcultures, green dots exhibited budding and bipolar embryos were obtained (Fig. 1H-K). The highest number of somatic embryos per petri dish (81) with shoot and root apical meristems was observed on the medium containing 1 mg L^{-1} ABA + 45 g L^{-1} maltose after 60 days (Fig. 4). Different concentrations of ABA had no significant effect on the callus greening. However, calluses with excellent greening were observed in the presence of 45 g L^{-1} maltose (Fig. 5).

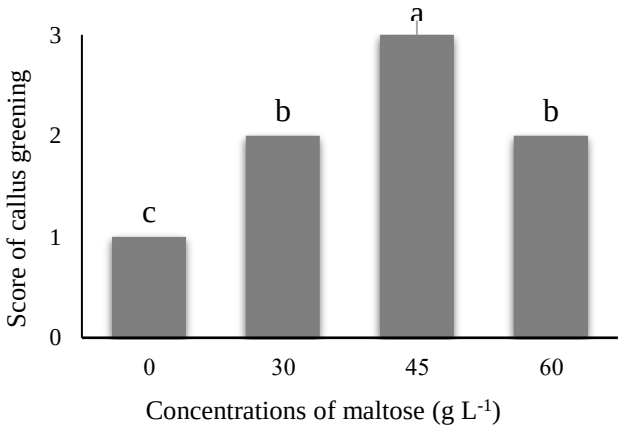

Fig. 4. Effects of different concentrations and combinations of maltose (g L^{-1}) and ABA (mg L^{-1}) on the number of somatic embryos per petri dish in *Agave amica.* Error bars indicate the SE (n=40) and different letters on bars are significantly different at P ≤ 0.05 (Duncan's multiple range test).

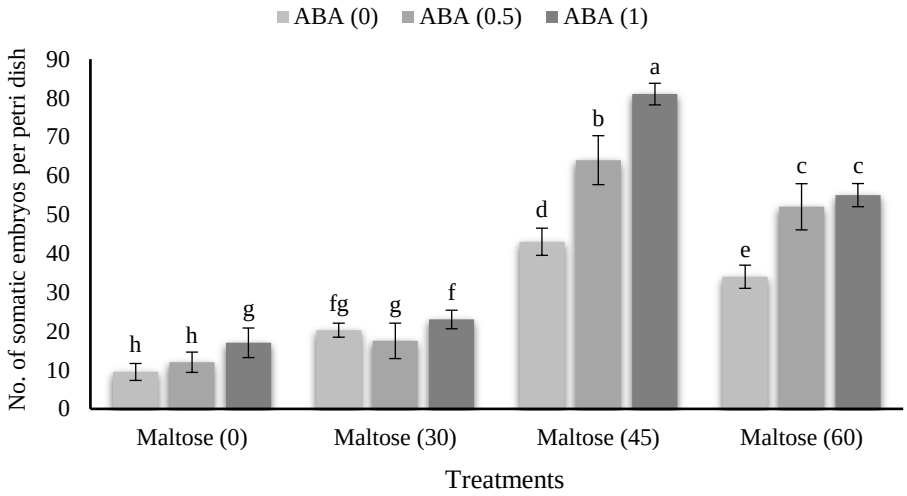

Fig. 5. Effects of different concentrations of maltose on callus greening in *Agave amica*. Scores 1, 2, and 3 represent three levels of callus greening including poor, moderate, and excellent, respectively. Mean values followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan's multiple range test).

Germination of somatic embryos

We realized that bipolar embryos were not able to finish their developmental process and exhibited successful germination. Therefore, we tried to find a beneficial natural additive to improve this stage. Among candidates, we selected goat colostrum as a novel organic additive with rich composition to improve embryo germination. Thus, after maturation of somatic embryos, best germination was only observed on MS medium containing goat colostrum (Fig. 6). The highest number of germinated embryos with regenerated leaves per culture vessel (14) was obtained at the concentration of 15 ml L^{-1} goat colostrum after 90 days of subculturing (Fig. 1L, M). The cultures were kept under illumination on the same medium to grow up and produce roots and shoots (Fig. 1N).

