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ORIGINAL ARTICLE

Influence of auxin and its polar transport inhibitor on the development of somatic embryos in *Digitalis trojana*

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Abstract

The present study reports the role of auxin and its transport inhibitor during the establishment of an efficient and optimized protocol for the somatic embryogenesis in *Digitalis trojana* Ivan. Hypocotyl segments (5 mm long) were placed vertically in the Murashige and Skoog medium supplemented with three sets [indole-3-acetic acid (IAA) alone or 2,3,5-triiodobenzoic acid (TIBA) alone or IAA–TIBA combination] of formulations of plant growth regulators, to assess their differential influence on induction and proliferation of somatic embryos (SEs). IAA alone was found to be the most effective, at a concentration of 0.5 mg/l, inducing ~ 10 SEs per explant with 52% induction frequency. On the other hand, the combination of 0.5 mg/l of IAA and 1 mg/l of TIBA produced significantly fewer (~ 3.6 SEs) and abnormal (enlarged, oblong, jar and cup-shaped) SEs per explant with 24% induction frequency in comparison to that in the IAA alone. The explants treated with IAA–TIBA exhibited a delayed response along with the formation of abnormal SEs. Our study revealed that IAA induces high-frequency SE formation when used singly, but the frequency gradually declines when IAA was coupled with increasing levels of TIBA. Eventually, our findings bring new insights into the roles of auxin and its polar transport in somatic embryogenesis of *D. trojana*.

Keywords Auxin · Foxglove · In vitro rooting · Polar auxin transport inhibitor · Somatic embryogenesis

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Abbreviations

DMRT	Duncan multiple range test
IAA	Indole-3-acetic acid
MS	Murashige and Skoog (1962)
PGR	Plant growth regulator
SE	Somatic embryo
TIBA	2,3,5-triiodobenzoic acid

Introduction

The process of a directional cell-to-cell transport of the plant growth regulator auxin is named as 'polar auxin transport' and it is one of the main processes that determine the spatial distribution of auxin in the plant. Local auxin metabolism and polar auxin transport lead to the asymmetric distribution of auxin caused by auxin gradients and auxin maxima, which eventually results in significant plant growth and development (Liu et al. 2017). Moreover, it plays a key role in the establishment of embryonic axis, organogenic development like root, stem and their respective cell niche formation and maintenance (Vanneste and Friml 2009; Liu et al. 2017). In addition, the polar auxin transport also plays a central role

in the plant embryogenesis (Cooke et al. 1993). Friml (2003) and Friml et al. (2003) had opined that changes in auxin fluxes in the polar auxin transport might be linked with the exogenous effects, which can induce a new distribution of auxin in the plant. In early embryo development, the formation of the apical meristem is regulated by auxin. The polar auxin efflux and the auxin response result in the apical–basal axis formation of the embryo (Liu et al. 1993; Dodeman et al. 1997; Friml 2003), suggesting an analogous regulation of zygotic and somatic embryos (SEs). However, the study on auxin transport inhibitor-affected growth (Liu et al. 1993) involving the use of Murashige and Skoog (1962) (MS) basal medium containing 2,3,5-triiodobenzoic acid (TIBA) and indole-3-acetic acid (IAA) is limited for SEs. Thus, in the absence of a general consensus on the roles played by auxin and its polar transport inhibitor, there is a need to determine the effect of auxin alone, inhibitor alone and in a combination of the two. For the present work, we have chosen *D. trojana* as the specimen plant that is medicinally important but vulnerable and endemic in the Turkish region (Verma et al. 2012).

