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Influence of plant growth regulators on *in vitro* regeneration of *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk

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Abstract

Saposhnikovia divaricata (Turcz. ex Ledeb.) Schischk is a medicinal plant with a broad spectrum of pharmacological activities, which are attributed to the presence of various bioactive compounds, including chromones. The uncontrolled harvesting of this plant has resulted in the depletion of its natural resources. The ability of *S. divaricata* to regenerate *in vitro* was studied. Aseptic culture was established by sterilizing seeds and germinating them, both with and without the pericarp. Adventitious shoot formation through direct morphogenesis was achieved on Murashige and Skoog medium in the presence of different cytokinins: benzylaminopurine, *meta*-topolin, and thidiazuron, at concentrations of 0.1 µM, 0.5 µM, and 1.0 µM. It has been determined that the most preferable explants were the cotyledonary nodes of aseptic seedlings, as they enable the rapid production of 1.4 ± 0.5 to 10.2 ± 2.0 microshoots per explant, depending on the type and concentration of the cytokinin used. Successful regeneration also occurred from the first true leaves of seedlings *in vitro*. Microshoots were formed directly from the explant tissues, with their quantity ranging from 1.7 ± 0.7 to 4.4 ± 0.9. The highest number of microshoots was induced by thidiazuron, but after cultivation with *meta*-topolin, the regenerated plants showed the highest frequency of rooting, ranging from 66 to 97%. Somatic embryoids were obtained when the first true leaves were cultivated with 2,4-dichlorophenoxyacetic acid (10.0 µM). Morphogenesis occurred *via* the indirect pathway (through the callus formation stage), accompanied by the formation of up to 24.2 ± 6.1 somatic embryoids, with a rooting frequency reaching 96%. These studies provide a preliminary basis for developing a protocol for the clonal micropropagation of *S. divaricata.*

Keywords *Saposhnikovia divaricata* · Fangfeng · Direct organogenesis · Indirect somatic embryogenesis · Cotyledonary nodes · First true leaves

Introduction

Saposhnikovia divaricata (Turcz. ex Ledeb.) Schischk is a perennial herbaceous plant of the *Umbelliferae* family that exhibits a wide range of pharmacological activities due to the accumulation of polyacetylene compounds, coumarins, polysaccharides, and chromones primarily in its roots (Kreiner *et al*. 2017). *S. divaricata* has been demonstrated to exhibit anti-inflammatory, antimicrobial, antifungal, antitumor, antiallergic, and antioxidant activities (Kreiner *et al*. 2017; Wang *et al*. 2017; Yang *et al*. 2017; Kim *et al*. 2018). Currently, *S. divaricata* is threatened in its natural populations due to uncontrolled harvesting of its roots, coupled with the difficulty of propagating it using traditional methods. At present, seed propagation is the main method used to cultivate this plant. However, this method is characterized by low seed germination rates (less than 50%), which can be slightly improved (57 to 75%) by the use of scarification, thermal stratification, or cold stratification (Zhou *et al*. 2009; Dou *et al*. 2010; Ahn *et al*. 2012). Another challenge to outdoor cultivation of *S. divaricata* is root rot caused by *Fusarium equiseti*, which affects more than 15 to 20% of plants (Zeng 2017). Consequently, the development of an *in vitro* regeneration system for *S. divaricata* using biotechnological methods

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is of great significance. The studies focused on the *in vitro* regeneration of *S. divaricata* have predominantly described an indirect pathway of morphogenesis (through a callus formation stage) leading to the development of somatic embryos (Sheng and Chen 1990; Ma *et al.* 2005; Wang 2005; Qiao *et al.* 2009). Nevertheless, there is currently no available data on direct regeneration (directly from explant tissues) or the production of adventitious microshoots for this plant. The efficacy of clonal micropropagation is contingent upon the specific plant growth regulator (PGR) selected and its concentration, which must be optimized for each plant species and genotype. This paper presents the results of investigating the effects of various cytokinins, as well as auxin on the morphogenesis and regeneration of *S. divaricata.*