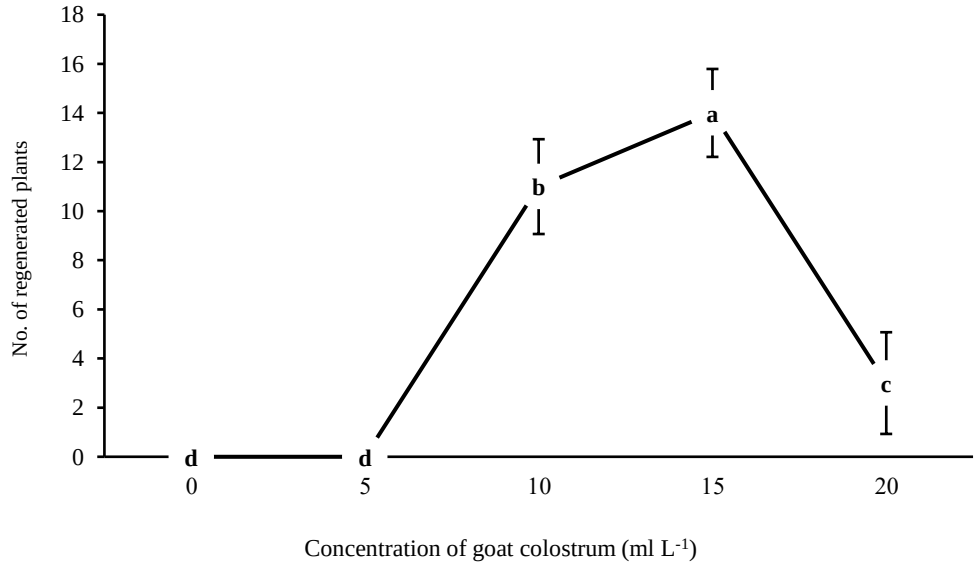

Fig. 6. Effect of different concentrations of goat colostrum on the number of regenerated plantlets in *Agave amica*. Mean values followed by the same letters are not significantly different at $P \leq 0.05$ (Duncan's multiple range test).

Table 3. Effects of different concentrations and combinations of plant growth regulators on the percentage of callus induction (PCI) and callus quality (CQ) using pedicel explants of *Agave amica.*

2,4-D (mg L^{-1})	NAA (mg L^{-1})	BAP (mg L^{-1})			
		0		0.5	
		PCI	CQ	PCI	CQ
0	0	0 d	NR	0 d	NR
	0.5	0 d	NR	0 d	NR
	1	0 d	NR	0 d	NR
	1.5	0 d	NR	0 d	NR
0.5	0	31 cd	+	31 cd	++
	0.5	100 a	+++	56 bc	++
	1	75 b	++	56 bc	++
	1.5	81 ab	++	69 b	+

Mean values followed by the same letters are not significantly different at the 5% level (Duncan's multiple range test). Values are data after arcsine transformation because the records were percentage. Signs +, ++, and +++ represent callus quality of grade 1 (poor), 2 (moderate), and 3 (excellent), respectively.

Plantlet growth, hardening, and survival

After 60 days of culturing germinated embryos, plantlets with 2-3 leaves and a well-developed root system were regenerated. During this culture, bulbs started to form at the base of the shoots on the medium containing 0.75 mg L-1 IBA (Fig. 1O). The whole regenerated plantlets (with shoot, bulb, and root) were subjected to the acclimatization process (Fig. 1P). Finally, a survival rate of 88% was observed (Fig. 1Q).

Discussion

Callus formation and subsequent organ regeneration or somatic embryogenesis are one of the best methods for mass propagation and production of plant materials after genetic transformation which is a useful technique for improvements and breeding of many crops. However, the efficiency of *in vitro* organogenesis is still low in many plant species. Embryogenic callus formation has a great potential to encourage somatic cells to form embryos developing into new plants and therefore overcomes this shortcoming. In this work, an efficient plantlet regeneration pathway via indirect somatic embryogenesis was established for tuberose.

Explant type is one of the most important factors affecting *in vitro* regeneration of plants. We found that pedicels exhibited better results compared to leaf explants. Literature review shows that inflorescence parts are among the most amenable explants for the production of high-quality calluses (Ascough et al., 2009). Kahrizi et al. (2008) reported that pedicel was the best explant for embryogenic callus induction in tuberose. Moreover, Hernández-Mendoza et al. (2015) showed that flower bud segments were suitable for massive *in vitro* regeneration of *Polianthes tuberosa*. They indicated that the decontamination problem of bulbs decreased their efficiency as a source of explant. The origin of callus determines its type, which in turn determines the profile of gene expression and subsequent regeneration pathway (Iwase et al., 2011). Stirn et al. (1995) reported that two embryo-related genes are expressed in embryogenic calluses of barley. Thus, the pattern of gene expression in calluses derived from floral parts may be more suitable for the induction and development of somatic embryoids.