Digitalis trojana Ivan (Plantaginaceae) is commonly known as Helen of Troy Foxglove. Several other *Digitalis* species have been well known for their therapeutical use, being a primary source of cardio-protective glycosides (Verma et al. 2016). Cardenolides, extracted from various *Digitalis* species are extensively used in the therapy of cardiac insufficiency (Mutschler et al. 2008). The *Digitalis* cardenolides do not play a major role in the current cardiovascular drug market; instead, they are used in the treatment of other diseases, such as cancer and viral infection (Kreis 2017). The susceptibility of cancer cells to cardenolides in tumor therapy is of special interest (Calderón-Montaño et al. 2014) in the field of medicinal chemistry. The cardenolides of *Digitalis* species including *D. trojana* have a high economical value. However, due to an unrestricted exploitation of this important plant species to meet the ever-increasing demand of the pharmaceutical industries, the plant population has been markedly depleted, which is declining furthermore owing to its limited cultivation and insufficient attempts to replenish it in the wild. Natural propagation of *D. trojana* through seeds is possible, but this method is not effective in producing a sufficient number of planting stocks, owing to its poor germination frequency (Verma et al. 2012, 2016). In such backdrop, in vitro mass multiplication emerges as a better alternative approach (Gantait and Kundu 2017). In practice, the cardenolides are isolated from the *Digitalis* plants, since the structural complexity of the cardenolides impedes an easy chemical synthesis in the laboratory (Verma et al. 2016). Cell culture techniques are extensively used for the production of cardenolides (Munkert et al. 2017). In addition, somatic embryogenesis offers a significant substitute for achieving in vitro mass production of essential secondary metabolites such as digoxin and digitoxin (Kuberski et al. 1984).

In the present article, for the first time, we describe a new, simple, and highly effective method for the induction of direct SEs from vertically held hypocotyl segments of *D. trojana* and evaluated the effect of auxin and its transport inhibitor during the induction of SEs. To achieve our objectives, we have used the MS medium supplemented with IAA alone, TIBA alone or IAA–TIBA combinations at different concentrations for establishing direct somatic embryogenesis and subsequent organogenesis.

Materials and methods

Plant material

Seeds were harvested from the wild populations of *D. trojana* Ivan from the Edremit–Kaz (Ida) mountain range (at the altitude of 345 m, N39°38.885 and E026°57.402), Canakkale, Turkey. Identification of species was made according to Davis (1978), and the voucher specimens (Eker-1905) were deposited at the Abant Izzet Baysal University Herbarium (Bolu, Turkey).

Surface sterilization and culture conditions

Collected seeds of *D. trojana* were surface-sterilized following the previously reported procedure of Verma et al. (2012). In each plastic Petri dish (90 mm × 15 mm), 25 seeds were placed onto plant growth regulator (PGR)-free MS medium (each Petri dish containing 25 ml medium) with 3% (w/v) sucrose. The medium was semi-solidified by adding 0.8% (w/v) agar and then the pH was adjusted to 5.8 prior to autoclaving the medium at 1.06 kg/cm^2 pressure for 15 min at 121 °C. The culture was kept for the first 2 days in the dark at 23 ± 1 °C and then transferred to 16-h light:8-h dark period (provided by cool white fluorescent light, irradiance at 50 µmol/m^2/s). Hypocotyl segments (5 mm long) from the 1-month-old in vitro germinated seedlings were used as explants for root and SE initiation.

Somatic embryogenesis: composition of plant growth regulators

The hypocotyl explants were vertically placed in plastic Petri dishes containing 30 ml semi-solidified MS media supplemented with different concentrations of IAA alone (0.1, 0.5, and 1 mg/l), TIBA alone (0.1, 0.5, and 1 mg/l) and IAA–TIBA combinations, respectively. Experiments were repeated three times, using 25 replicates/explants per replication. Both the frequency (%) of explants directly

inducing SEs and roots, the mean numbers of SEs and roots per explant were recorded after a 5-week culture period.

Conversion of somatic embryos into plantlets

Hypocotyl-derived SEs were kept in the same medium containing the PGRs (IAA alone) or PGR-free-half-strength MS medium for another 4 weeks for further growth of the SEs. After 4 weeks of culture, the SEs initiated organogenesis and developed into complete plantlets (with 4–5 leaves).

Sample preparation and observation through scanning electron microscope

SEs at their different developmental stages were selected for scanning electron microscope (SEM) analysis to confirm the observation at ultra-structural level. Initially, the SE samples were prefixed with 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) at 4 °C for 48 h. After that, the samples were postfixed using 1% osmium tetroxide at 4 °C for 2 h. The postfixed samples were washed with 0.1 M sodium cacodylate buffer (pH 6.8) for three times for 30 min each. Thereafter, they were gradually desiccated in a graded acetone series, and dried at the critical point, for 30 min (using CO_2 as the transient fluid). Ultimately, the dried samples were observed via scanning electron microscope with integrated Oxford Instruments INCA® image capture system software.