Benzyladenine (BA) is one of the most commonly utilized PGRs in the *in vitro* cultivation of a diverse range of plants (Zhang *et al.* 2016). For this reason, its influence on the morphogenesis of *S. divaricata* was also investigated. However, BA is known to have the potential to negatively impact the proliferation, growth, and quality of microshoots in some plant species (George *et al.* 2008; Van Staden *et al.* 2008). As an alternative to this compound, its hydroxylated derivatives such as 6-(3-hydroxylbenzylamino)-purine or *meta*-topolin (*m*-T) can be used. It was first isolated from the leaves of *Populus canadensis* (Strnad *et al.* 1997), which is considered a stable form of cytokinin. These derivatives can rapidly convert to active cytokinins when needed. Such reversible changes in the *O*-glucosides allow the cytokinin to remain physiologically active for a longer period of time, resulting in a better culture response (Werbrouck *et al.* 1996; Strnad 1997). Thidiazuron (TDZ) is of interest because it has potent cytokinin-like activity even though, unlike natural cytokinins, it is not an adenine derivative. Its unique property is the ability to exhibit both cytokinin- and auxin-like activities simultaneously (Murthy *et al.* 1998). TDZ has a high morphogenic potential, including the proliferation of axillary and adventitious shoots as well as somatic embryoids (Dewir *et al.* 2018). 2,4-Dichlorophenoxyacetic acid (2,4-D) is a synthetic auxin that is also one of the most commonly used PGRs in the *in vitro* regeneration of various plants. 2,4-D can be used alone or in combination with other PGRs (Gaj 2004). The objectives of the study were to (1) establish a well-growing aseptic *in vitro* culture of *S. divaricata* using mature seeds as explants; (2) assess the effects of various cytokinins, including benzyladenine (BA), *meta*-topolin (*m*-T), and thidiazuron (TDZ), as well as an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), on the morphogenesis and regeneration of *S. divaricata* from different types of explants (cotyledonary and first true leaves, cotyledonary nodes) under *in vitro* conditions.

Materials and methods

Plant materials Mature second-generation seeds (harvested in 2022) were collected from an experimental plot of Central Siberian Botanical Garden SB RAS. These seeds were used as explants for introduction into an *in vitro* culture. Initially, this species was introduced from seeds collected in 2016–2017 in the vicinity of Mount Spyashchy Lev (Russia, Buryatia, Tarbagatai District). The plant material was sterilized using a 0.1% $AgNO_3$ (LenReaktiv, Saint Petersburg, Russia) for 10 min, followed by triple rinsing with sterile water. Seeds were germinated *in vitro* either with or without pericarp. Cultivation was carried out on hormone-free ½ Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with 3.0% sucrose (Shostka Chemical Reagents Plant, Shostka, Ukraine) and 0.6% agar (PanReac, Castellar del Vallès, Spain).

Initiation of direct regeneration of microshoots Aseptic seedlings with two cotyledon leaves and one pair of first true leaves were dissected using forceps and a scalpel and subsequently used to obtain explants. Cotyledonary nodes with apical growing point, cotyledonary leaves, and first true leaves were separated and cultivated on MS medium, which was supplemented with growth regulators. The following growth regulators were used: BA, TDZ (or N-phenyl-N′-1,2,3-thiadiazol-5-ylurea), and *m*-T at concentrations of 0.1, 0.5, and 1.0 µM. All cytokinins were purchased from Sigma-Aldrich (St. Louis, MO). A control experiment was conducted by incubating explants on hormone-free MS medium. The duration of the passage was 30 to 45 d.

Induction of callus formation and somatic embryogenesis Callus formation was induced by cultivating the first true leaves of aseptic seedlings on MS medium supplemented with 10.0 µM 2,4-D (Sigma-Aldrich) and 0 or 5 µM BA in a dry-air thermostatically controlled chamber TV-80–1 ("Smolensk SKTB SPU", Smolensk, Russia) in the dark at a temperature of 24 ± 1 °C. Cultivation in the dark was conducted over four 30-d passages. As a control experiment, first leaves were cultivated on hormone-free MS medium. The obtained calluses were transferred to MS medium supplemented with either 0 or 5 µM BA and cultivated under light conditions over three 30-d passages.

