ORIGINAL ARTICLE

In vitro morphogenetic responses from obligatory apomictic *Taraxacum belorussicum* Val. N. Tikhom seedlings explants

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Abstract

Taraxacum belorussicum Val. N. Tikhom, a poorly known and obligatory apomictic species, is an attractive plant material for studying the embryological, genetic and molecular mechanisms of apomixis. This work aims to obtain an efficient protocol for *Taraxacum belorussicum* regeneration. Four types of explants (cotyledons, hypocotyls, meristems and roots) that were taken from 2-weeks-old seedlings were used for in vitro cultures, and a fast and efficient protocol of *T. belorussicum* regeneration was obtained. Various ½ MS-based media containing IAA (5.71 μM), TDZ (4.54 μM) and PSK (100 nM) were chosen to assess the morphogenetic abilities of selected *T. belorussicum* explants. Studies on the role of PSK were done in three independent experiments, where the most significant factors were always light and darkness. All explants produced callus by the third day of culture and adventitious shoots after 7 days, although in an asynchronous indirect manner, and with different intensities for all explant types. The most preferred medium culture for hypocotyl, cotyledon and meristem explants was ½ MS + TDZ, and ½ MS + IAA + TDZ + PSK for roots which were the only explant sensitive to PSK. A short darkness pretreatment (8 days) in PSK medium was found suitable to enhance organogenesis. Secondary organogenesis was observed for regenerated plants on meristem explants from the ½ MS + IAA + TDZ + PSK medium. A weak somatic embryogenesis was observed for hypocotyl and cotyledon explants from ½ MS + IAA + TDZ and ½ MS + IAA + TDZ + PSK media. Histological and scanning electron microscope images (SEM) of *T. belorussicum* confirmed indirect organogenesis and somatic embryogenesis. Plant material treated with aniline blue solution revealed the presence of callose in the cell walls of cotyledon and hypocotyl explants. The presence of extracellular matrix (ECM) and heterogenic structure of callus was also verified by scanning electron microscopy and light microscopy, confirming the high morphogenetic ability of *T. belorussicum*.

Key message

An efficient regeneration protocol for *Taraxacum belorussicum*, the obligatory apomict, was obtained. Application of PSK revealed the significance of light and darkness as a key factor in the morphogenetic response. A short darkness pretreatment (8 days) in PSK medium was found suitable to enhance organogenesis. Histological and scanning electron microscope images confirmed indirect organogenesis and somatic embryogenesis presence. The protocol can enhance the knowledge about this species in studying the embryological, genetic and molecular mechanisms of apomixis.

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Abbreviations

BAP	6-Benzyl amino purine
ECM	Extracellular matrix
IAA	Indole-3-acetic acid
BA	Indole-3-butyric acid
MS	Murashige & Skoog
NAA	Naphthalene acetic acid
PAS	Periodic acid schiff
PGRs	Plant growth regulators
PSK	Phytosulfokine
SE	Somatic embryogenesis
SEM	Scanning electron microscope
TDZ	Thidiazuron

Introduction

Taraxacum belorussicum Val. N. Tikhom or dandelion, belongs to the genus *Taraxacum* (Asteraceae) (Tikhomirov 2003). This genus includes many species which were used for the analysis of embryological, genetic and molecular aspects of apomictic mechanisms (Janas et al. 2016). In general, dandelions have also been a part of the natural medicine tool kit because of their medical qualities as diuretic, choleric, anti-inflammatory, anti-oxidative, anti-carcinogenic and melliferous. *T. belorussicum* Val. is also a raw material for the cosmetic industry (Hu and Kitts 2003; Jamshieed et al. 2010). Based on their specific features and heavily urbanized areas of growth (Erofeeva 2014) some *Taraxacum* species can play a role as temperate climate bio-indicators (Jamshieed et al. 2010). *T. belorussicum* is a *Palustria* triploid species (Marciniuk et al. 2010) and comes from the area of Minsk in Belarus where it was described for the first time by Tikhomirov (Tikhomirov 2003). It is an obligatory apomict that can produce embryos in a parthenogenetic manner, and can thus be used for the comparative study of parthenogenesis and somatic embryogenesis.

Apomixis is a type of plant reproduction during which the embryo is formed without full meiosis and syngamia, or omitting either of these two processes (Noyes 2007). *Taraxacum* is characterized by the occurrence of diplospory apomixis (Koltunow 1993; Hand and Koltunow 2014), where a meiotic prophase is observed, but chromosomes do not create bivalents or metaphase tiles. Instead, a restitution nucleus is formed which leads to generation of unreduced embryo sac cells after successive mitotic divisions (Hand and Koltunow 2014; Rodkiewicz et al. 1996). Despite this, some apomictic species often maintain the ability to sexually reproduce, and certain seed parts have a zygotic origin (Rodkiewicz et al. 1996).

One of the common morphogenetic pathways leading to in vitro plant regeneration is organogenesis (Huang et al. 2014), which can be indirect via callus and direct by the creation of adventitious shoots from explant tissue (Woźny and Przybył 2004). A second possible path of in vitro morphogenesis is somatic embryogenesis, with somatic embryo induction from a somatic cell without fertilization (Williams and Maheswaran 1986). Somatic embryos can be formed directly from the initial explant (Kuo et al. 2005) or indirectly from callus cells (Jasrai et al. 2003). Both morphogenetic processes are under the control of several factors, one of which is the explant's origin (Ebida and Hu 1993), while others are the growth factors, the auxins, cytokinins and polysaccharides, amino acids, vitamins and proteins (Nic-Can et al. 2015) to which it is exposed. The ratio between concentration of auxins and cytokinins has a crucial influence on in vitro morphogenesis (Isah 2015). Phytosulfokine (PSK), which was isolated for the first time from the *Asparagus officinalis* mesophyll (Matsubayashi and Sakagami 1996), being a peptide hormone, plays a role of regulatory molecule in the growth and development of plant cells, especially in somatic embryogenesis induction (Lorbiecke et al. 2005; Igasaki et al. 2003; Kobayashi et al. 1999; Maćkowska et al. 2014; Ochatt et al. 2018).

Plant cell predisposition to undergo one of the two developmental pathways in vitro can be predicted using callose identification, as callose deposited in the cell walls is a marker of somatic embryogenesis. In addition, the presence of cuticle and the loss of plasmodesmata connections are attributed to this process (Fiuk and Rybczyński 2006). Callose may be also deposited in the place of damage as protection against microorganism infection (Ellinger and Voigt 2014).

According to the latest data, the presence of the Extracellular Matrix (ECM) in in vitro culture is a specific feature of morphogenesis or germinal tissues that is associated with ECM signaling functions. In some cases, ECM may perform a protective function or is seen as a specific feature of callus surfaces formed under in vitro conditions (Popielarska-Konieczna et al. 2013). The formation of ECM may be the result of stress factors or exposure to chemical agents that protect callus cells (Bevitori et al. 2013) and can create a pathway for receiving and transducing signals that determine the recognition and fate of cells, and the morphogenesis of plant.

Apomixis is a phenomenon in which embryos arise by bypassing the process of fertilization, as also observed in embryo formation from somatic tissue cultured in vitro. To what extent this similarity has its justification at the molecular or physiological level so far is not known and is an interesting research challenge, for which use of apomictic

Taraxacum species seems to be the right choice. Therefore, tracing the morphogenetic abilities with the hope of obtaining an efficient somatic embryogenesis of this species is the first and necessary step for later comparative studies of these two developmental processes. Based on the growing evidence suggesting that PSK has impact on acquisition of SE and morphogenesis, while there are no reports concerning these processes in *T. belorussicum,* we have attempted to identify problems related to the development of an efficient regeneration system for selected types of explants in in vitro conditions that included histological and SEM analysis.

Materials and methods

Plant material

All experiments were performed with seeds of apomict *T. belorussicum* Val. N. Tikhom kindly received from Professor Jolanta Marciniuk (Institute of Biology, University of Natural Sciences and Humanities at Siedlce in Poland). All seeds were sterilized by immersion in 70% ethanol (Sigma, St. Louis, MO, USA) for 30 s and then in 2% solution of sodium hypochlorite (Sigma) for 15 min. Next, they were rinsed three times in sterile water and placed in Petri dishes (Corning Incorporated, Corning, NY) containing ½ MS (Murashige and Skoog 1962) medium supplemented with 30 g/l sucrose and 8 g/l agar.

In vitro plant culture conditions

All used media were based on MS medium (Murashige & Skoog Medium, Duchefa Biochemie B.V., Haarlem, The Netherlands). Seeds were cultured onto 20 ml of ½ MS medium culture under a 16-h light photoperiod (cool white fluorescent light with an intensity of $39\ \mu M \times m^{-2} \times s^{-1}$) at 23 ± 2 °C for 2 weeks. Afterwards, four types of explants: cotyledon (Fig. S1b), meristem (Fig. S1c), hypocotyl (Fig. S1d) and root (Fig. S1e) were obtained from the 2-week-old seedlings (Fig. S1a) and cultured on several media according to different experimental systems with 25 explants per Petri dish, in three repetitions. As a control, ½ MS medium was used.

Organogenesis model regenerated plants with clearly formed leaves were isolated from explants after 40 days of culture and transferred into test-tubes (Equimed, Cracow, Poland) containing 50 ml ½ MS, supplemented with 4.92 μM IBA, under conditions as above for 90 days without subculturing. Then, regenerated well rooted plantlets were transferred into pots filled with experimental soil media, covered by transparent plastic boxes and kept in the same culture conditions for the next 14 days. Within this time, all plantlets acclimatized successfully.