Statistical analysis

The data were recorded and finally the statistical analysis of variance (ANOVA) was done using SPSS (version 17.0, SPSS Inc., Chicago, IL, USA) software. Treatment data (i.e., mean ± standard error) differing significantly were evaluated using Duncan's multiple range test at $P < 0.05$ level (Duncan 1955). Data expressed as percentage were transformed using arcsine prior to ANOVA and converted back to the original scale for demonstration in the table and figures (Compton 1994).

Results and discussion

Effect of auxin alone on root induction

During the vertical placement of hypocotyl segments in the in vitro media, the bottom cut ends of the explants were submerged into the media and top cut ends were held apically. Such arrangement obviously causes wounding of the hypocotyl segments. Although roots might occasionally be induced by auxin on the intact hypocotyl, wounding is a mandatory requirement for the development of roots. De Klerk et al. (2011) found that wounding-related compounds play an important role in the generation of roots. In our study, 1 week after the culture initiation, the hypocotyl explants became swollen at the basal cut end while the apical cut end did not show any change. At the beginning of the second week, tiny dot-like structures were visible at the basal cut end, which by the end of that week itself developed into root primordia directly, without any sign of callus intervention near the cut ends and also from the middle part of the explants. The root formation continued for the entire period of 60 days in the same medium without any subculture (Fig. 1a–c). Roots were induced well ahead of somatic embryogenesis, from the lower cut surface of the hypocotyl explants from 15 days onwards, in the MS medium supplemented with IAA. In the present study, root induction was achieved with all the concentrations of auxin (0.1, 0.5 and 1 mg/l IAA alone) used and the mean number of roots per hypocotyl explant was 5.7, 7.4 and 8.9, respectively (Table 1). When IAA (0.5 mg/l) was combined with TIBA (0.1, 0.5, and 1 mg/l), it was found to induce only 3.4, 2.9 and 1.8 mean number of roots per explant, respectively (Table 1). If the auxin concentration was increased up to 2 mg/l, it resulted in callus formation from the hypocotyl explants (data not shown).

Effect of auxin alone on direct somatic embryogenesis

When the explants were cultured in a PGR-free MS medium, no embryo was found (Table 1). In the presence of exogenous auxin (IAA) in the medium, the root induction started first from the basal cut end of the hypocotyl segment (Fig. 1a). It is apparent that the exogenous IAA induced the root formation first, and then the endogenous level of IAA in the tissue might have increased, which favored the somatic embryogenesis. On the other hand, a unifying role of exogenous auxin seems to be implemented in the *D. trojana* system of somatic embryogenesis, characterized by (1) an initial high concentration of the free auxin for root induction in basal cut end and (2) a much lower concentration of auxin for the organized development of the bipolar embryos. Not only is the exogenous auxin important for the initiation of somatic embryogenesis, but it also initiates changes in endogenous auxin concentration that may be critical for the later stages of embryogenesis (Cooke et al. 1993). Several reports indicate that high level of endogenous IAA is associated with an increased embryogenic response (Pasternak et al. 2002; Hamasaki et al. 2005). In carrot cells, exogenous 2,4-dichlorophenoxy acetic acid (2,4-D) stimulates the accumulation of large amounts of endogenous IAA (Kumar and Shekhawat 2009). The exogenous auxins might act indirectly by affecting the endogenous IAA metabolism (Morris 2000).