Histological analysis Histological analysis was conducted exclusively on leaf explants. For this purpose, three samples were taken for each of the studied media (containing the PGRs at different concentrations). The material for analysis was fixed in formalin (40%), glacial acetic acid (99.9%), and ethyl alcohol (96%) in proportions 7:7:100 (v/v/v) for 5 to 7 d, then rinsed and stored in 70% ethyl alcohol. The

samples were dehydrated by successively immersing them in ethanol, a mixture of ethanol and chloroform, and then pure chloroform. Finally, the samples were immersed in Paraplast (Sigma-Aldrich). Morphogenesis was studied in permanent mounts (Pausheva 1988). Thin Sects. (7 μm) were cut on a rotary microtome (Microm HM-325, Walldorf, Germany), mounted on slides, and stained with Ehrlich's hematoxylin, followed by counterstaining with Alcian Blue. Histological analysis was performed using a software-controlled light microscope Axioskop-40 (Carl Zeiss, Gottingen, Germany) equipped with a digital camera. Morphogenetic structures obtained during *in vitro* cultivation were analyzed using a stereomicroscope SteREO Discovery V 12 (Carl Zeiss).

Microshoot elongation and rooting *in vitro* Microshoot elongation and rooting *in vitro* were performed on hormone-free MS medium for 30 to 45 d. The degree of regeneration, rooting frequency (%), and the number of shoots per explant were recorded at the end of each stage. All cultures, except the callus induction stage, were incubated under a 16-h photoperiod, with a light intensity of 50 μmol m^{-2} s^{-1} and a temperature of 23 ± 2 °C.

Statistical analysis Each medium, containing either of the PGRs at varying concentrations, was employed to cultivate between 10 and 50 explants. All experiments were conducted in triplicate. In each experimental variant, 10 to 50 explants were used. Multiple comparisons were performed using one-way ANOVA followed by Tukey's HSD test to assess the significance of differences among the means. Statistical analysis and data interpretation were performed using Microsoft Excel 7.0 and Statistica 8. The accepted significance level was set at $P \leq 0.05$, and the data were presented as means ± standard errors (M ± m).

Results and Discussion

Seed germination *in vitro* Germination of sterile intact seeds and seeds with removed pericarp was characterized by different rates of seedling emergence. Intact seeds began to germinate only on the 29th d of cultivation (total germination rate was 8.8%), while for seeds with removed pericarp, germination was observed on the 14th d (total germination rate was 66.7%). The data on germination period *in vitro* were consistent with the findings of another study on the germination of the same seeds under laboratory conditions on a combined substrate (quartz sand and paper filter) (Elisafenko *et al*. 2023). In that study, the period was also approximately 30 d. However, the germination rates of intact seeds differed significantly between *in vitro* and laboratory conditions, with the latter being 4.7 to 8.1 times higher. At the same time, the germination rate was, on average, comparable to that of seeds with the pericarp removed *in vitro*. According to the study by Milentyeva *et al*. (2021), *S. divaricata* seedlings emerged from intact seeds *in vitro* after 6 to 8 wk. Removal of the pericarp accelerated the germination process by eliminating the influence of exogenous dormancy factors, removing mechanical barriers, and maximizing water availability. At the same time, the seedlings exhibited a normally developed hypocotyl, epicotyl, two cotyledons, apex, and root.

Development of microshoots To evaluate the regenerative capacity of explants in response to various PGRs, the resulting seedlings were divided into parts (cotyledonary nodes with growing point, cotyledonary leaves, and first true leaves) (Table 1). Cotyledonary nodes were used as explants because they contain intercalary meristems with primary embryonic cells, which can directly induce a large number of shoots through organogenesis (Paz *et al*. 2006), even in the absence of PGRs. In this study, the effects of the following compounds on *in vitro* morphogenesis were investigated: BA and *m*-T (cytokinins of the aminopurine series), TDZ (a derivative of diphenylurea), 2,4-D (a herbicide with auxin activity, a derivative of phenoxyacetic acid; used alone or in combination with BA).