SE/PSK model/short darkness explants were cultured in ½ MS medium supplemented with 5.71 μM IAA, 4.54 μM TDZ and 100 nM PSK (phytosulfokine-α, PeptaNova GmbH, Germany) at 23 ± 2 °C for 8 days in the dark (short darkness), then explants were transferred to the light regime above for 60 days, with two subcultures (Fig. S2). Next, the regenerated plants were test tube cultured (Equimed, Cracow, Poland) in 50 ml ½ MS, supplemented with 4.92 μM IBA under the photoperiodic light and temperature conditions as above.

SE/PSK model/long darkness Explants were cultured in ½ MS medium, supplemented with 5.71 μM IAA, 4.54 μM TDZ and 100 nM PSK at 23 ± 2 °C for 55 days in the dark (long darkness), and were then transferred to the light for 35 days without subculturing (Fig. S3). Six types of media were used in this experimental model (P1—½ MS − control, P2—½ MS + IAA + TDZ, P3—½ MS + IAA + TDZ + PSK, P4—½ MS + PSK, P5—½ MS + TDZ, P6—½ MS + TDZ + PSK). Next, the regenerated plants were test tube cultured in 50 ml ½ MS, supplemented with 4.92 μM IBA under the same photoperiodic light conditions as above at 23 ± 2 °C.

Histological analysis of in vitro morphogenesis

Samples of each kind of explant after 50 days of culture were fixed in a mixture of 5% glutaraldehyde in phosphate buffer, pH 7.2 (Sigma) in 5 °C overnight, washed four times in 0.1 M phosphate buffer (pH 7.2), then dehydrated in an ethanol series (10%, 30%, 50%, 70%, 96%; 15 min each) and kept overnight in absolute ethanol. The fixed tissue samples were subsequently embedded in Technovit 7100 (Heraeus Kulzer, South Bend, Indiana, USA). Next, histology blocks of plant material were made using a solution of Technovit B under vacuum for 24 h. Plant material histology blocks slices (7 μm of thickness) were obtained by using a rotational microtome—Microm HM 355 S I Microtome (MICROM International GmbH, Walldorf, Germany).

Histological samples were stained using 0.1% toluidine blue (Sigma), 0.05% aniline blue (Sigma), 0.5% naphthol blue black (Sigma) and PAS—Periodic Acid Schiff (Sigma). Next, sample slides were closed by histological medium (Entellan; Merck Millipore, USA). To detect cell structures such as the cytoplasm or nuclear cell, the slides were first warmed, then stained with 0.1% toluidine blue for 30 s and afterwards, washed in distilled water. The callose fluorescence was observed in UV light (370 nm) after staining with 0.1% toluidine blue for 30 s and being incubated with 0.05% aniline blue for 10 min.

Scanning electron microscope (SEM)

For examination utilizing the scanning electron microscope, plant material of each type of explant was collected at the 50th day of culture and pre-fixed in 5% buffered glutaraldehyde (0.1 M phosphate buffer, pH 7.2) for 3 h at room temperature. After dehydration in a graded ethanol series, samples were dried at the critical point of CO_2 by use of a Quorum Technologies E3000 (Quorum Technologies Ltd., Lewes, UK). Afterwards, samples were coated with fine-grained gold using automated sputter coater Jeol JFC—1100 E (JEOL, Peabody, MA, USA) for imaging via scanning electron microscope Jeol JSM 5410 (JEOL).

Observation and documentation of results

Images of in vitro cultures were made utilizing a Canon PC 1089 (Canon, Uxbridge, Middlesex, United Kingdom) connected to a binocular loupe Stemi SV 11 (Carl Zeiss Microscopy, Jena, Germany) at 7-day intervals. Symptoms of somatic embryogenesis were observed on selected types of explants, while organogenesis was scored as an average amount of shoots per explant for all types of explants, after 20 and 45 days of culture for SE/PSK model/short darkness and after 30 and 65 days of culture for SE/PSK model/long darkness. Regenerated plants and ex vitro culture evolution were documented by Canon PC 1089 (Canon, Uxbridge, Middlesex, United Kingdom). Histology samples were analyzed using a phase contrast microscope Nikon Eclipse E400 (Nikon Corporation, Tokyo, Japan) with Olympus 10x/0.25 (Olympus Corporation, Tokyo, Japan) and Olympus 40x/0.55 (Olympus Corporation) objective lenses. Samples stained with aniline blue were analyzed with an EX330-380 (Nikon Corporation) excitation filter. Images were processed by EOS Utility version 3 software (Canon).

Statistics

Statistical analyses were performed by One-way ANOVA test with Tukey test for multiple comparisons and test t with using the PRISM GraphPad 6.01 software (GraphPad Software Inc., San Diego, California, USA). Results were expressed as mean $\pm$ SEM per one explant for all types of explants. Statistical significance of differences between groups were considered at p values: $*p < 0.01$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Results

Induction and development of callus

All types of explants (meristem, hypocotyl, cotyledon, root), after mechanical damage, produced callus at different efficiency rates after 3 days of culture in all experimental models (Fig. 1). Observation of root explants (Fig. 1a) showed that callus formation begins initially at the ends of explants, in the place of scalpel cutting (Fig. 1b). Next, the entire explant swells (Fig. 1c) and callus formation subsequently covers the whole or the most part of the explant surface (Fig. 1d). Indeed, 100% of the explants started overgrowing with callus after 8 days of culture. At this time all explants showed two callus types, non-morphogenetic (Fig. 1d) and morphogenetic (Fig. 1e). Morphogenetic callus was identified by the formation of adventitious shoots (Fig. 1f)—a trait that is not characteristic for non-morphogenetic callus. Observation of hypocotyl and root explants provided similar information about the callus formation and development, albeit with some important differences. Initially, the callus had a compact structure and was visible on one part of the explants (Fig. 1c). The callus color concentrated around various shades of green, while white and yellow colors were also noticed.

During the culture of selected *T. belorussicum* explants, the most advanced formation and development of callus was observed on root (Fig. 1 I) and hypocotyl (Fig. 1 II) explants. Meristem explants also produced callus, but in a moderate amount, while callus was formed with the slowest and the least efficiency on cotyledon explants. Callusing was most intensive in the experimental model with a complete medium: ½ MS + IAA + TDZ + PSK (Fig. 1). Explants cultured on the control medium did not produce callus (data not shown). Results obtained from other experimental models (½ MS + IAA + TDZ and ½ MS + TDZ + PSK) revealed a varied intensity of callusing as a phase preceding the induction of adventitious shoots, observed on hypocotyl and root explants (Fig. 1). The weakest development of callus was noted on cotyledon explants. The callus color was from light to dark green, as well as white, yellow and brown. Meristem, cotyledon and hypocotyl explants showed the greenest shades, thus the largest amount of chlorophyll in callus cells, while shades of brown were visible on root explants.

Morphogenetic response of selected explants depending on plant growth regulators treatment

The intensity of morphogenetic response (organogenesis) of selected types of explants was expressed as the average number of adventitious shoots produced during in vitro culture. The anecdotal presence of somatic embryogenesis did not allow for its proper quantification.