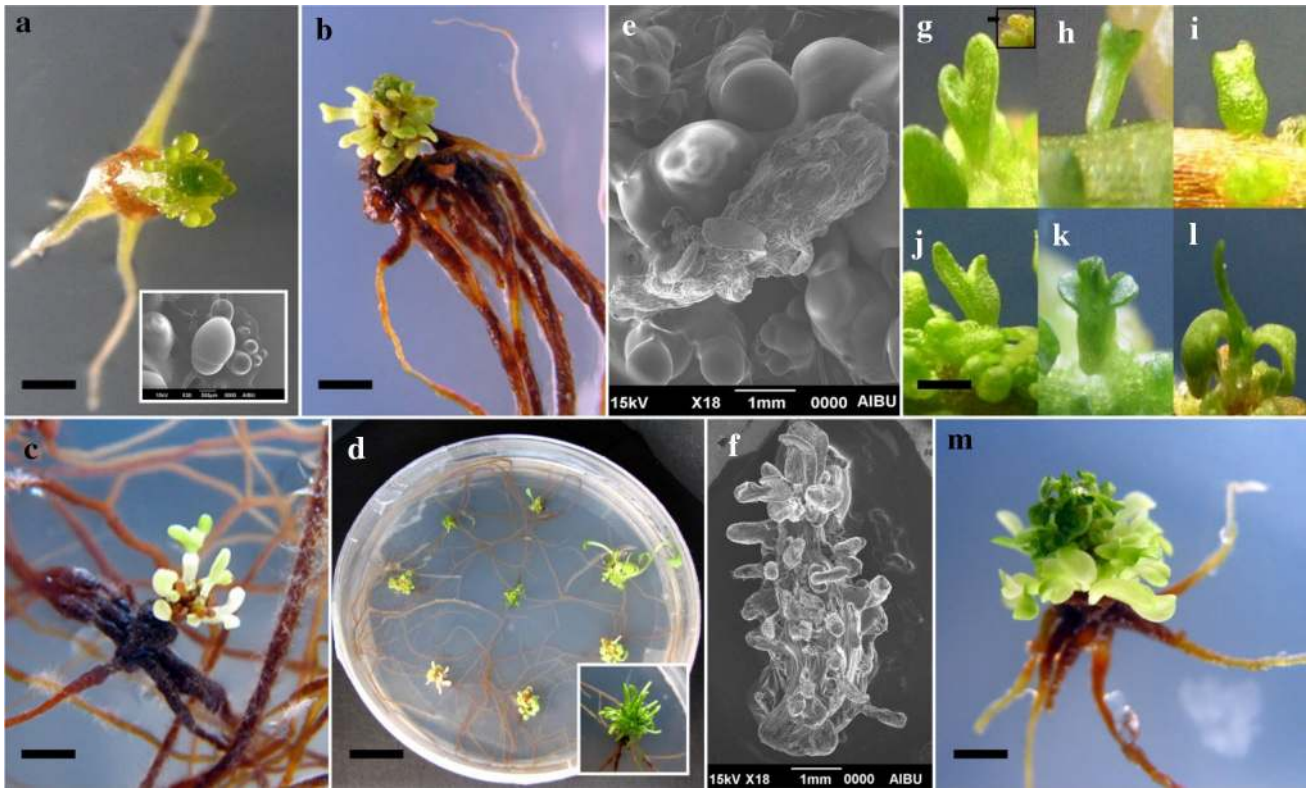

Fig. 1 Plant regeneration in *Digitalis trojana* through direct somatic embryogenesis from the hypocotyl segments (kept in vertical position) submerged in MS medium, supplemented with 0.5 and 1 mg/l IAA. **a** Somatic embryos (SEs) formed from a cut surface (apical) of hypocotyl explants after 15 days of culture and root initiation within a week from lower cut (basal) surface of the hypocotyl segment (submerged in the media) (Bar 3 mm), scanning electron microscopy (SEM) of SEs (INSET), **b** somatic embryogenesis (globular, heart and torpedo stages) from a hypocotyl explant cultured in MS medium supplemented with 1 mg/l IAA (Bar 3 mm), **c** well-developed SEs from a hypocotyl explant cultured in MS medium, supplemented with 0.5 mg/l IAA (the arrowhead indicates cotyledonary stage) (Bar 5 mm), **d** hypocotyl-derived SEs developed into shoot organogenesis after 6 weeks of culture in the same medium (Bar 10 mm), **e** SEM of globular and late globular SE stages (Bar 1 mm), **f** SEM of somatic embryogenesis showing the different stages with early organogenesis stages (Bar 5 mm), **g** heart-shaped SE (arrowheads indicating globular), **h** jar shaped SE, **i** early torpedo stage, **j** late torpedo stage, **k** cotyledonary SE (started leaf primordium with shoot apical meristem), **l** the transformation of the small leaf primordium to a mature leaf, **m** a complete plantlet regenerated from the SEs (Bar 10 mm)

Table 1 Direct root induction from hypocotyl explants of *Digitalis trojana* excised from the 45-day-old in vitro-germinated seedlings and cultured in MS medium containing different concentrations of IAA alone, TIBA alone and IAA–TIBA combinations