A morphogenic response was observed in 100% of the explants when cotyledonary nodes with growing point were cultivated using all types and concentrations of PGRs tested. Increasing the concentration of BA in a medium was accompanied by an increase in the number of microshoots per explant (Table 1), as well as increased callus formation at the base of the microshoots. Increasing BA to 1 μM resulted in purple pigmentation, abnormal development of 42% of the microshoots, and their vitrification.

Increasing the concentration of *m*-T in a medium when cultivating cotyledonary nodes led to an increase in the number of microshoots per explant from 1.4 to 6.4. Incubation of cotyledonary nodes on media with *m*-T resulted in the formation of long thin leaves. The formation of callus at the base of the microshoots was not observed in the medium containing *m*-T, but spontaneous rhizogenesis increased with increasing concentration of this substance. At the same time, a large number of drying leaves on the microshoots was observed from around the middle of the passage, both on media with BA and with *m*-T.

It is believed that *m*-T enhances the reproduction rate and quality of *in vitro* regenerated plants (Ptak *et al*. 2013; Jayaprakash *et al*. 2021). Our data indicated that at low concentrations of a PGR (0.1 and 0.5 μM), the reproduction rate was higher on media supplemented with BA, whereas at higher concentrations (1.0 μM), it was higher with *m*-T. This suggests that the effects of higher concentrations of these PGRs on the micropropagation of *S. divaricata* should be further investigated. The development of the highest number

Table 1. Regeneration of microshoots and somatic embryos from different types of explants of *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk under the influence of various types of plant growth regulators (PGRs): benzyladenine (BA), *meta*-topolin (*m*-T), thidiazuron (TDZ), and 2,4-dichlorophenoxyacetic acid (2,4-D) at different concentrations

PGR treatments (μM)	Explant used	Morphogenic response (%)	Structure type	Number of structures per explant*	Frequency of rooting (%)**
0 (control)	Cotyledonary node	100	Microshoot	1.2 ± 0.5^{f}	28
0.1 BA	Cotyledonary node	100	Microshoot	3.2 ± 1.2^{e}	14
0.5 BA	Cotyledonary node	100	Microshoot	$5.1 \pm 1.4^{c,d}$	-
1 BA	Cotyledonary node	100	Microshoot	5.4 ± 1.6^{c}	-
0.1 m-T	Cotyledonary node	100	Microshoot	1.4 ± 0.5^{f}	44
0.5 *m*-T	Cotyledonary node	100	Microshoot	$2.2 \pm 0.8^{e,f}$	50
1 *m*-T	Cotyledonary node	100	Microshoot	6.4 ± 1.6^{c}	69
0.1 TDZ	Cotyledonary node	100	Microshoot	3.5 ± 0.8^{e}	12
0.5 TDZ	Cotyledonary node	100	Microshoot	10.2 ± 2.0^{a}	21
1 TDZ	Cotyledonary node	100	Microshoot	8.4 ± 1.5^{b}	-
0 (control)	Cotyledonary leaf	-	-	-	-
0.1 BA	Cotyledonary leaf	-	-	-	-
0.5 BA	Cotyledonary leaf	-	-	-	-
1 BA	Cotyledonary leaf	27	-	-	-
0.1 m-T	Cotyledonary leaf	-	-	-	-
0.5 *m*-T	Cotyledonary leaf	-	-	-	-
1 *m*-T	Cotyledonary leaf	-	-	-	-
0.1 TDZ	Cotyledonary leaf	-	-	-	-
0.5 TDZ	Cotyledonary leaf	15	-	-	-
1 TDZ	Cotyledonary leaf	-	-	-	-
0 (control)	First true leaf	-	-	-	-
0.1 BA	First true leaf	10	Microshoot	2 ± 0.6^{d}	-
0.5 BA	First true leaf	34	Microshoot	$3.8 \pm 1.0^{a,b,c}$	-
1 BA	First true leaf	48	Microshoot	$3.9 \pm 1.0^{a,b}$	-
0.1 m-T	First true leaf	100	Microshoot	1.7 ± 0.7^{d}	97
0.5 *m*-T	First true leaf	73	Microshoot	$3.1 \pm 0.8^{b,c}$	66
1 *m*-T	First true leaf	54	Microshoot	$3.2 \pm 1.5^{b,c}$	74
0.1 TDZ	First true leaf	58	Microshoot	$2.8 \pm 0.8^{c,d}$	-
0.5 TDZ	First true leaf	62	Microshoot	$3.4 \pm 0.9^{b,c}$	11
1 TDZ	First true leaf	30	Microshoot	4.4 ± 0.9^{a}	-
0 (control)	First true leaf	-	-	-	-
10 2,4-D	First true leaf	66	Somatic embryo	24.2 ± 6.1	96
10 2,4-D + 5 BA	First true leaf	72	-	-	-

