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SHORT PROTOCOL COMMUNICATION

Induction of somatic embryogenesis and ectopic proliferation in *Tecoma stans* (L.) Juss. ex Kunth cell suspension culture

Rowida Omar[1] · Ivan Kushkevych[2] · Mohamed Abd El-Salam[1,3]

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Abstract
Somatic embryogenesis is a developmental pathway where somatic cells of plants generate embryogenic cells that subsequently mature into somatic embryos under favorable conditions. This process is one of the most important *in vitro* techniques for plant propagation, with diverse practical implications. In this study, ectopic proliferation and somatic embryos from *Tecoma stans* (L.) Juss. ex Kunth cell cultures were induced by employing primary conditioning Murashige and Skoog medium supplemented with 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid. Subsequently, a secondary induction medium supplemented with a combination of 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid with various concentrations of 6-benzyladenine cytokinin (1 to 5 mg L^{-1}) was used to promote embryogenesis. The results revealed the successful formation of pre-embryonic and embryonic stages, including globular, heart, torpedo, and cotyledon stages within a 2-wk incubation period under the specified hormonal conditions, leading to subsequent development into the mature vegetative phase after an additional 4 wk. Significant embryo production (16 ± 2.0 torpedo stage embryos per 50 mL culture media) was observed in Murashige and Skoog medium enriched with 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 2.0 mg L^{-1} 6-benzyladenine, surpassing the results observed with other concentrations (p-value < 0.0001). The generated somatic embryos can serve as a potential *in vitro* tool for the propagation, generation, and organogenesis of *T. stans*, contributing to its role as both an ornamental and medicinal plant. Moreover, the induction of somatic embryogenesis opens avenues for the potential production of *T. stans* bioactive secondary metabolites and diverse applications in biotechnology, biotransformation, and biocatalysis, particularly in the conversion of both exogenous and endogenous substrates, such as tecomine—the principal antidiabetic alkaloid in the leaf extract.

Keywords *Tecoma stans* · Somatic embryogenesis · Plant cell culture · Plant regeneration · Organogenesis

Introduction

Somatic embryogenesis is the formation of embryos in tissue culture from vegetative tissue. It holds paramount significance in the realm of plant biotechnology due to its versatile applications in crop improvement, propagation, and conservation of genetic resources (Egertsdotter *et al*. 2019; Bandyopadhyay *et al*. 2022). It offers a controlled and efficient method for the mass production of genetically identical plants, providing an alternative to conventional seed propagation. Additionally, somatic embryogenesis plays a pivotal role in the preservation of endangered or economically valuable plant species (Bandyopadhyay *et al*. 2022; Castander-Olarieta *et al*. 2022).

The pathway is a distinctive biological process in plants through which embryogenesis occurs from a somatic cell, and development of techniques allows for various applications (Spinoso-Castillo and Bello-Bello 2022). It offers a controlled and efficient method for generating somatic embryos from plant cells, ultimately leading to the development of entire plantlets (Castander-Olarieta *et al*. 2022). This intricate process is regulated by numerous external

✉ Ivan Kushkevych
kushkevych@mail.muni.cz

Mohamed Abd El-Salam
mohamed.abdelsalam@deltauniv.edu.eg

1 Department of Pharmacognosy, Faculty of Pharmacy, Delta University for Science and Technology, Gamasa 11152, Egypt

2 Department of Experimental Biology, Faculty of Science, Masaryk University, 62500 Brno, Czech Republic

3 School of Pharmacy and Biomolecular Sciences, Royal College of Surgeons in Ireland, Dublin D02 VN51, Ireland

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and internal stimuli (Karami, *et al*. 2009; Spinoso-Castillo and Bello-Bello 2022). In plant tissue culture, somatic embryogenesis is a process wherein embryonic cells develop from an explant, resulting in the generation of somatic embryos that can subsequently germinate and develop into entire plantlets (Desai *et al*. 2022; Muguerza *et al*. 2022). The induction of somatic embryogenesis is significantly influenced by plant growth regulators, such as auxins (JimÉNez 2001; Wójcik *et al*. 2020). It is considered an important tool for medicinal plant cloning and the production of bioactive compounds.