Medium + plant growth regulator (concentrations are in mg/l)	Mean frequency (%) of explants developing roots	Mean number of roots per explant[a]	Root length (cm)
MS (control)	0^{b}	0^{g}	0^{g}
MS + IAA (0.1)	100^{a}	5.7 ± 0.4^{c}	2.7 ± 0.2^{c}
MS + IAA (0.5)	100^{a}	7.4 ± 0.5^{b}	3.2 ± 0.6^{b}
MS + IAA (1.0)	100^{a}	8.9 ± 0.6^{a}	5.7 ± 0.8^{a}
MS + TIBA (0.1)	0^{b}	0^{g}	0^{g}
MS + TIBA (0.5)	0^{b}	0^{g}	0^{g}
MS + TIBA (1.0)	0^{b}	0^{g}	0^{g}
MS + IAA (0.5) + TIBA (0.1)	100^{a}	3.4 ± 0.3^{d}	2.1 ± 0.4^{d}
MS + IAA (0.5) + TIBA (0.5)	100^{a}	2.9 ± 0.6^{e}	2.0 ± 0.7^{e}
MS + IAA (0.5) + TIBA (1.0)	100^{a}	1.8 ± 0.5^{f}	1.9 ± 0.4^{f}

Data expressed as percentage were transformed using arcsine prior to ANOVA and converted back to the original scale for demonstration in the table (Compton 1994)

A total of 25 explants were used in each treatment and the data were recorded up to 6 weeks

Mean values ($n = 3$) ± standard error followed by different lower case letters denote a statistically significant difference at $P < 0.05$, as determined by Duncan's multiple range test (Duncan 1955)

In the current work, we found that, in the presence of IAA, tiny structures developed directly from the upper cut end (apical) of hypocotyl explants after 30 days of culture (Fig. 1a). After incubating the same culture for an additional 25–30 days, the same tiny structures grew into enlarged embryos, with a simultaneous increase in their number (Fig. 1b). Several studies have shown that the process of embryogenesis takes place with the induction of cells with embryogenic competence in the presence of high concentration of auxin. The development of embryogenic competence cells into SEs takes place in the absence of auxin or in the presence of a low concentration of auxin (Halperin and Wetherell 1964; Thorpe 1994). It was reported that somatic embryogenesis in cultures proceeds from those cells which are already committed to embryogenic development (Sahrawat and Chand 2001), yet the exact point of origin of embryo development still remains unclear (Soriano et al. 2013). This requires PGRs, especially auxin, and additional favorable conditions to allow these pre-determined cells to undergo cell division and expression of embryogenesis (Sharp et al. 1980). Moreover, after SE induction, the role of auxin changes: the SEs start to synthesize their own auxin (Michalczuk et al. 1992).

The fact that a proper polar transport of auxin is a prerequisite for normal embryogenesis, beyond the globular stage, has already been established by several authors (Schiavone and Cooke 1987; Liu et al. 1993). In the case of *D. trojana*, the SEs turned green and advanced in different developmental stages (heart, torpedo, and cotyledons) after 60 days of culture (Fig. 1c, e–j). Henceforth, cotyledonary embryos further developed in shoot apical meristem with primordium development (Fig. 1k, l) and finally developed into normal plantlets (Fig. 1d) with subsequent plant regeneration (Fig. 1m).

The maximum percentage of embryogenic explants that transformed into SEs was recorded to be 52% (Fig. 2a). This was achieved in an MS medium supplemented with 0.5 mg/l IAA. The highest number of SEs per explants was 10 (Fig. 2b). However, the percentage of embryogenic explants and the number of SEs gradually increased up to 60 days; during this period the production of SEs was maximum but, however, longer time periods caused necrosis in some explants, which could explain the decrease in the number of SEs obtained from the embryogenic explants.

Effect of IAA and TIBA on somatic embryogenesis

In the present study, TIBA was added at various concentrations either individually or in combination with IAA, to examine its effect on SE formation. No induction of SE in the presence of only TIBA (or in control experiments) was detected. Following 1 month of culture, the hypocotyl explants exhibited necrotic effect under TIBA treatment. The combination of IAA with TIBA was examined by determining the frequency of embryogenesis (Fig. 3a) at the apical region of the explants and also by counting the mean number of SE per explant (Fig. 3b). The highest frequency (48%) of somatic embryogenesis was obtained in the MS medium with 0.5 mg/l IAA + 0.1 mg/l TIBA. As expected, this combination had shown the largest mean number of embryos (7.8 SEs per explant). The 0.5 mg/l IAA + 0.5 mg/l TIBA combination resulted in 30% embryogenesis frequency and induced ~ 6 (5.6) SEs per explant; while the 0.5 mg/l IAA + 1 mg/l TIBA treatment yielded an embryogenesis