*Means followed by *different letters* in *the same column* are significantly different ($P \leq 0.05$) according to Tukey's HSD

**Rooting of microshoots occurred on hormone-free MS medium used for their elongation

of microshoots induced by *m*-T (compared to all cytokinins tested, including BA) was observed in the propagation of *Stevia rebaudiana* Bertoni (Ptak *et al*. 2023). Additionally, unlike BA, *m*-T did not cause vitrification (Aremu *et al*. 2012), that is consistent with the results presented in this study.

When cotyledonary nodes (Fig. 1*A*) were cultivated on media with a low concentration of TDZ (0.1 μM), the number of microshoots per explant was almost similar to that observed with BA at equimolar concentration (Table 1). The maximum number of microshoots (10.2) was observed at intermediate (0.5 μM) concentrations of TDZ (Fig. 1*B*). An increase in the concentration of TDZ in the medium resulted in a decrease in the number of microshoots with undeveloped leaf blades. This could be attributed to the fact that high concentrations of TDZ (> 2 μM) often led to morphological, physiological, and cytogenetic developmental abnormalities and vitrification of regenerated plants (Dewir *et al*. 2018). Cultivation of microshoots on media with all tested concentrations of TDZ resulted in the formation of short, thickened petioles. Tissue proliferation was observed at the interface between the plant material and the medium (Fig. 1*A*, *B*). In control samples on medium