Fig. 1 Two paths of callus development, indirect organogenesis and somatic embryogenesis (arrow) of *T. belorussicum* in in vitro culture: **I** the development of callus from the root explant, **II** the development of callus from hypocotyl explant and the first symptoms of somatic embryogenesis (marked as an oval structures); a—explant on the first day of culture, b—beginning of callus formation on the cut edges of explant, c—swelling callus and visible changes in the explant (after 14 days of culture), d—morphogenetic structures after a few days of culture, e—explant overgrown with developing callus (60th day of root explant culture), f—adventitious shoots on root explant on the 80th day of culture and hypocotyl after 28 days (indirect organogenesis). Photos came from SE/PSK model/short darkness; explants were cultured on ½ MS medium supplemented with 5.71 μM IAA, 4.54 μM TDZ and 100 nM PSK. Scale 1 mm
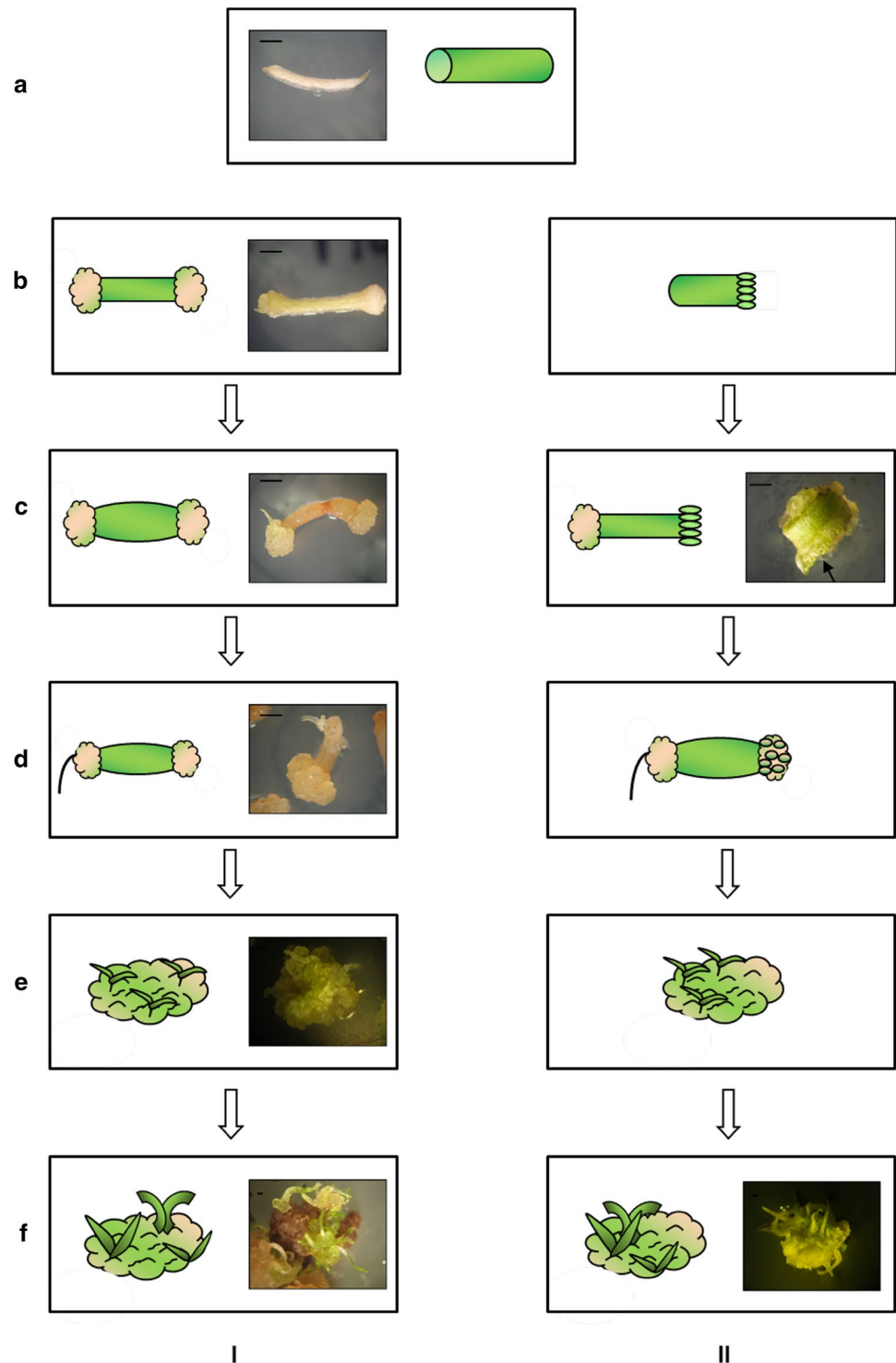

Organogenesis

In the organogenesis model, the first morphogenetic response occurred after 7 days of culture on ½ MS medium with TDZ and IAA, where analyses revealed callus formation on all types of explants, but meristem and root explants degenerated over time. Furthermore, the presence of adventitious shoots was evident only on hypocotyl and meristem explant surfaces (Table S1). Hence, the regenerative efficiency in vitro was calculated only for these explants (Fig. 2a), with an average of regenerated plants of 8.48 ± 1.7 and 17.8 ± 4.94 for cotyledon and hypocotyl explants, respectively, whereby hypocotyl explants were selected for subsequent studies. Noteworthy, 140 out of 251 regenerated

Fig. 2 Organogenesis efficiency of *T. belorussicum* explants. **a** hypocotyl and cotyledon after 40 days of culture on ½ MS with 5.71 µM IAA and 4.54 µM TDZ. **b** hypocotyl, meristem, cotyledon and root cultured on ½ MS with 5.71 µM IAA, 4.54 µM TDZ and 100 nM PSK during 8 days without access to light and after the transfer of the explants to light for the subsequent 60 days. Number of adventitious shoots per one explant were assessed twice after 20 days (T1) and 45 days (T2) of culture. **c** hypocotyl, meristem, cotyledon and root cultured on: P1—½ MS (control), P2—½ MS + PSK, P3—½ MS + TDZ, P4—½ MS + IAA + TDZ, P5—½ MS + TDZ + PSK, P6—½ MS + IAA + TDZ + PSK during 55 days without access to light and after transferring the explants to light for the subsequent 35 days. The number of adventitious shoots per one explant were assessed twice after 30 days (T1) and 65 days (T2) of culture. Data are presented as mean ± SEM per one explant for all types of explants. Statistical significance of differences between groups were considered at p values: *p < 0.1, **p < 0.01, ***p < 0.001, ****p < 0.0001 (**a** test *t*, **b** and **c** one-way ANOVA test). The figures show the results from 25 independent explants per one Petri dish in three repetitions
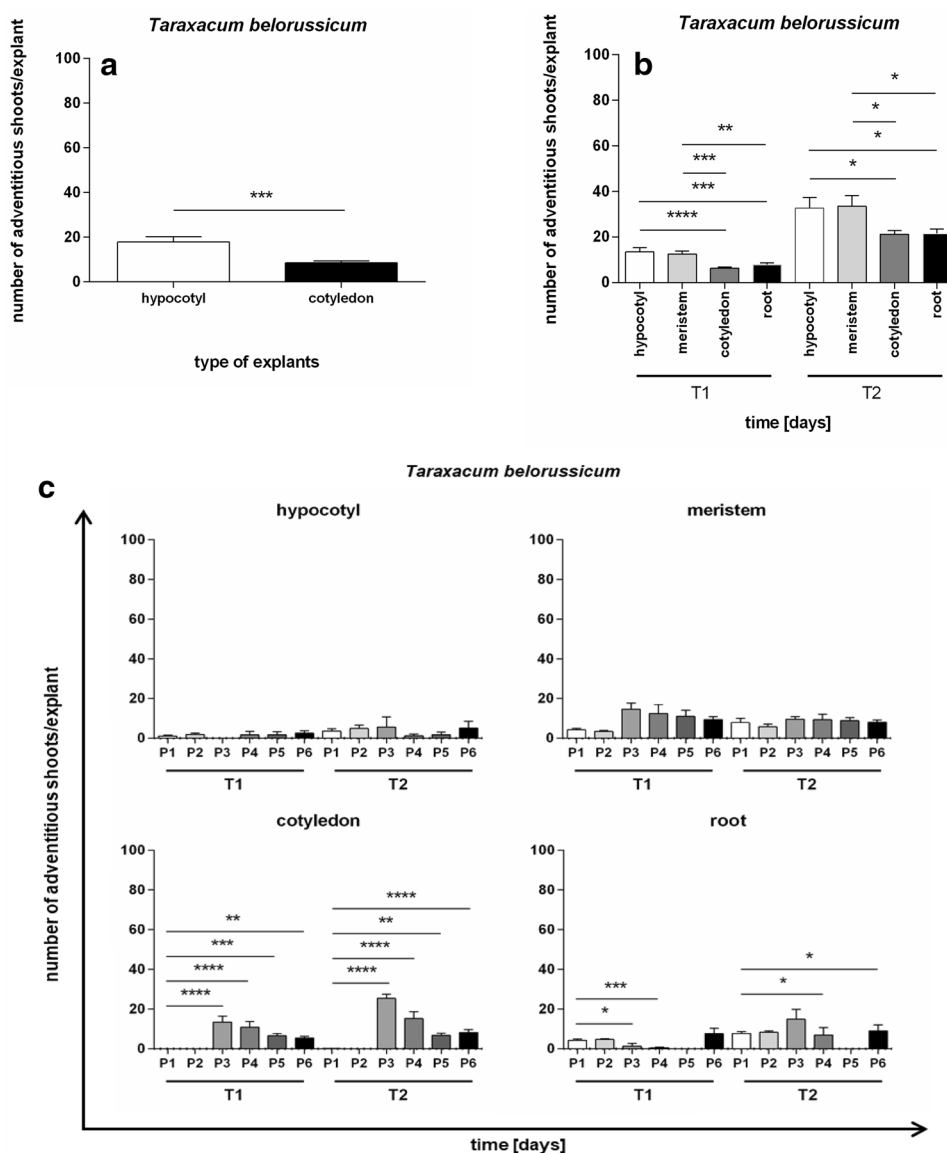
plants obtained from hypocotyl explants were successfully rooted and ultimately able to function in ex-vitro conditions.

In SE/PSK model/short darkness, hypocotyl and meristem explants revealed better morphogenetic response than did cotyledon and root explants after 20 and 45 days of culture on ½ MS medium supplemented with IAA, TDZ and PSK, with a short time under dark conditions (8 days). The presence of adventitious shoots was observed on the surface of all type of explants and thus their regenerative efficiency was calculated. Analyses also showed that organogenesis efficiency in terms of average amount of regenerated plants was highest on meristem and hypocotyl explants, while that of root and cotyledon explants was the lowest (Fig. 2b). In addition, indirect organogenesis proceeded with different efficiency on the majority of all types of explants. However,

all the regenerated plants obtained were successfully rooted after 28 days on ½ MS medium supplemented with IBA.