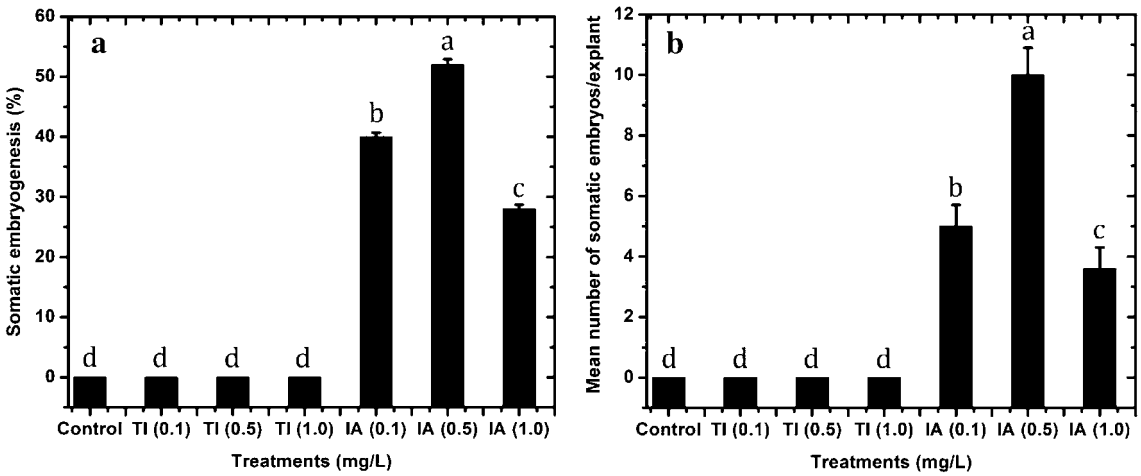

Fig. 2 Individual effects of TIBA (0.1–1 mg/l TI) or IAA (0.1–1 mg/l IA) on somatic embryogenesis of *Digitalis trojana*. **a** Frequency of somatic embryogenesis. **b** Number of somatic embryos induced per explant. Mean values ($n = 3$) ± standard error followed by different lower case letters denote a statistically significant difference at $P = 0.05$, as determined by Duncan's multiple range test (Duncan 1955). Data expressed as percentage were transformed using arcsine prior to ANOVA and converted back to the original scale for demonstration in the figure (Compton 1994)

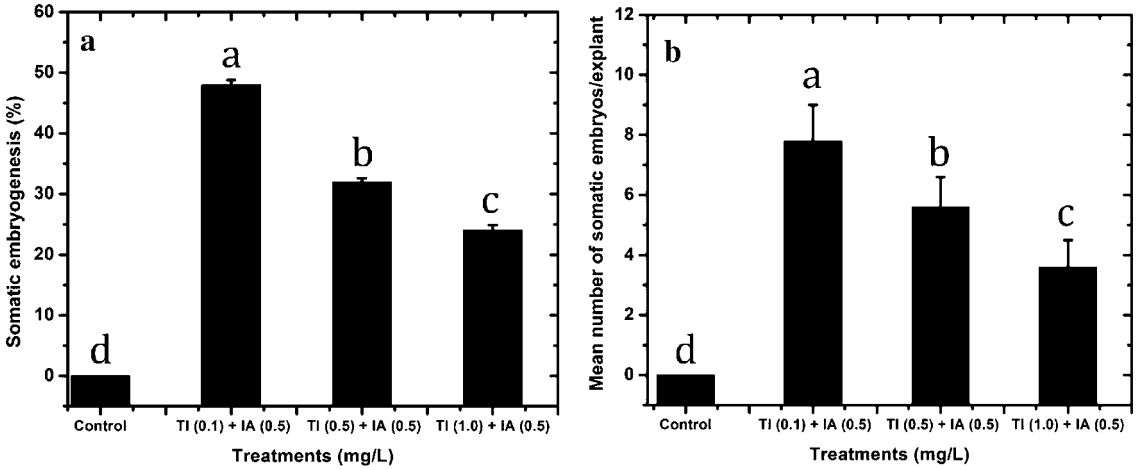

Fig. 3 Combined effect of TIBA (0.1–1 mg/l) and IAA (0.5 mg/l) on the somatic embryogenesis of *Digitalis trojana*. **a** Frequency of somatic embryogenesis, **b** number of somatic embryos induced per explant. Mean values ($n = 3$) ± standard error followed by different lower case letters denote a statistically significant difference at $P = 0.05$, as determined by Duncan's multiple range test (Duncan 1955). Data expressed as percentage were transformed using arcsine prior to ANOVA and converted back to the original scale for demonstration in the figure (Compton 1994)