Tecoma stans (L.) Juss. Ex Kunth (Bignoniaceae) is a promising medicinal plant native to the drier habitats of North America and the high-altitude regions of South America. It is a flowering perennial shrub naturalized in tropical and subtropical areas of Africa, Asia, and Oceanica (Anand and Basavaraju 2021). The plant is traditionally used for the control of diabetes (Shapiro and Gong 2002; Alonso-Castro *et al*. 2010; Anand and Basavaraju 2021) and in alleviating various ailments (Anand and Basavaraju 2021; Morales-Ferra *et al*. 2022). The leaves are commonly used in traditional Mexican medicine for urinary tract and gastrointestinal disorders (Anand and Basavaraju 2021), jaundice, flu, skin infections, and snake bites (Anand and Basavaraju 2021). Current research focused on the biological screening of its bioactive phytochemicals and therapeutic potential. Tecomine is one of the major therapeutically bioactive alkaloids that was isolated and identified to exhibit antidiabetic effect (Hammouda and Motawi 1959; Hammouda *et al*. 1963; Hammouda *et al*. 1964; Hammouda and Amer 1966). Other diverse secondary metabolites, such as glycosides, flavonoids, saponins and tannins, quinones, and mono- and tri-terpenes, were also identified in the plant extracts (Anand and Basavaraju 2021).

In the present study, the aim was to optimize the induction conditions of somatic embryogenesis in *T. stans* cell suspension culture. By delving into the intricacies of this process, the aim was also to contribute valuable insights that can potentially enhance the efficiency of *in vitro* propagation methods for *T. stans*. The induction of somatic embryogenesis in *T. stans* represents a pivotal exploration to harness the unique capabilities of this biological pathway for the benefit of medicinal plant propagation and potential bioactive compound production. This method holds immense promise as it allows the production of plantlets with desired traits, contributing to the conservation and sustainable utilization of this valuable medicinal plant. Furthermore, the success of this methodology could pave the way for enhancing the potential production of bioactive compounds present in *T. stans*, including the well-studied alkaloid tecomine and other secondary metabolites. This may open avenues for the development of advanced biotechnological approaches to support the cultivation and utilization of *T. stans* for medicinal purposes. To the best of our knowledge, there are no prior reports on the somatic embryogenesis and cell culture in *T. stans*.

Material and Methods

Tecoma stans (L.) Juss. ex Kunth leaves were collected and identified at the Botanical Garden, Faculty of Science, University of Alexandria, Egypt. Voucher samples were preserved at the Department of Pharmacognosy, Faculty of Pharmacy, Delta University for Science and Technology, and University of Alexandria, Egypt. Murashige and Skoog (MS) basal medium (Caisson Laboratories, Smithfield, UT, CAT# MSP09), sucrose (Sigma-Aldrich, St. Louis, MO, CAT#S9378), and agar (Sigma-Aldrich, Burlington, MA, CAT #A7921) were used for cell culture. Plant growth regulator solutions, including 6-benzyladenine (BA) (Sigma Chemical Company, St. Louis, MO, CAT#63,178) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Eastman Kodak Chemical Company, Rochester, NY, CAT#14,650), were prepared and stored at 4 °C until needed. Sodium hypochlorite 5% solution (Chlorox) Cairo, Egypt and Tween 80 (Sigma-Aldrish, Burlington, MA, CAT# P4780) were used for the explant surface sterilization. All other chemicals and solvents were of analytical grade.