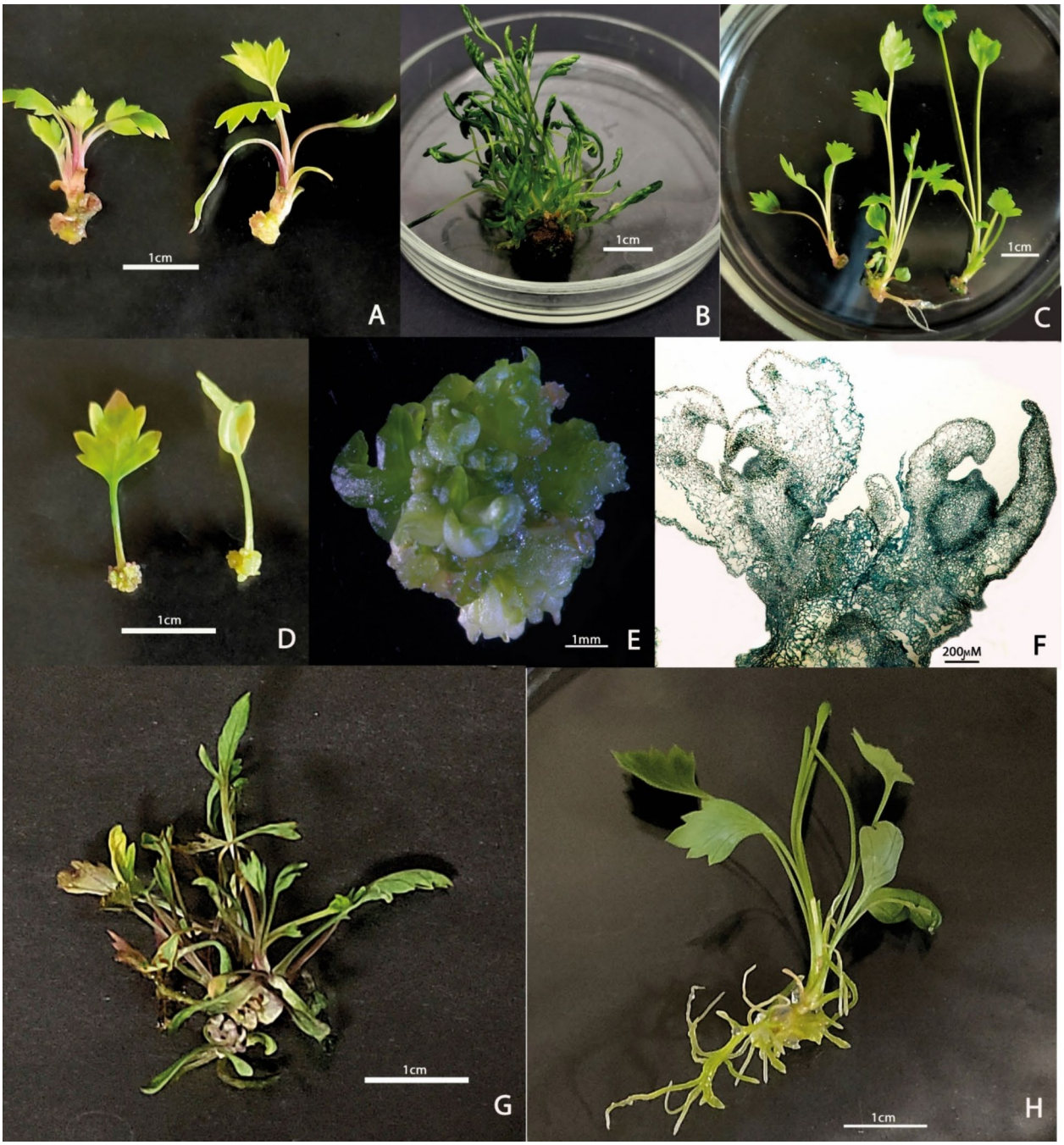

Figure 1. Direct regeneration of *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk from cotyledonary nodes (*A–C*) and first true leaves (*D–H*): cultivation of cotyledonary nodes with growing point for 14 (*A*) and 30 (*B*) d on Murashige and Skoog (MS) medium supplemented with 0.5 μM thidiazuron (TDZ); rooting of microshoots (*C*) on hormone-free MS medium; first true leaves (*D*), thickening at the end of their petioles (*E*), and formed microshoots (*F*) with primary vascular system after 45 d of cultivation on medium with 0.5 μM TDZ; elongation (*G*) and rooting of microshoots (*H*) obtained from leaf explants on hormone-free MS medium.

without PGRs, development of cotyledonary nodes with growing point was slow, with mostly only one (occasionally two) microshoots formed. Callus formation at the base of the shoot was not observed, but spontaneous rhizogenesis was observed in 28% of cases.

The use of cotyledonary nodes as explants had several advantages, such as rapid growth of adventitious shoots *in vitro*, low mutation rates, and ease of operation (Behera *et al.* 2019). In our studies on direct regeneration of microshoots, the selection of cotyledonary nodes as explants proved

successful for all types and concentrations of PGRs tested, which is consistent with the results of other studies. For instance, in the regeneration of *Toona ciliata*, cotyledonary nodes were found to be more effective explants than cotyledonary leaves (Song *et al*. 2021). Similarly, the regeneration of *Tectona grandis* from cotyledonary nodes was more efficient than from hypocotyls (Tambarussi *et al*. 2017).

The ability of cotyledonary and first true leaves to regenerate under the influence of the same PGRs at identical concentrations was also studied. It was found that when cotyledonary leaves were cultivated on medium containing BA at low and medium concentrations (0.1 and 0.5 μM), 100% of the explants died. However, as the concentration of this hormone increased, tissue proliferation at the base of the cotyledons was observed in 27% of the explants, with the formation of individual microshoots on the surface of the explants (Table 1). These microshoots subsequently failed to produce viable regenerated plants. Cultivation of cotyledonary leaves on medium containing *m*-T did not elicit a morphogenic response at any of the concentrations studied. Death of 80% of the explants was observed during passage. Incubation of cotyledons on medium with 0.1 μM or 1.0 μM TDZ resulted in chlorosis and necrosis of the explants, with no signs of regeneration observed. However, at a concentration of 0.5 μM, rather large (approximately 5 to 7 mm in diameter) vitrified outgrowths were observed at the base of 15% of the cotyledons. Cotyledonary leaves, similar to cotyledonary nodes, are a common type of explant used for regeneration (Gambhir *et al*. 2017; Zimik and Arumugam 2017; Sivanandhan *et al*. 2019; Song *et al*. 2021). In contrast to our results, previous studies have demonstrated effective regeneration from cotyledonary leaves, which was performed for *Sesamum indicum* L. (Zimik and Arumugam 2017) and *Toona ciliata* (Song *et al*. 2021). In both cases, the regenerated plants were subsequently adapted to *ex vitro* conditions. Notably, the authors utilized a variety of PGR types, combinations, and concentrations.