Pedicels cultured on 2,4-D-free media were not able to produce callus. We found that 2,4-D possesses a crucial role in tuberose callus induction. Many cases of research proved that 2,4-D is a suitable auxin for somatic embryogenesis in the genus *Agave*. Martínez-Palacios et al. (2003) produced indirect somatic embryos using stem segments of *A. victoria-reginae* seedlings in the MS medium supplemented with 0.5 mg L^{-1} 2,4,-D. Nikam et al. (2003) reported that the most effective medium to induce indirect somatic embryos of A. sisalana contained 0.25 mg L^{-1} 2,4-D. Portillo et al. (2007) indicated that 2,4-D (1-2 mg L^{-1}) was the best auxin for somatic embryogenesis in *A. tequilana*. In a relevant research, embryogenic calluses of *A. vera-cruz* were produced on the MS medium containing 1 mg L^{-1} 2,4-D and 1 mg L^{-1} NAA (Tejavathi et al., 2007). Auxins are among the most widely used plant growth regulators for callus formation. Some auxin-induced transcription factors participate in callus induction by inducing the transcription of genes required for DNA replication (Inzé and De Veylder, 2006; Okushima et al., 2007). In addition to activation of core cell regulators, auxins downregulate cell cycle inhibitors and helps callus formation. Moreover, we realized that 2,4-D was essential to produce nodular, friable, semi-compact embryogenic calluses and subsequent induction of polyembryogenic masses. In fact, 2,4-D improved

the induction of *in vitro* unorganized cell clusters and resulted in polyembryogenic masses which are aggregates of organized cells and will continue to be produced on a 2,4-D medium.

The induction of somatic embryos usually requires an auxin, in particular 2,4-D, while the development stage is inhibited by auxins and is often enhanced by ABA. In our work, the inclusion of ABA was necessary for the maturation of somatic embryos. However, Reyes-Díaz et al. (2017) showed that ABA did not improve embryogenesis response in *Agave angustifolia*. It seems that the efficiency of ABA in the maturation of somatic embryos is completely genotype-dependent. Fidalgo et al. (2005) reported that 0.25 mg L-1 ABA improved embryo maturation and germination of *Iris hollandica* Hort. 'Bronze Queen'. In contrast, Kim et al. (2009) found that ABA had no significant effect on the maturation of *I. pseudacorus* somatic embryos. Although Verma et al. (2016) reported that ABA enhanced somatic embryogenesis in Turkish crocus species, Taheri-Dehkordi et al. (2020) indicated that ABA was not necessary for indirect somatic embryogenesis of *Crocus sativus* L., *C. caspius*, and *C. speciosus*. However, the maturation of somatic embryos by ABA occurred through different pathways. For example, the accumulation of reserve substances in somatic embryos is encouraged by ABA. The expression of genes involved in the biosynthesis of late embryogenesis abundant (LEA) proteins during somatic embryogenesis is usually regulated by ABA and osmotic stress (Garcia-Martin et al., 2005). Moreover, ABA promotes synchronized germination of somatic embryos (Stasolla et al., 2003).