Static Culture Induction Callus cultures of *T. stans* were induced according to our previously reported method (Abd El-Salam *et al*. 2015) with some modification. Briefly, the leaves were exposed to surface sterilization using a 1.25% (v/v) sodium hypochlorite solution and 0.1% Tween 80 for 15 min. Subsequently, leaves were rinsed three times with sterile distilled water and dried by blotting on sterile filter paper. The pH of the MS medium was adjusted to 6.0 before autoclaving at 121 °C and 15 psi for 20 min. Agar (0.9% w/v)-based MS medium with sucrose (30.0 g L^{-1}) was supplemented with 1.0 mg L^{-1} 2,4-D and used to establish and grow static cultures. The leaves, nodes, and internodes of *T. stans* were scored on their dorsal sides with a sterile scalpel blade and cut into pieces, about 1.0 cm^3 size. The explants were placed onto 9-cm Petri dishes containing 20 mL of MS static medium supplemented with 1.0 mg L^{-1} 2,4-D and incubated for a duration of 4 wk, during which the growth of callus was continuously monitored.

Initiation of Cell Suspension Culture and Somatic Embryogenesis Induction Cell suspension cultures were initiated by transferring 2.0 g of fresh weight from 4-wk-old formed callus into 100 mL of MS liquid medium supplemented with 1.0 mg L^{-1} of BA. The cultures were incubated under light at 23 °C $\pm$ 1.0 °C on a gyratory shaker at 100 rpm for a duration of 4 wk. Then, the cell suspension was sub-cultured on MS liquid media (50 mL) supplemented with varying concentration ratios of a mixture containing 1.0 mg L^{-1} 2,4-D and 1.0 to 5.0 mg L^{-1} BA for the induction of somatic embryogenesis. The suspension cultures continued to grow under the previously mentioned conditions for an additional 4 wk. During this

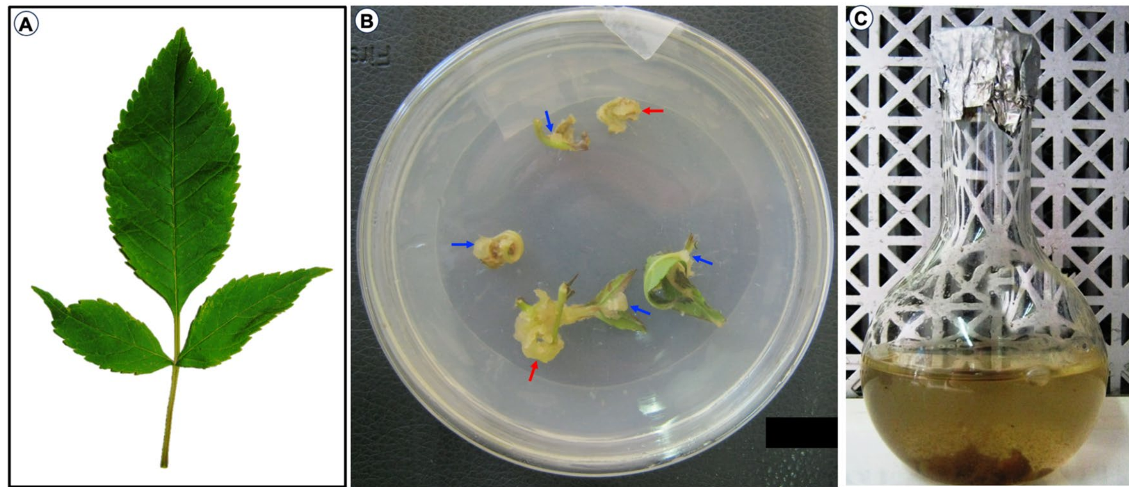

Figure 1. Tissue culture of *Tecoma stans* (L.) Juss. ex Kunth. (*A*) Fresh collected leaf. (*B*) Callus was induced from the leaves, nodes, and internodes of the explants grown on solid Murashige and Skoog (MS) medium containing 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid. After 3 to 4 wk, a friable light yellowish-white callus was formed. (*C*) Cell suspension culture was initiated by transferring the callus into liquid medium supplemented with 1.0 mg L^{-1} 6-benzyladenine. The *red arrow* indicates the induced callus resulting from the cut areas of the internodes in contact with the MS medium. The *blue arrow* indicates the induced callus at the scored areas of the leaves.

period, cell suspension samples were collected every 4 d and examined using a phase-contrast microscope at magnifications of 20 × and 40 ×.