The cultivation of the first true leaves resulted in a similar response of the explants. After incubation with PGRs, tissue proliferation was observed at the tips of the petioles (Fig. 1*D*), ranging in size from 3 to 10 mm depending on the type and concentration of hormones (Fig. 1*E*). When culturing leaf explants *in vitro*, a wide range of morphogenic responses (such as somatic embryogenesis and adventitious shoot formation) can occur due to the absence of apical meristems in leaves (Woo and Wetzstein 2008). Additionally, the use of certain PGRs, such as TDZ, can induce morphogenesis in leaf explants through direct (Tomsone and Gertnere 2003) or indirect organogenesis (Pavingerova 2009; Qiao *et al*. 2009), as well as indirect somatic embryogenesis (Vejsadová and Petrova 2003; Qiao *et al*. 2009). Therefore, we conducted histological studies to clarify the morphogenetic processes occurring in the leaf explants. It revealed the presence of formed microshoots (Fig. 1*F*) with vessels of the primary vascular system in these proliferations.

During the cultivation of the first true leaves, an increase in the concentration of BA in the medium led to an enhanced morphogenic response (10 to 48%), an increase in the number of microshoots per explant (Table 1). Additionally, the appearance of purple pigmentation (and its intensity) in the explants was observed. Upon transfer of the proliferated tissues of the explants to a hormone-free MS medium, elongation of the microshoots was observed. Incubation of the first true leaves on a medium with a low concentration (0.1 μM) of *m*-T resulted in a 100% morphogenic response of the explants. However, as the concentration of *m*-T increased, this indicator decreased to 54%. The number of microshoots per explant was found to be significantly lower with *m*-T compared to other cytokinins tested at similar concentrations. The cultivation of leaves with low (0.1 μM) and medium (0.5 μM) concentrations of TDZ showed a morphogenic response of 58% and 62%, respectively. However, increasing the concentration to 1.0 μM resulted in a decrease of the morphogenic response to 30%. The number of microshoots per explant increased with increasing concentrations of TDZ in the medium, ranging from $2.8 \pm 0.8^{c,d}$ to 4.4 ± 0.9^{a}. Thus, TDZ was found to be the most effective hormone among those studied for the regeneration of *S. divaricata*. However, in the propagation of other plants, TDZ was not always the most effective PGR. For example, during the clonal micropropagation of *Salvia guaranitica*, it was observed that the multiplication factor was 2.5 times higher when using BA at a concentration of 0.5 mg L^{-1} compared to using TDZ at the same concentration (Echeverrigaray *et al*. 2010). However, a direct comparison of the effects of TDZ to those of aminopurine cytokinins (such as BA and others) used at identical concentrations may not be entirely valid. This is because aminopurine cytokinins have an operational range of activity in concentrations 1 to 10 μM (Guo *et al*. 2011), whereas TDZ is effective at significantly lower concentrations (< 1 μM) (Dewir *et al*. 2018).