In SE/PSK model/long darkness, meristem and cotyledon explants revealed better morphogenetic response than did hypocotyl and root explants after 30 and 65 days of culture under an extended period of time in dark conditions (55 days) (Fig. 2c). In this model, ½ MS medium supplemented with TDZ turned out to be more efficient than medium with PSK added, for cotyledon, hypocotyl and meristem explants, while ½ MS medium supplemented with IAA, TDZ and PSK showed the highest efficiency for root explants. Conversely, root explants cultured on ½ MS medium with TDZ and PSK and cotyledon explants cultured on ½ MS medium with PSK died out after 103 days of culture (Figs. 3 and 4). I was also observed that indirect organogenesis proceeded with diverse efficiency on the majority
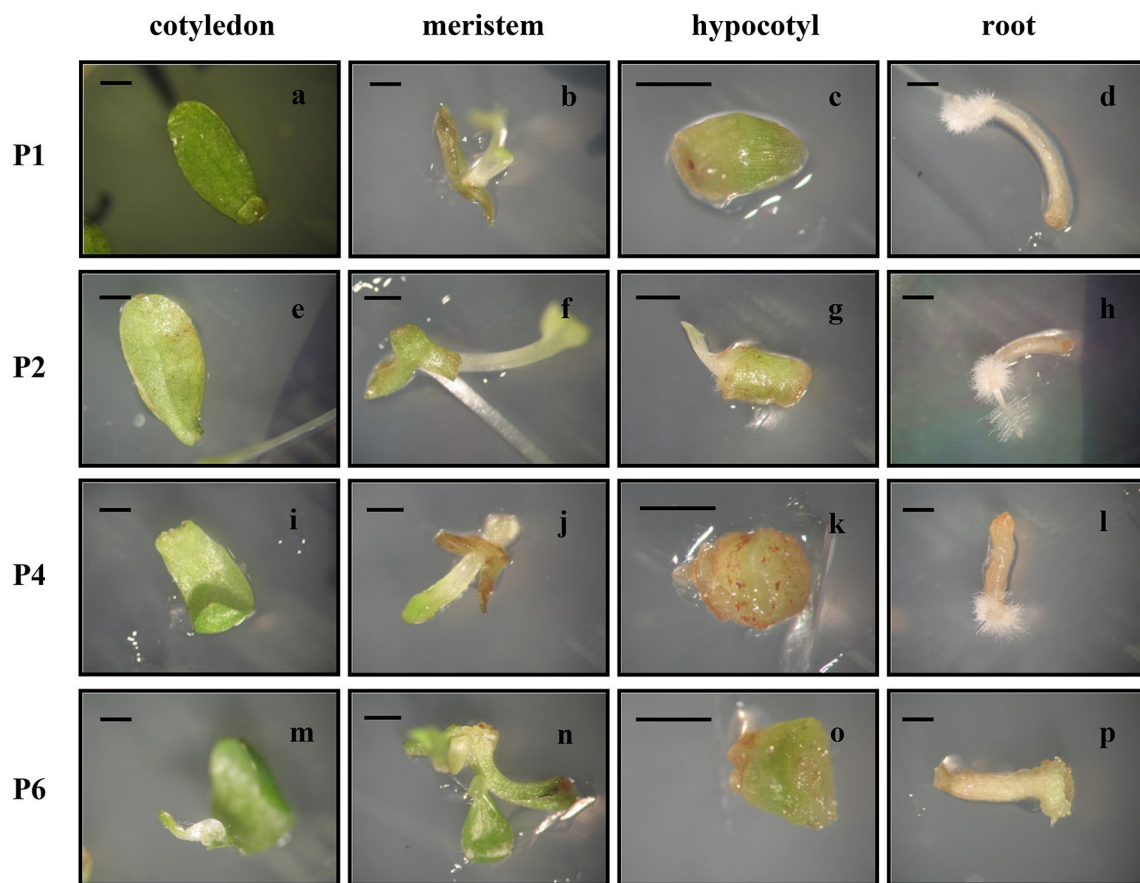
cotyledon	meristem	hypocotyl	root

Fig. 3 The development of *T. belorussicum* explants (**a**, **e**, **i**, **m** cotyledon; **b**, **f**, **j**, **n** meristem; **c**, **g**, **k**, **o** hypocotyl; **d**, **h**, **l**, **p** root) cultured on: P1—½ MS (control), P2—½ MS+PSK, P4—½ MS+IAA+TDZ, P6—½ MS+IAA+TDZ+PSK in 23±2 °C after 7 days of culture (SE/PSK model/short darkness). Scale: 1 mm

of explants, while secondary organogenesis (adventitious shoots formation on the regenerated plants) was noticeable on regenerated plants from meristem explants cultured on ½ MS medium supplemented with IAA, TDZ and PSK (Fig. 4n). All regenerated plants obtained were successfully rooted after 28 days on ½ MS medium supplemented with IBA.

Somatic embryogenesis

In SE/PSK model/short darkness, symptoms of somatic embryogenesis were observed on hypocotyl and cotyledon explants (Fig. 1c II). Somatic embryos were formed directly from explant tissue cultured on all type of ½ MS media supplemented with PSK. The highest efficiency of somatic embryogenesis, in the form of small, light-green globular structures, was observed on ½ MS medium supplemented with TDZ, IAA and PSK with a short time period under darkness (8 days). Formation of somatic embryos was not observed in the organogenesis model and SE/PSK model/

long darkness on all types of explants and in SE/PSK model/short darkness on meristem and root explants.

Role of phytosulfokine (PSK) in the process of organogenesis

The applied conditions in SE/PSK model/short darkness were designed to enhance PSK activity by pre-incubation of explant cultures in the dark for 8 days. Results obtained clearly indicate that the addition of PSK to the medium increased the intensity and efficiency of the organogenesis process. However, the explants were in a poor condition: part of them began to quickly degenerate and, under these conditions, most explants were overgrown with callus (especially the root explants). To improve this, explants were subjected to light after 8 days of incubation in dark. During culture under light conditions, a significant improvement was evident, the color of explants changing from brown yellow to bright and dark green.

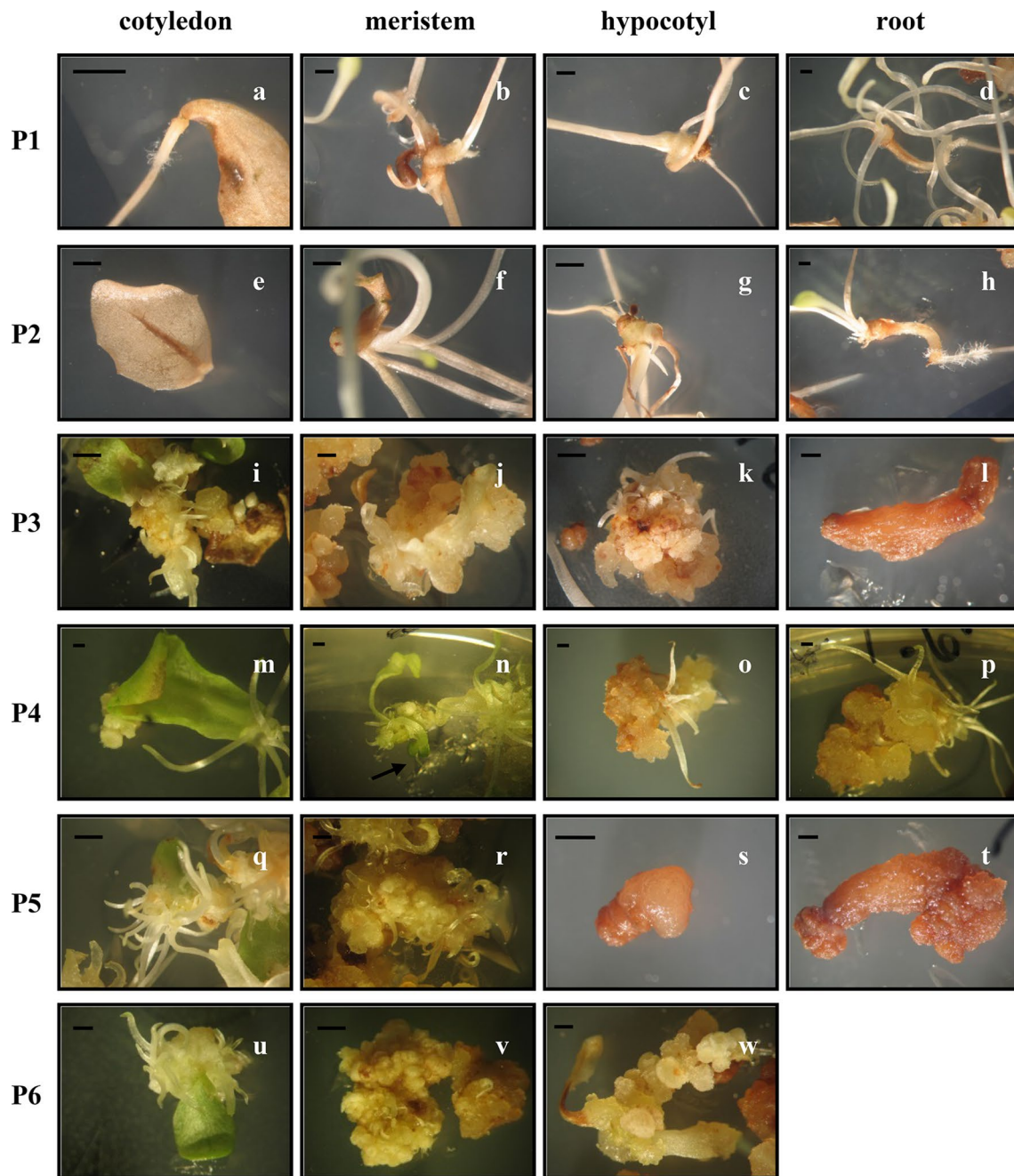The combination of PSK with auxins and cytokinins enhanced the process of indirect organogenesis and had