Results and Discussion

In the not-too-distant past, the zenith of micropropagation technology was epitomized by axillary shoot production. However, a discernible surge in scholarly interest in somatic embryogenesis has been noted, particularly since the mid-1980s (Bornman 1993). Currently, the cultivation of somatic embryos *in vitro* and their subsequent transformation into artificial or synthetic seeds stands as the forefront of micropropagation potential, presenting an efficacious mechanism for vegetative propagation (Conger *et al*. 1989). As delineated by Gray, the capability to vegetatively propagate exemplary individuals with efficiencies comparable to traditional seed propagation holds the promise of revolutionizing crop production, encompassing both traditionally seed-propagated and vegetatively propagated plants through the controlled cultivation and conversion of somatic embryos *in vitro* (Gray and Conger 1985; Gray 1989).

Within the array of *in vitro* cultivation methods, somatic embryogenesis stands out as a pivotal advancement in plant tissue culture (Egertsdotter *et al*. 2019; Simonović *et al*. 2020). This innovation has facilitated large-scale propagation and the creation of biotechnological instruments aimed at augmenting both the quantity and quality of plantation forestry (Egertsdotter *et al*. 2019). The combination of somatic embryogenesis and cryopreservation forms the foundation for diverse varietal forestry practices (Fehér 2019; Castander-Olarieta *et al*. 2022). Plant cells have the potential to undergo somatic embryogenesis when

Table 1. Effect of plant growth regulator on the production of somatic embryos (torpedo stage) of *Tecoma stans* (L.) Juss. ex Kunth leaves cell suspension cultures harvested at day 27 of culture incubation under the specified conditions

Cell suspension culture flask (F)	Auxin to cytokinin hormonal ratio of 2,4-D and BA (mg L^{-1})	Average number of induced somatic embryos per 50 mL volume of suspension culture
F1	1:1	0 ± 0.0
F2	1:2	16 ± 2****
F3	1:3	10 ± 1.1####
F4	1:4	6 ± 0.5####
F5	1:5	5 ± 0.1####

Data are expressed as the mean of three replicates ± SD. One-way ANOVA Tukey *post hoc* test was performed. *p*-value (****$p < 0.0001$) indicates statistical significance from all groups; ####$p < 0.0001$ indicates statistical significance from F1. *2,4-D* is 4-dichlorophenoxyacetic acid, an auxin, while *BA* is 6-benzyladenine, a cytokinin.