frequency of 24% along with a mean number of ~ 4 (3.6) SEs per explant. In the presence of TIBA, the hypocotyl explants displayed different embryogenic responses as compared to IAA (0.5 mg/l) alone. When the TIBA concentrations were increased from 0.1 to 1 mg/l, embryogenic response decreased and abnormal SEs formed (Fig. 4a–d). TIBA at 1 mg/l hindered the frequency of direct SE formation. The variable performance generated by TIBA (0.1, 0.5, and 1 mg/l) with the combination of IAA (0.5 mg/l) on direct SE formation may be the result of their different levels of auxin inhibition. However, further investigations are needed to establish any connection between the inhibitory effects of TIBA on auxin and on the resultant somatic embryogenesis of *D. trojana*. It is to be noted that auxin (0.5 mg/l) combined with TIBA (0.1, 0.5, and 1 mg/l) significantly impeded direct somatic embryogenesis. Auxin concentrations (0.1, 0.5, 1 mg/l IAA) when used individually, exhibited a significant inductive effect on direct SE formation. At this stage, the role of endogenous auxin on the direct embryogenesis of *D. trojana* remains unclear. In *D. trojana*, the abnormality of SE was detected, as shown in Fig. 4a–c, when a higher concentration of TIBA (0.5–1 mg/l) was used. TIBA was found to be inhibitory for SE formation (Chée and Cantliffe 1989).

Exposure of IAA on hypocotyl explants transferred to TIBA-containing media and vice versa

In the first set of experiments, the hypocotyls were cultured in MS medium containing 0.5 mg/l IAA for 1 week and then transferred to another medium containing only 1 mg/l TIBA. The frequency of somatic embryogenesis at the apical part of the explants and the mean number of SEs per explant were determined. In the second set of experiments, hypocotyls were cultured in MS medium containing 1 mg/l TIBA for 1 week and then transferred to a 0.5 mg/l IAA-containing medium and kept in the above conditions to determine the somatic embryogenesis. In the TIBA-containing medium, the basal and apical cut ends of the hypocotyl explants gave comparable responses similar to that of the IAA-supplemented medium. But in the second set of experiments SEs were found to be abnormal and suppressed. It may be argued that the initial exposure to TIBA could have imparted some inhibitory effects, including the blockage of the polar auxin transport process that eventually resulted in abnormal SEs. On the other hand, the initial exposure to IAA for a sufficiently long duration, so as to reach the optimum level of auxin in the tissues, was required for the induction of SEs. Venkatesh et al. (2009) also reported that immature zygotic embryos of groundnuts, cultured in MS medium with 4 mg/l TIBA did not respond to somatic embryogenesis; but when cultured in medium supplemented with TIBA (2 and 4 mg/l) + 2,4-D (4 mg/l), induction of somatic embryogenesis was observed. In the present study, the *D. trojana* hypocotyls were exposed to (either before or after) TIBA to examine its effect on SE formation. In both the cases, there was an induction of somatic embryogenesis; in the former case, the SEs grew normally, while, in the latter, the growth was morphologically abnormal. An increase in the frequency of morphologically abnormal types coupled with a delayed response in the presence of TIBA might have occurred because of the interference in the auxin polar transport. Choi et al. (1997) observed the sporadic development of SEs which had a normal embryonic axis but with jar-shaped cotyledons in a medium containing 5–10 µM TIBA.