Fig. 4 The development of *T. belorussicum* explants (**a**, **e**, **i**, **m**, **q**, **u** cotyledon; **b**, **f**, **j**, **n**, **r**, **v** meristem; **c**, **g**, **k**, **o**, **s**, **w** hypocotyl; **d**, **h**, **l**, **p**, **t** root) cultured on: P1—½ MS (control), P2—½ MS + PSK, P3—½ MS + IAA + TDZ, P4—½ MS + IAA + TDZ + PSK, P5—½ MS + TDZ, P6—½ MS + TDZ + PSK in 23 ± 2 °C after 28 days of culture (SE/PSK model/long darkness). Scale: 1 mm

a positive influence on somatic embryogenesis induction. Results obtained from SE/PSK model/long darkness with an extended period of explant culture in the dark (55 days), indicated that selected types of explants had undergone a varied intensity of morphogenesis process depending on the medium used (Fig. 2c). For cotyledon, hypocotyl and meristem explants, the use of a medium without PSK was much more favorable. However, root explants developed better on

a medium with PSK. It should be noted that the used media differed not only in the presence or absence of PSK, but also in the auxin and cytokinins content. Still, the interaction between PSK, auxins and cytokinins did not significantly affect the development of explants. In the case of hypocotyl, meristem and root explants, the use of PSK without
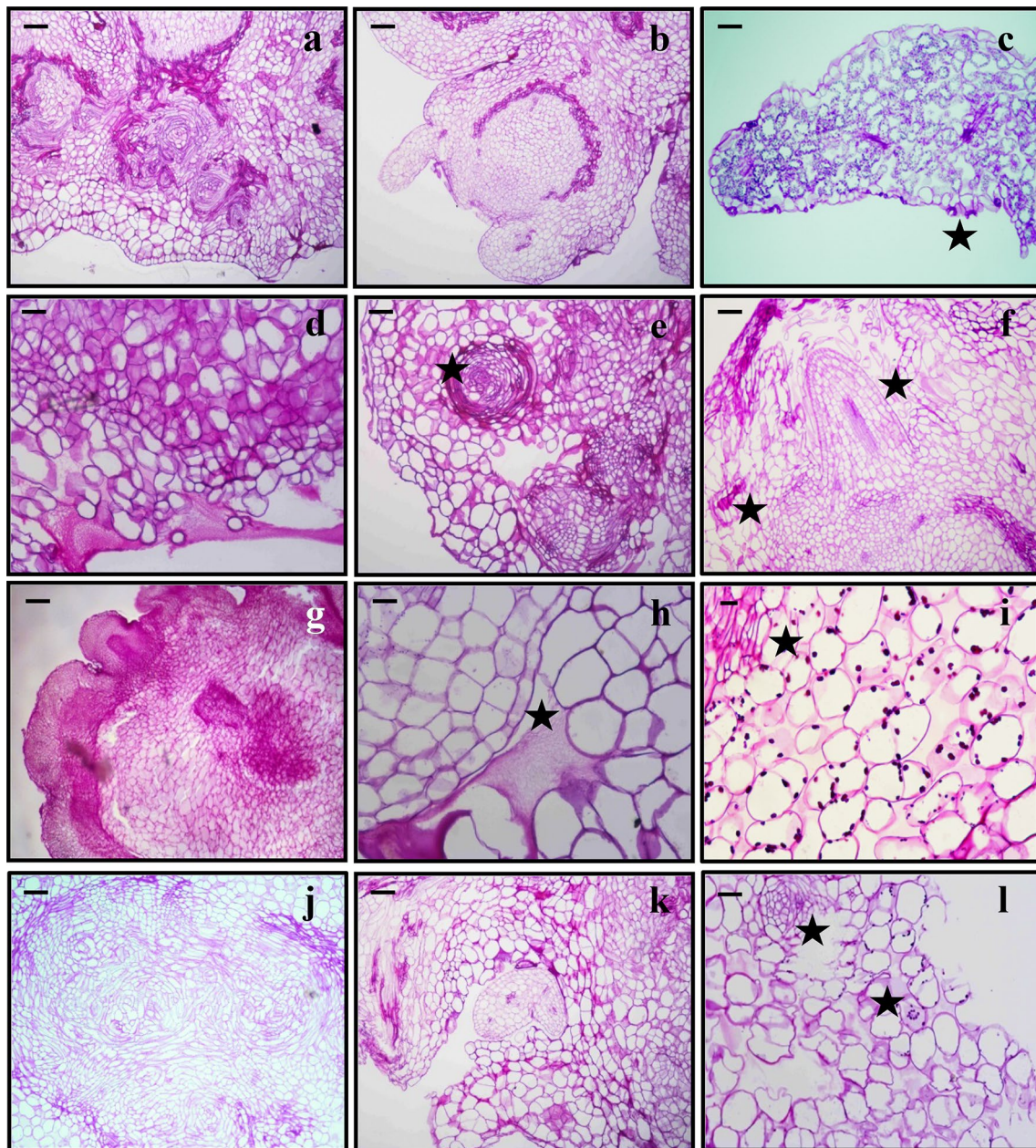
Fig. 5 Cross-section of *T. belorussicum* explants from SE/PSK model/long darkness: cotyledon (**a**, **b**), meristem (**d**, **e**), hypocotyl (**g**, **h**) and root (**j**, **k**) cultured on ½ MS medium supplemented with 5.71 µM IAA, 4.54 µM TDZ and 100 nM PSK in 23±2 °C after 55 days of culture: **a** explant with xylogenic centers, **b** somatic embryo from the cotyledon explant, **d** presence of ECM between cells of the meristem explant, **e** xylogenic centers (asterisk) in meristem explant, **g** morphogenesis on the edges of the explant, **h** ECM between hypocotyl cells with fragment of regenerated plant, **j** adventitious shoots as deformed relicts in the root explants, **k** cross-section through the explant tissue in advanced culture and leaf of regenerated plant, and cross-section of *T. belorussicum* explants from the organo-genesis model: cotyledon (**c**), meristem (**f**), hypocotyl (**i**) and root (**l**) cultured on ½ MS medium supplemented with 5.71 µM IAA and 4.54 µM TDZ in 23±2 °C after 47 days of culture: **c** leaf of regenerated plant with fragment of the vascular bundle, stomata (asterisk) and starch grains in the mesophyll cells, **f** young stage of regenerating leaf (asterisk) with vascular bundle inside and the xylogenic center (asterisk), **i** numerous starch grains in callus cells and the xylogenic center at the young stage of its development (asterisk), **l** varied shape of callus cells, presence of ECM (asterisk) and xylogenic centers (asterisk) at the early stage of formation. PAS reaction. Scale: 100 µm (**a–g**, **j**, **k**), 25 µm (**h**, **i** and **l**)
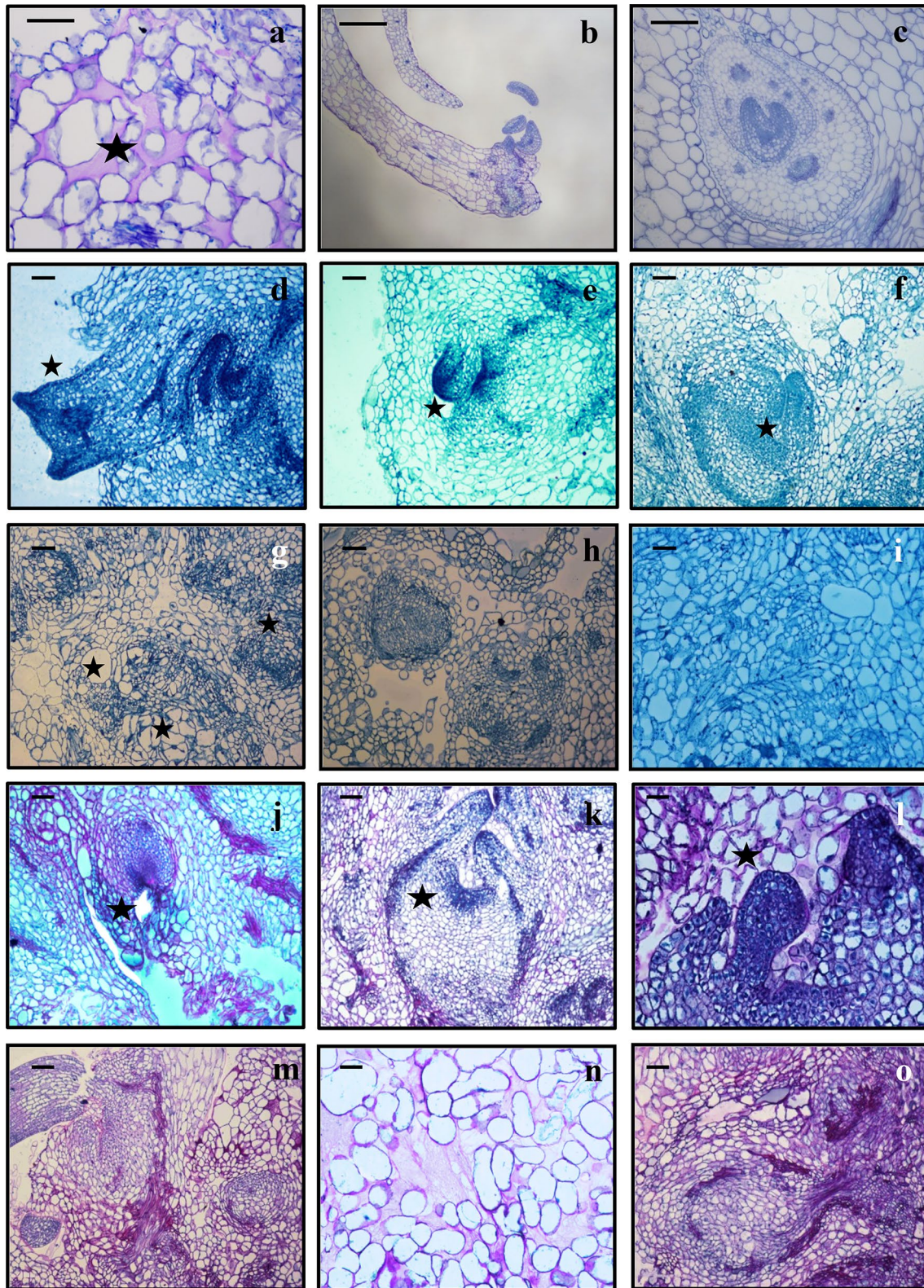
other agents, slightly stimulated the organogenesis process. However, in the case of cotyledon explants, the use of PSK without auxins and cytokinins did not bring a desired effect.