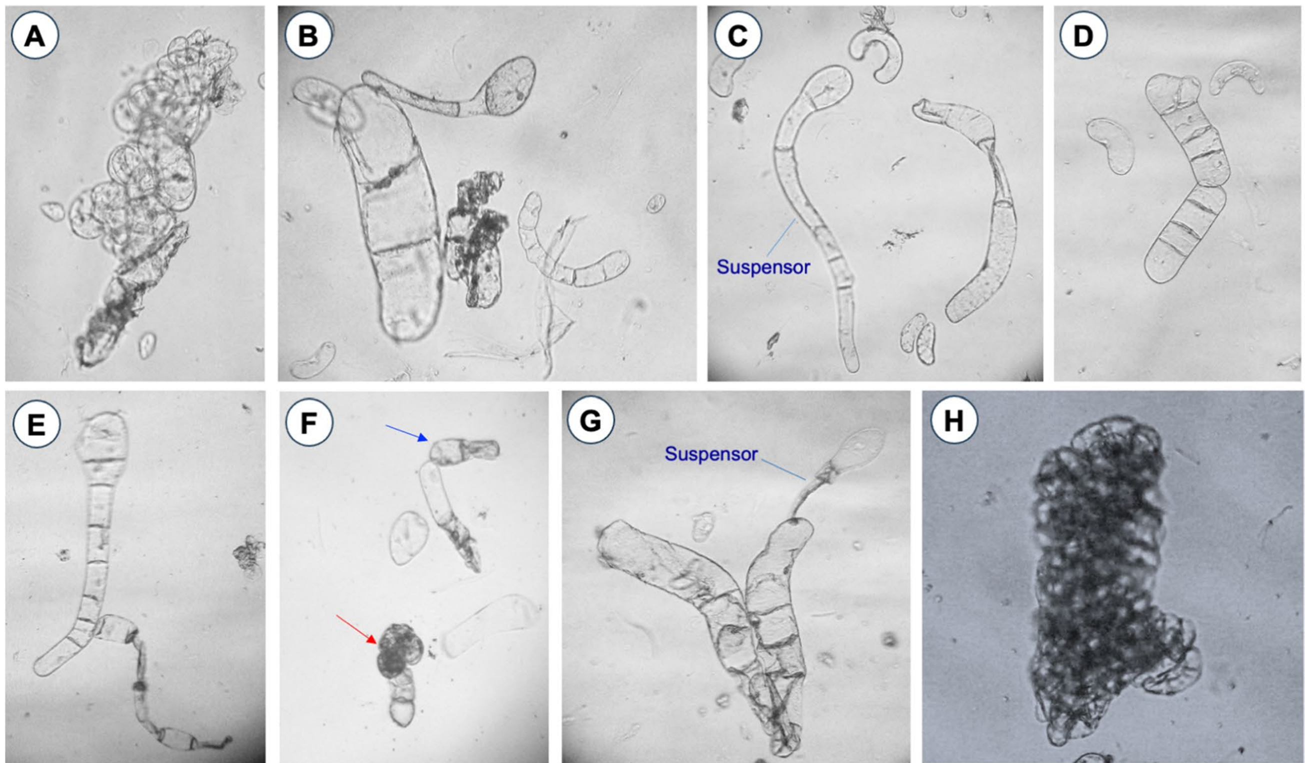

Figure 2. Phase-contrast microscopical examination of the cell suspension culture of *Tecoma stans* (L.) Juss. ex Kunth in Murashige and Skoog liquid medium supplemented 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 2.0 mg L^{-1} 6-benzyladenine at day 14 of culture incubation. (*A*) Cell division, isolated cells, and clusters. (*B*) Earlier embryogenic stages (one-cell proper). (*C*) Pre-embryonic stages (two-cell embryo and two-cell proper). (*D*, *E*) Four-cell embryo; quadrant stage. (*F*) Octant (*red arrow*), dermatogen, and early globular stages (*blue arrow*). (*G*, *H*) Early heart-stage embryos (×40).

appropriately stimulated with plant growth regulators, specific incubation conditions, and supplementation of the culture medium. Somatic embryogenesis in plants involves two pathways: direct and indirect. In direct somatic embryogenesis, embryos form directly from isolated cells, bypassing the formation of callous tissue (Fehér 2019). On the other hand, indirect somatic embryogenesis involves the formation of a callus as a preliminary stage before the development of somatic embryos. It is worth mentioning that not all plant cells possess this morphogenic capacity. Therefore, identifying the factors influencing this response has proven to be a challenging task (Ramírez-Mosqueda 2022).