We realized that a suitable carbohydrate at a high concentration could improve somatic embryogenesis of tuberose. In fact, maltose significantly promoted callus greening under the effect of light. Other works also show that maltose significantly induces the highest percentage of callus greening in *Phyllostachys heterocycla* var. *pubescens* (Yuan et al., 2013) and *Freesia hybrida* (Pourkhaloee and Khosh-Khui, 2015). Ruvalcaba-Ruíz (2003) produced somatic embryos of *A. tequilana* on a medium containing 90 g L^{-1} maltose. Tejavathi et al. (2007) found that 40 g L^{-1} sucrose was essential for the conversion of *A. vera-cruz* somatic embryos to plantlets. In *A. angustifolia*, somatic embryos were achieved under osmotic conditions induced by 60 g L^{-1} sucrose. Plantlets were obtained 140 days after the beginning of the experiment (Arzate-Fernández and Mejía-Franco 2011). Besides providing energy and carbon source, carbohydrates are involved in the regulation of osmotic conditions in tissue culture. Carbon source plays a key role in callus greening as a good sign of bud (shoot apical meristem of embryoids) formation in embryogenic calluses. On the other hand, osmoregulators mimic the water stress conditions which occurred during the late stages of seed maturation on plants. Stasolla et al. (2003) reported that in white spruce the transcript levels of genes involved in the control of shoot and root apical meristems and formation of the embryo architecture increased in immature PEG-treated embryos. Moreover, in fully matured PEG-treated embryos the transcript levels of genes involved in nitrogen metabolism (assimilation of NH_4^+) were higher than in the control. Therefore, the type and concentration of carbohydrates are two main factors for the maturation of somatic embryos.

In the present work, the conversion of somatic embryos to plantlets failed on different media. Therefore, we attempted to find a new additive to improve the conversion rate. We found that the application of goat colostrum (i.e. the initial milk produced in the mammalian glands during parturition and the first few days after birth) in the culture medium had a dramatic impact on the germination of somatic embryos. This effect can be related to a rich composition of colostrum. On the other hand, due to its high density, the colostrum could provide osmotic conditions as a promoting factor for the conversion of somatic embryos in the plantlets. In a relevant research, the protein contents in the first days of colostrum and mature milk production by goats were reportedly 14.18 and 3.44% (w/v), respectively (Rashid et al., 2012). Kehoe et al. (2007) reported that the mean concentration of calcium in colostrum was approximately 4-folds higher than its concentration in milk. Glutamine and its counterpart, glutamic acid, are the most abundant amino acids in milk protein (Meijer et al., 1993). L-glutamine provides a situation like natural embryogenesis and thus is a suitable nitrogen source for the maturation of somatic embryos (Tejavathi et al., 2007). Moreover, casein is a phosphoprotein found abundantly in most mammalian milk which is an excellent source of amino nitrogen and free amino acids. L-glutamine and casein hydrolysate are natural additives that have been widely used in plant tissue culture. Ruvalcaba-Ruíz (2003) produced somatic embryos of *A. tequilana* on the medium supplemented with 300 mg L^{-1} casein hydrolysate. For *A. tequilana* genotypes, MS medium supplemented with 500 mg L^{-1} L-glutamine and 250 mg L^{-1} casein hydrolysate was suitable for the maturation of somatic embryos (Portillo et al., 2007). Delgado-Aceves et al. (2021) used 500 mg L^{-1} glutamine and 250 mg L-

1 casein hydrolysate to induce somatic embryos on calluses of *A. tequilana* Weber 'Chato'.

Milk and its counterparts (like milk whey) have been used as a biostimulant and biofertilizer in agriculture. In fact, milk contains proteins rich in nitrogen, minerals, and calcium which have been proved to enhance nutrient uptake and plant growth. In a relevant research, perennial ryegrass plants were foliar sprayed with diluted raw milk and significantly produced greater above-ground biomass compared to non-treated plants (Hilshey, 2014). Dawood et al. (2019) reported that foliar application of milk whey significantly enhanced seed yield of flax (*Linum usitatissimum* L.) cultivars. Haroun and Ibrahim (2003) found that milk whey could enhance photosynthesis, total carbohydrates, and total nitrogen content in wheat. Therefore, it could be concluded that goat colostrum contains all growth-promoting compounds that were mentioned above and, thus, plays a role as a biostimulant in tissue culture of tuberose, thereby promoting somatic embryo conversion into plantlets.

Conclusion

In using inflorescence segments, we developed an efficient system for somatic embryogenesis in tuberose (*Agave amica* L.). The presence of 2,4-D was necessary for callogenesis and induction of embryogenic calluses. Exogenous ABA and maltose were essential for the maturation of somatic embryos. We discovered that the application of milk (goat colostrum) completed the conversion of somatic embryos into plantlets. This impact could be attributed to the rich composition of milk as previously reported because milk could be a biostimulant in agriculture. The present work provides a reliable somatic embryogenesis system that may be used for *in vitro* propagation and for the breeding of tuberose.

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Conflict of interest

The authors indicate no conflict of interest for this work.

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