The applied light conditions in SE/PSK model/long darkness were also designed to enhance PSK activity. After 55 days, explants showed a poor condition, most of them

◄**Fig. 6** Cross-section of *T. belorussicum* explants cultured on ½ MS medium supplemented with 5.71 μM IAA and 4.54 μM TDZ from the organogenesis model: meristem (**a**, **e**, **j**), cotyledon (**d**), hypocotyl (**f**, **k**) and root (**l**) after 47 days of culture, and cross-section of *T. belorussicum* explants cultured on ½ MS medium supplemented with 5.71 μM IAA, 4.54 μM TDZ and 100 nM PSK: cotyledon (**b**) and meristem (**c**) after 20 days of culture from SE/PSK model/short darkness, meristem (G), hypocotyl (H, M) and root (I, N, O) after 55 days of culture from SE/PSK model/long darkness. **b** fibrous structure of ECM (asterisk) between callus cells and xylogenic centers, **b**, **c** organogenesis on the 20th day of culture at one of the poles, **d** regenerated plant (asterisk) growing from cotyledon explant and secondary meristematic center, **e** regenerated plant (asterisk) growing from the sub-surface layer of the meristem explant with meristematic center and one leaf, as well as an xylogenic center on the right, **f** regenerated plant from hypocotyl explant, meristematic center (asterisk), two growing leaves and arch of the vascular tissue, **g** cytological differentiation within the meristem, **h** secondary meristematic and xylogenic centers, **i** compact structure of explant with the varied shape of cells, **j** young adventitious shoot, content of protein and the rest of explant epidermis, **k** regenerated plant, apical meristem, two growing leaves and vascular bundles, **l** different shapes of regenerated young leaves, **m** enlargement of the fragment from which adventitious shoot grow, meristematic and xylogenic centers (asterisks), vascular bundles, **n** ECM between callus cells, **o** morphogenesis from pericycle. **a–c** Staining by 0.1% toluidine blue. Scale: 100 μm (**a–c**); **d–i** Protein localization by staining with 0.5% naphthol blue black. Scale: 100 μm (**d–h**), 500 μm (**i**); **j–o** PAS reaction for the presence of starch in explants and staining with 0.5% naphthol blue/black to determine the protein localization. Scale: 25 μm (**n**), 100 μm (**k–o**), 500 μm (**j**, **l**)

began to die. So, all surviving cultures were transferred to the light for the subsequent 35 days. However, the effect of PSK on the formation of callus was noticed while under dark conditions. Observation of explants in the light showed a significant improvement in their condition as they turned from brown yellow to green (due to the restoration of chlorophyll). PSK had a positive effect on somatic embryogenesis (visible on cotyledon explants), but deformed somatic embryos (which were not able to convert) were observed. Such tissues did not produce regenerated plants and the culture was terminated at this stage.

Histological evaluation of selected explants

Explants from the organogenesis model and SE/PSK model/long darkness were selected for histological analysis.

In the organogenesis model, histological analysis confirmed the presence of two types of callus tissue. This differed in the shape and size of cells. The first consisted of loosely distributed cells with various shapes and intercellular spaces (Fig. 5l). The second type was distinguished by a compact structure with meristematic centers and adventitious shoots were formed (Fig. 6e). These centers were mainly present in the subsurface layers, hence confirming that organogenesis proceeded indirectly via callus tissue. Cells in the meristematic centers were characterized by a dense cytoplasm, clearly visible nuclei and thin

cell walls. In addition, to the adventitious shoots, somatic embryo-like structures were observed on the hypocotyl explants. Moreover, in some explants, different stages of regeneration process were observed at the same time of culture (Fig. 6d, e, k). Analyses also documented non-synchronized development of regenerated plants leaves from the meristem explants (Fig. 6l). Still, explants maintained tissue integrity—made evident by the presence of anticlinal cell divisions in the epidermis of meristem explants (Fig. 6j). However, the majority of callus cells were highly vacuolated, in some cases, nuclei with well-visible nucleoli could be observed, as well as various cytokinesis phases. Xylogenic centers were also commonly present among the explants and callus cells (Figs. 5f, i, l, 6a). Vascular tissue was observed in the adventitious shoots at advanced stages of regeneration (Figs. 5f, 6d) and in the root explants as deformed relicts (Fig. 5j). PAS staining showed, in all types of explants, the presence of starch granules accumulating around the meristematic centers, near the cell walls, mainly in the callus cells (Fig. 5i, l). These granules were also quite noticeable in the stomates and the mesophyll tissue of regenerated leaves, as well as in the vascular tissue. This suggests a properly functioning photosynthetic system has developed (Fig. 5c). Their protein content was identified through naphthol blue black staining. The aforementioned granules were highly concentrated in the cells of meristematic centers, at various stages of development (Fig. 6d–f, j–l). Both PAS and toluidine blue staining revealed the presence of an extracellular matrix (ECM) with fibrous, reticular or mucoid structures filling the spaces between the callus cells (Fig. 6a). In contrast to the successful application of these two methods of staining, the ECM was not visible under naphthol blue/black conditions, which makes evident its polysaccharide and non-protein character.

In the SE/PSK model/long darkness, histological analysis of the cotyledon explants confirmed indirect organogenesis and the induction of indirect somatic embryogenesis (Fig. 5a, b). Results revealed structures similar to somatic embryos formed from explant tissues overgrown with endogenous callus (Fig. 6b). These structures were deformed at the later stage of development. The PAS staining showed clearly visible vascular bundles in the embryo cotyledon explant and the absence of starch in explant and embryo tissues (Fig. 5b). Secondary xylogenic centers were seen within the mesophyll, the explant epidermis was noted to be continual (Fig. 5a) and lobed nuclei cells were noted in explant tissues and regenerated plants. Naphthol blue/black staining showed a concentration of the dye in the cells of the developing regenerated plant, in the top region of explant. This effect may indicate formation of the apical meristem. Double staining with PAS and naphthol blue/black revealed the presence of secondary meristematic centers next to
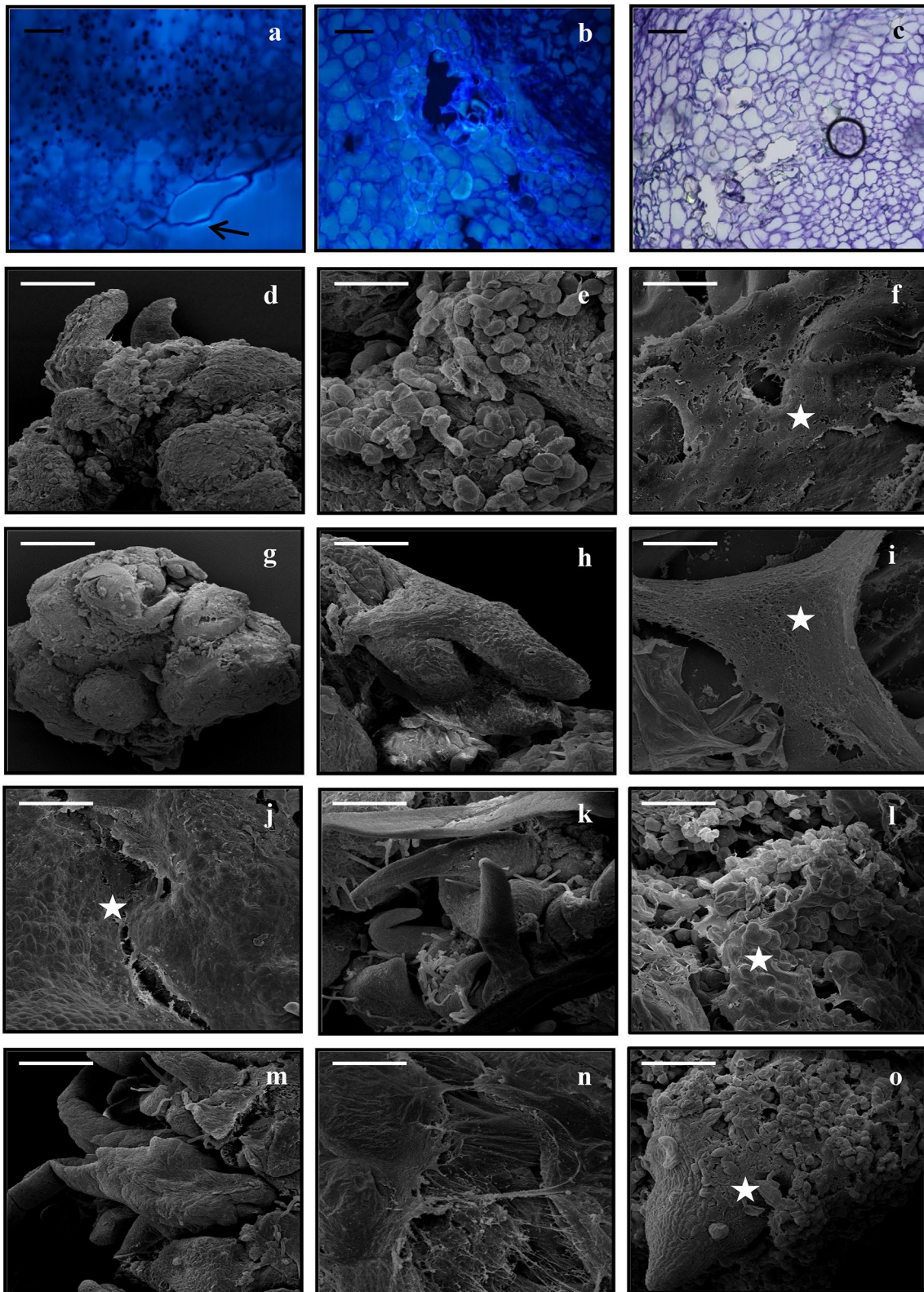
the formed adventitious shoot on the explant during callus formation. Based on the continuity of the epidermis, the presence of endogenous callus was indicated. Moreover, well-formed long vessels were observed in the place where secondary meristematic centers were formed, while well-visible nuclei and dense cytoplasm were reported in small