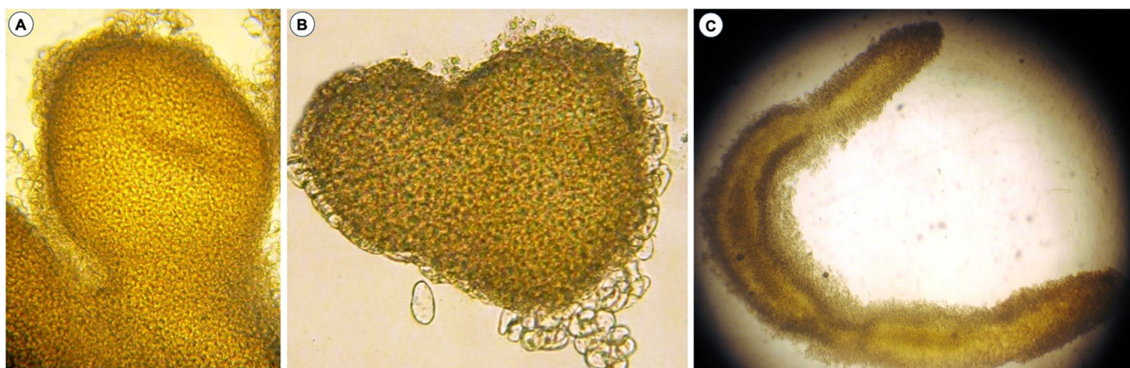

Figure 3. Phase-contrast microscopical examination of (*A*) globular, (*B*) late heart, and (*C*) cotyledon stages of *Tecoma stans* (L.) Juss. ex Kunth embryos induced in Murashige and Skoog liquid medium supplemented with 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 2.0 mg L^{-1} 6-benzyladenine, at day 21 of culture incubation (×20).