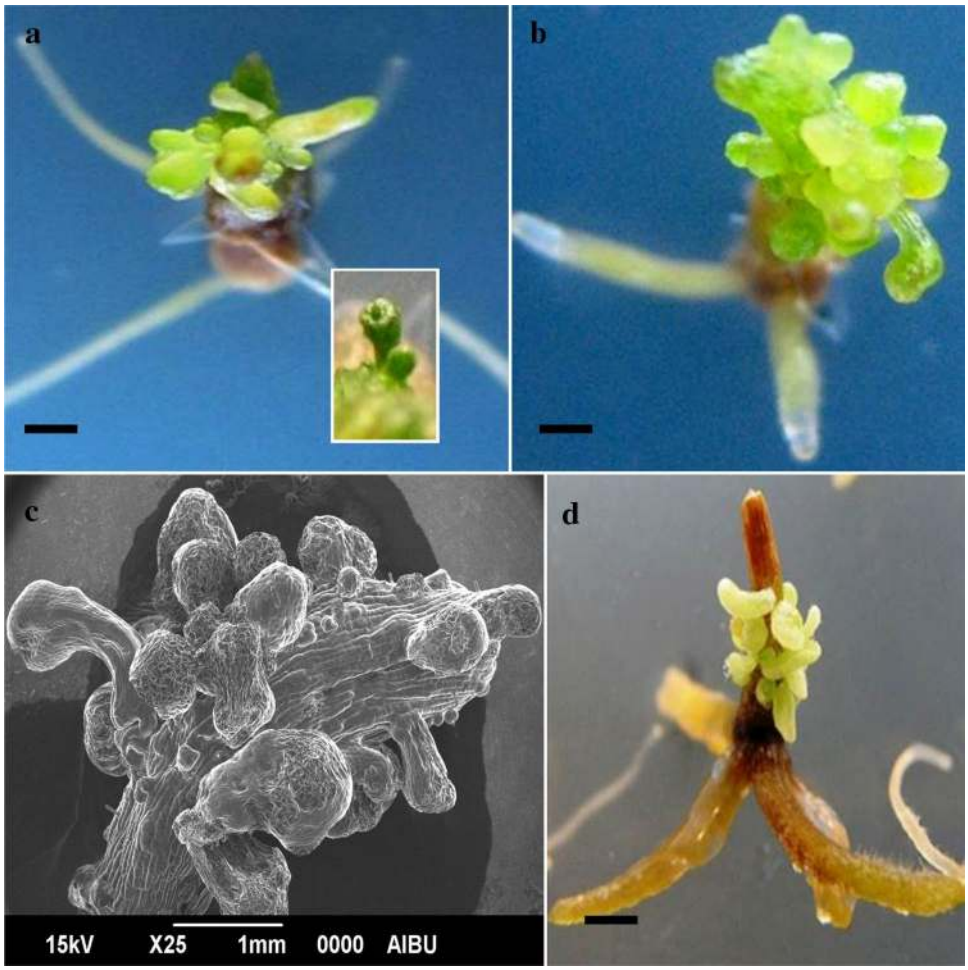

Fig. 4 Effect of auxin (IAA) and auxin polar transport inhibitor (TIBA) on induction of somatic embryos from hypocotyl segments (inoculated in vertical position) in the MS medium supplemented with variable levels of plant growth regulators. **a–c** Morphologically abnormal embryos: cup, trumpet and fused cotyledon, in 0.5 mg/l IAA and 1 mg/l TIBA (Bar 2, 1 mm), **d** sickle-like embryos formation in 0.5 mg/l IAA and 1 mg/l TIBA (Bar 2 mm)

In the present work, enlarged, oblong globular embryos, cup, and jar-shaped cotyledonous embryos were also found, when the treatment was carried out using combined TIBA + IAA (Fig. 4a–c). In case of *Brassica juncea*, the results showed that auxin polar transport plays a vital role in the formation of cotyledon during embryo development (Liu et al. 1993). In the present work, SE development was found to be normal when auxin was used singly, while a combination or exposure to TIBA-induced abnormal SEs. These observations confirmed that auxin polar transport plays an important role in the morphology of somatic embryogenesis.

Conclusion

In this article, a novel protocol was developed to elucidate the effect of exogenous auxin on hypocotyl segments of *D. trojana* to induce direct somatic embryogenesis. Somatic embryogenesis was promoted at all the concentrations of IAA used. In the presence of an auxin polar transport inhibitor (such as TIBA), SE formation from the embryogenic cells was suppressed, resulting in the development of abnormal embryos. The polar auxin transport inhibitor impeded the adventitious root formation as well. The inhibitor disrupted the somatic embryogenesis and thus specific spatial auxin distributions probably got affected. The protocol reported here is a simple easy-to-carryout procedure that could be used to understand the role of auxin and auxin polar transport in somatic embryogenesis, in other plants as well.

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Compliance with ethical standards

Conflict of interest The authors jointly declare no conflicts of interest.

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