◄**Fig. 7** Cross-section of *T. belorussicum* explants: hypocotyl (**a**–**c**, **j**–**k**), cotyledon (**d**–**f**), meristem (**g**–**i**) and root (**m**–**o**) cultured on ½ MS medium supplemented with 5.71 µM IAA, 4.54 µM TDZ and 100 nM PSK in 23±2 °C after 20 days (**a**–**c**) and 55 days (**d**–**o**) of culture from SE/PSK model/short darkness: **a** peripheral cells with callose in the cell wall (arrow), **b** the presence of callose in the cell wall (arrows), **c** control (from the light microscope), and from SE/PSK model/long darkness: **d** organogenesis, **e** callus cells, **g** regenerating plantlets from endogenic callus, **h** organogenesis at various stages of development, **j** extensive amounts of ECM covering cells of explant, **k** adventitious shoots and trichomes, **m** indirect organogenesis, **n** fibrous structure of ECM, and cross-section of *T. belorussicum* explants: cotyledon (**f**), meristem (**i**), hypocotyl (**l**) and root (**o**) cultured on ½ MS medium supplemented with 5.71 µM IAA and 4.54 µM TDZ in 23±2 °C after 45 days of culture from organogenesis model: **f** the lobe structure of ECM, **i** dense network of ECM, **l** abundant callus coated with the ECM, **o** regenerated plantlet growing from callus tissue of explant. **a**–**c** Staining with 0.1% toluidine blue and 0.05% aniline blue to detect the callose. Scale: 100 µm. **d**–**o** Scanning electron microscope. Scale: 13 µm (**i**), 30 µm (**f**), 180 µm (**j**, **l**, **n**), 200 µm (**k**), 300 µm (**e**, **g**), 400 µm (**o**), 600 µm (**d**), 1000 µm (**h**, **m**)

cells surrounding vessels. This effect indicates the meristematic nature of these cells. Such aforementioned changes observed on the cross-sections are mostly noticeable in the cotyledon's mesophyll. Still, despite many alterations, the tissue maintained compact nature and cells adhered closely to each other.

Histological analysis of the meristem explant showed the presence of secondary meristematic centers, but the continuity of tissues was not maintained. Many intercellular spaces were ECM enriched (Fig. 5d). However, continuity of epidermis was not maintained, and an exogenous callus was observed next to endogenous callus (Fig. 5e). Differences in size and shape of cells, the presence of many cytokinesis at various stages of advancement and the formation of xylogenic centers were observed (Fig. 6g). Cells stained with PAS and naphthol blue/black showed that these were viable. This was confirmed by the presence of proteins and the accumulation of starched in these cells, as well as their abundant cytoplasm. The appearance of shoots from the meristematic tissue and the presence of the first younger leaves were observed (Fig. 6c), while double staining with PAS and naphthol blue/black revealed the structure of the tissue surrounding the meristematic tissue. Herein, circular abundant meristematic and xylogenic centers, dense cytoplasm and large dividing nuclei were noted in the meristematic tissue—indicating high mitotic activity. The differentiation of callus parenchyma cells into meristematic tissues was also seen. This is a natural development.

Histologic analysis of the hypocotyl explant revealed changes in the axial roller and parenchyma of the primary cortex cells (Fig. 5g and h). Endogenous callus with large spaces between cells was also present within the primary cortex (Fig. 6h). Double staining with PAS and naphthol blue/black showed lack of epidermis continuity, abundant endogenous and exogenous callus. Moreover, meristematic and xylogenic centers had formed from subepidermal cells of callusing explant and were located inside and on the edge of the callus. The formation of adventitious shoots and somatic embryos on the explant was also noted in the meristematic centers formed in the callus (Fig. 6m). In addition, the callus was heterogenous, with different size and irregular shapes of cells. At the base of the forming adventitious shoots, new young shoots were seen to have sprouted—indicating the unsynchronized nature of indirect organogenesis.

Histological analysis of the root explant revealed a disorder in its anatomical structure, as well as large intercellular spaces, lack of epidermis continuity and presence of vascular bundles. The structure of parenchyma of the primary cortex was of compact nature. Changes in the explant's construction, the presence of meristematic centers, cell aggregates and formation of callus were observed at the later stage of explant development (Fig. 5k). Furthermore, well-developed vessels arranged in a concentric way and the explant being overgrown with callus were evident. The cross-section through the root explant also showed cytological changes characterized by different size and shapes of cells (Fig. 6i). Double staining with PAS and naphthol blue/black revealed the presence of ECM between the callus cells (Fig. 6n), while formation of adventitious shoots and the connection of explant's vascular bundle with the regenerated plant's vascular bundle were observed (Fig. 6o).

The presence of callose in explant cells

Analysis of histological samples revealed a weak presence of callose only in tissues of cotyledon and hypocotyl explants, but not in cells of root and meristem explants. In the hypocotyl explant, callose fluorescence was noted in the cell wall of peripheral cells of callus (Fig. 7a) and in the cell wall of the few parenchyma of the primary cortex cells (Fig. 7b). However, the signal of callose was uneven—this being associated with progressive deposition of callose in the cell wall.

Analysis of explant organogenesis using scanning electron microscope (SEM)

Analysis of cotyledon's explants surface indicated the presence of adventitious shoots, showed the lack of continuity of epidermis (Fig. 7d) and revealed a variety of callus cell shapes formed in some parts of the explant (Fig. 7e, l). On the surface of the adventitious shoot growing out from the meristem explant, clearly visible stomata and various callus cells were noticeable. Furthermore, the extracellular matrix with a mantle structure was seen where adventitious shoots were forming (Fig. 7f, g, i). Observation also indicated the presence of cotyledons on the surface of the explant at an advanced stage of development, with cells visible on its

surface (Fig. 7h). Moreover, organogenesis and ECM formation on callus surface were noted on the hypocotyl explant (Fig. 7j), while single, hairline and single-celled structures were visible on the regenerated plants and explants, as were shoots at various stages of development. Apical meristem structures and single fibers at the youngest stage of development were observed (Fig. 7k). What is more, follicular callus, fibers, well visible fibrous of ECM and regenerated plants at various stages of development were observed on the root explant (Fig. 7m, n). In addition, analysis confirmed the occurrence of indirect organogenesis via callus (Fig. 7o).

Discussion

Existing world literature devoted to representatives of the genus *Taraxacum* is rather poor and mostly focused on their therapeutic properties (Ahmad et al. 2000; Chunga et al. 2018), developmental and embryological processes (Janas et al. 2016), and their application in in vitro culture conditions (Ermayanti and Martin 2011; Tuleja et al. 2014a).

T. belorussicum is an interesting obligatory apomictic species (Rodkiewicz et al. 1996) and was the object of rare embryological analysis (Małecka 1965, 1982; Janas et al. 2016). Preliminary studies of in vitro plant cultures (Tuleja et al. 2014a) revealed direct organogenesis without forming callus, as well as slight somatic embryogenesis on explants from immature parthenogenetic embryos. However, the obtained results did not confirm the occurrence of direct organogenesis. The same effect was also observed in other species e.g. *T. officinale* Weber (Chen et al. 2005). One of the possible reasons for such result can be the difference in explant origin along with their developmental stage (Kumar and Chandra 2010). The aforementioned experiments were conducted on explants derived from 2-week-old seedlings. It seems that the age of *T. belorussicum* explants plays a crucial role in SE induction and this developmental pathway is involved mostly in younger plant material (Tuleja et al. 2014a). Even the application of PSK, the peptide responsible of somatic embryogenesis induction (Igasaki et al. 2003), did not break this recalcitrance. However, slight signals of SE activity were detected in 2-week-old seedlings explants, and this was additionally confirmed by the callose presence, treated as a SE prerequisite (Dubois et al. 1989; Tao et al. 2012), as well as the first symptom of embryogenic cell potential (Fiuk and Rybczyński 2006).