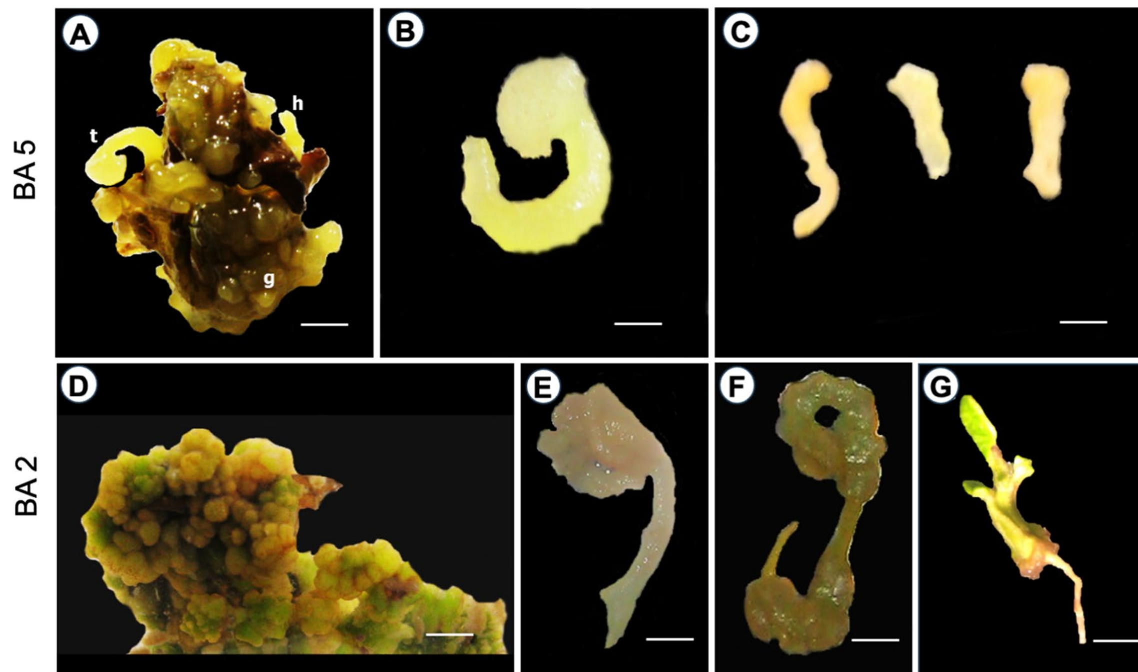

Figure 4. Macroscopical examination of somatic embryonic stages and ectopic proliferation of *Tecoma stans* (L.) Juss. ex Kunth induced in Murashige and Skoog (MS) liquid medium under 2.0 or 5.0 mg L^{-1} 6-benzyladenine (BA) with 1.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). (*A*) Embryonic phases (torpedo, heart, and globular stages denoted as t, h, and g respectively) were observed at the interface of the leaf explant. (*B*, *C*) Mature torpedo stages harvested at day 27 of suspension culture incubation. (*D*) Ectopic proliferation on the leaf explants. (*E*, *F*) Mature embryos harvested at day 31. (*G*) Vegetative phase grown on plain solid MS medium at day 38. *Scale bar* = 2 mm. BA2, 2.0 mg L^{-1} 6-benzyladenine; BA5, 5.0 mg L^{-1} 6-benzyladenine.

A previous study demonstrated the establishment of a micropropagation protocol for *T. stans* through the utilization of thidiazuron. The combined use of BA and naphthaleneacetic acid (NAA) along with thidiazuron (TDZ) in *T. stans* cultures yielded favorable and synergistic outcomes, resulting in heightened rates of shoot induction and proliferation (Hussain *et al*. 2019a, b).

Another investigation conducted by Hussain *et al*. (2019a, b) explored the influence of meta-topolin on *in vitro* organogenesis in *T. stans* L., assessed genetic fidelity, and performed phytochemical profiling on both wild and regenerated plants (Hussain *et al*. 2019a, b). However, the application of somatic embryogenesis in *T. stans* has not been explored, instigating the present authors' interest in this study. To achieve the induction of somatic embryos of *T. stans*, a primary or conditioning static medium having 1.0 mg L^{-1} 2,4-D for the redetermination and reprogramming stage was applied (Fig. 1), followed by a secondary induction medium containing low auxin and cytokinins ratio of 2,4-D and BA (1:1 to 1:5) where the unfolding of the developmental sequence induction of embryogenesis was observed.

Several stages of pre-embryonic and embryonic somatic embryos were detected; however, the 1:2 ratio of 2,4-D and BA growth regulators (Table 1) gave the best results of the number of mature somatic embryos (16 ± 2 torpedo embryos per volume) compared to the rest of the tested hormone concentrations. Microscopical examination showed (Fig. 2) isolated somatic cells and clusters, earlier embryogenic stages (one-cell proper and two-cell proper, quadrant stage, octant, dermatogen, early globular, and early heart-stage embryos). Moreover, mature globular, heart, cotyledon, and torpedo stages were also identified (Figs. 3 and 4). The torpedo stage further developed into the vegetative phase when utilizing a combination of 2,4-D and BA (in a ratio of 1:2). This concentration is deemed optimal for regulating cell growth and completing the somatic embryo growth cycle (Fig. 4). Namde and Wani (2014) have studied the effects of explants and plant growth hormones, 2,4-D, kinetin, and BA, on *T. stans* culture. Of interest, the 2:2 ratio of 2,4-D and BA gave 100% callus induction frequency, which was in accordance with the present study results upon using 2.0 mg L^{-1} BA.

Conclusion

In this study, a novel approach involving a low ratio of auxin and cytokinin was introduced, demonstrating its potential as a promising biotechnological alternative

for the *in vitro* propagation of *T. stans* through somatic embryogenesis. This method can be applied to produce *T. stans*, a significant medicinal plant known for its bioactive phytochemicals with antidiabetic and other biological activities. Further comprehensive tissue culture studies of *T. stans* are recommended to advance the scientific community understanding and utilization of its biotechnological applications.

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Author contribution R. O.: investigation; I.K.: reviewing; M.A. E-S.: investigation, conceptualization, experimental design, formal analysis, original draft writing, reviewing, overall project supervision and administration.

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Data availability The data that support the findings of this study are available from the corresponding author Dr. Mohamed Abd El-Salam upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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