The present study showed significant differences in suitability between the applied *T. belorussicum* explants. In the organogenesis model (under light condition), the best morphogenetic response was noted in hypocotyl explants, whereas, hypocotyl and meristem explants had greater response in the SE/PSK model/short darkness. The hypocotyl is employed as a promising type of explant in many studies for the induction of efficient organogenesis and somatic embryogenesis (Jach and Przywara 2000). On the other hand, cotyledon and meristem explants in the SE/PSK model/long darkness revealed better morphogenetic response than did hypocotyl and root explants after 30 and 65 days of culture under an extended period of time in dark conditions. These differences suggest that all types of explants, despite their common origin from one plant, could give various morphogenetic responses depending on the combination of growth conditions and the plant regulators used.

The high morphogenetic ability of all types of *T. belorussicum* explants was additionally confirmed by the noticeable presence of secondary organogenesis on the meristem-regenerated plants. However, it is worth noting that under the applied in vitro conditions, the time of culture for each type of explant was not a factor limiting their viability and morphogenetic potential. This indicated the high regenerative potential of this species and the suitability of this plant for research in tissue culture field experiments.

A characteristic feature of all types of *T. belorussicum* explants employed in the applied experimental models was the relatively short time of response to the used in vitro conditions. This effect is desirable for in vitro culture of that species. It has already been demonstrated in earlier studies on *T. belorussicum* (Tuleja et al. 2014a) and is not a common feature among plants exposed to such conditions, e.g. *Cephalotus follicularis* reacts scarcely after 47 days of in vitro culture (Tuleja et al. 2014b).

The in vitro plant regenerative capacity is influenced by genetic properties and by several environmental conditions e.g. the nutrient compositions and the exposure to light. Generally, the light impact on regeneration process appears to be highly context dependent (Ikeuchi et al. 2016), light can trigger organ regeneration (Saitou et al. 1992) or inhibit root or shoot regeneration in some plants (Bellini et al. 2014). The incubation in dark conditions increased the organogenetic capacity of *T. belorussicum* hypocotyls and especially of the cotyledons. A similar effect of light was reported in *Arabidopsis* cotyledons (Nemeth et al. 2013).

Enhancement of in vitro plant regeneration capacity can be achieved by the use of appropriate media supplemented with PGRs. The combinations of IAA and TDZ used in these experiments were sufficient to induce organogenesis. A similar combination of the same PGRs for *Cichorium intybus* L. also resulted in a high level of morphogenesis (Yucesan et al. 2007). Still, data shows that the use of ½ MS without growth regulators is sufficient for rooting *Plantago asiatica* (Makowczyńska and Andrzejewska-Golec 2003). However, this approach does not work in *T. belorussicum*. Therefore, the use of ½ MS medium supplemented with IBA to achieve the effective rooting of *T. belorussicum* regenerated plants turned out necessary. This fact was confirmed by studies on *Taraxacum officinale* Weber (Jamshieed et al. 2010).

The addition of PSK increased the efficiency of *T. belorussicum* organogenetic potential, as also reported from *Pisum sativum* and *Vicia faba* explants (Ochatt et al. 2018). Data showed that the use of PSK induced callus in *Oryza sativa* and *Beta vulgaris* protoplasts (Grzebelus et al. 2012), as well as cell proliferation in maize, rice, carrots or asparagus, and stimulated cell division in these plants (Yang et al. 2000). The obtained results from SE/PSK model/short darkness are associated with data on the positive impact of PSK on the morphogenesis process. In the case of *T. belorussicum*, the positive effect of PSK on the organogenesis process was not confirmed in SE/PSK model/long darkness. Therefore, the effect of PSK on *T. belorussicum* explants seems to be more favorable when the incubation time in the dark is reduced to a few days. This feature is perhaps characteristic for *T. belorussicum* and may be correlated with the preferences of this plant towards light conditions. Thus, the longer incubation time in the dark weakens the condition and morphogenetic potential of explants. PSK, used alone, stimulated mainly meristem, hypocotyl and root explants. In contrast, the use of PSK with auxins and cytokinins enhanced the morphogenesis process, mainly indirect organogenesis, in SE/PSK model/short darkness. This fact was confirmed by data showing that the expression of biological activity of PSK correlates with the pathway of PSK signaling via auxins and cytokinins (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1999). It was observed as the formation of adventitious roots on cucumber hypocotyls (Yamakawa et al. 1998a) and adventitious buds in *Antirrhinum majus* (Yang et al. 1999). However, this dependency did not apply to SE/PSK model/long darkness and did not enhance morphogenesis of *T. belorussicum*.

Observation of explants showed the formation of callus after the addition of PSK. This enhanced the development of callus and significantly weakened the condition of explants. Thus, PSK which promoted the synthesis of chlorophyll in cucumber cotyledons (Yamakawa et al. 1998b), could have allowed the explants to overcome the unfavorable conditions due to the lack of light. In addition, PSK is noted to affect cell viability (Yamakawa et al. 1999) and to reduce the occurrence of albino individuals, which confirms its participation in chlorophyll synthesis through increasing photosynthesis efficiency and assimilating production (Asif et al. 2014).

Incubation with PSK in darkness affects the induction of somatic embryogenesis on hypocotyl and cotyledon explants. These results confirm the role of PSK in increasing the intensity of somatic embryogenesis of *Cryptomeria japonica* (Igasaki et al. 2003), *Daucus carota* (Kobayashi et al. 1999; Maćkowska et al. 2014) and pea (Ochatt et al. 2018). However, it seems that the induced somatic embryos of *T. belorussicum* become deformed after longer culture times, probably as a result of callus development and do not generate plantlets. In the explants, the use of different exposure periods to light and dark conditions affects the variable level of PSK which, in turn, influences morphogenesis at initial stages of cell de-differentiation, proliferation and re-differentiation. Thus, the activity of PSK was similar to the activity of plant growth and development regulators (Matsubayashi et al. 2004). Moreover, the manner of PSK function in *T. belorussicum* cultures probably did not differ from the function presented in our data, subject to taking into account the short incubation time in the dark.

Induction of callus formation can occur from any initial explant. For this purpose, appropriate amounts of growth regulators should be applied (Sugiyama 2000). Previous studies showed that the use of a combination of IAA and TDZ for cotyledon explants of *Pelargonium rapaceum* has a positive effect on callus induction and proliferation (Sukhumpinij et al. 2010). This fact was confirmed in the present study, wherein callus formation occurred more or less intensively depending on the combination of IAA, TDZ and PSK.

Histological analysis confirmed the indirect course of organogenesis and showed an increased occurrence of starch and proteins in cells of explants near the conducting elements, the effect of which indicates the energetic role of these substances, especially starch, in organogenesis (Karim et al. 2006; Pei-Lang et al. 2006). The presence of starch in the leaves of regenerated plants also revealed their photosynthetic activity, especially in SE/PSK model/short darkness. The most significant accumulation of proteins was observed in the vicinity of secondary meristematic centers, which demonstrates the high metabolic activity of these cells (Fisher 1968). In this work, the histological analysis of meristem explants revealed the occurrence of two types of organogenesis: one is based on the native development and can be assigned to the meristematic cells of the apical meristem, while the second is released through the reprogramming of the differentiated somatic cells located in the tissue surrounding the apical meristem. Both processes rely on the phenomenon of cellular plasticity, which can be defined as the ability to re-specify cell fate (Ikeuchi et al. 2016).

The presence of ECM singled out by histological analyzes and SEM on the surface of the hypocotyls and roots of *T. belorussicum* may be the result of a stress response to applied in vitro culture conditions. According to current data, ECM can be formed in response to stress factors such as tissue culture conditions or the chemical agents used to protect callus cells (Bevitori et al. 2013). It seems that the extracellular matrix (ECM) was formed to create a path for the receiving and transmitting signals that determine the recognition of cells and morphogenesis process. Until recently, ECM was considered as a marker of somatic embryogenesis in *Cocos nucifera*, *Zea mays*, *Drosera* sp. and *Papaver* sp. (Namasivayam 2007), because the cells

acquired embryogenic competence in the presence of ECM. Currently, data suggest that the presence of ECM in in vitro culture is a specific feature of morphogenesis (Popielarska-Konieczna et al. 2013) and of morphogenetic callus formed in in vitro conditions. Of note, it may also have a protective function.

The plants obtained in in vitro cultures were in very good condition, thus they easily acclimated to the ground. The valuable result of these experiments is obtaining somatic embryos and enabling their comparative analysis with apomictic embryos.

Conclusions

Analyzes of selected *Taraxacum belorussicum* explants revealed that this plant showed morphogenetic abilities directed towards indirect organogenesis. Results have shown that the indirect organogenesis was not synchronized. Still, the in vitro culture conditions, through the application of IAA, TDZ and PSK factors, were sufficient for developing an efficient innovative regenerative system for each type of explants. Taking into consideration results of the study, hypocotyl explants seem to be the most promising in inducing somatic embryogenesis. In addition, observation of the culture effects revealed that callus formation is characteristic and separate for the particular type of *T. belorussicum* explant. Moreover, the time of *T. belorussicum* explant culture was not a limiting factor for their viability and morphogenetic potential. This indicates the high suitability of this species for use in in vitro experiments. Results from this study conducted on selected types of *T. belorussicum* explants also confirmed the role of PSK in the process of morphogenesis (organogenesis and somatic embryogenesis). Herein, the short incubation of *T. belorussicum* explants in PSK-enhanced culture, under short conditions of darkness, is most beneficial for organogenesis efficiency.

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