Somatic Embryogenesis (SE) When cultivating the first true leaves on a medium containing 2,4-D (or 2,4-D supplemented with BA), the formation of primary callus was observed within 30 d. In a medium containing 10.0 μM 2,4-D, white callus was formed in 66% of the explants, predominantly on the leaf petioles (Fig. 2*A*). Meanwhile, in a medium containing both 10.0 μM 2,4-D and 5.0 μM BA, 72% of explants exhibited the formation of light green callus across the entire surface of the leaf blade. A similar morphogenic response of leaf and root explants to equimolar concentrations of 2,4-D was reported by Qiao *et al*. (2009); however, the callus in their study had a brown color, with optimal callus formation observed at a concentration of 2.26 μM. Additionally,

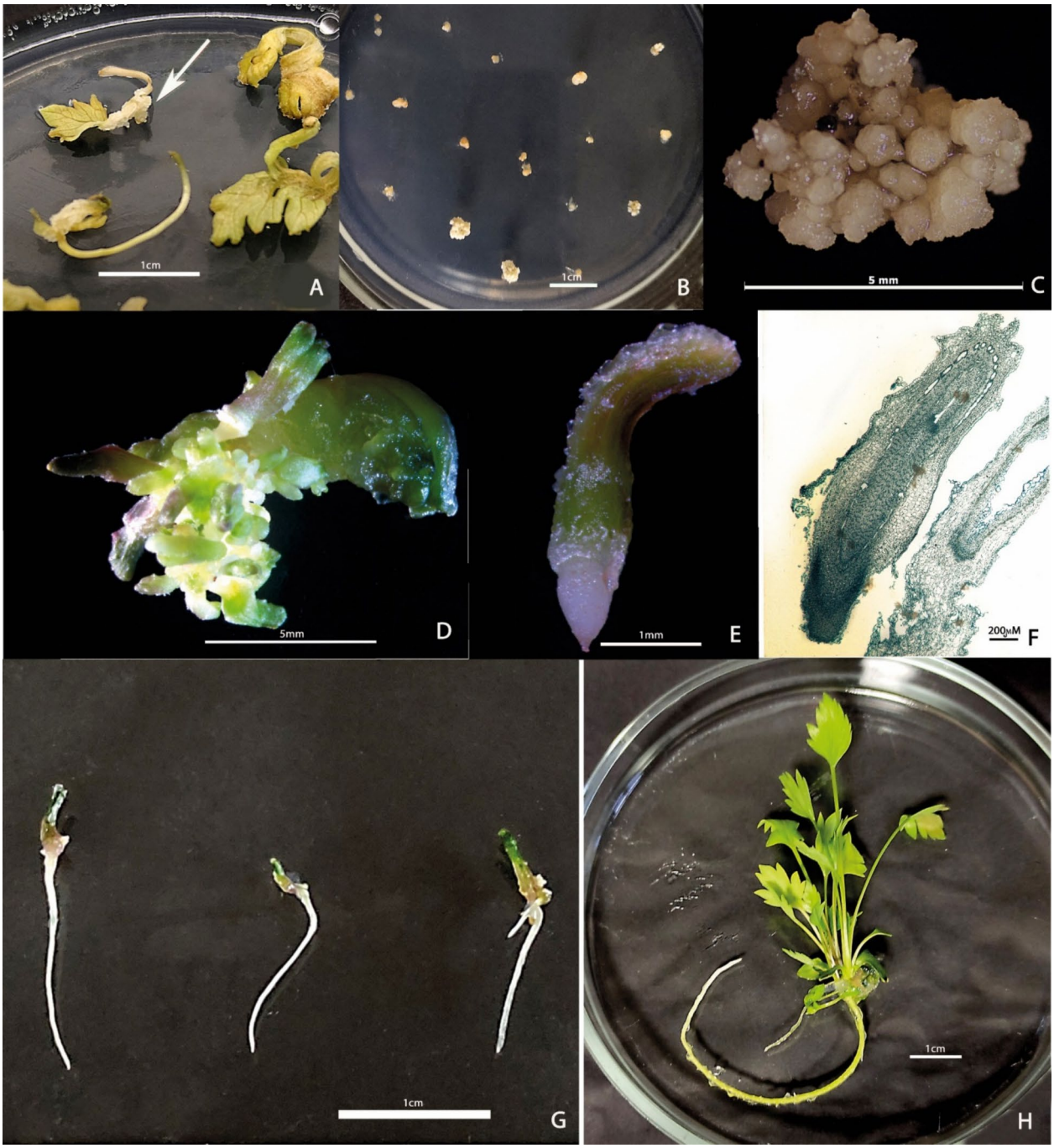

Figure 2. Indirect regeneration of *Saposhnikovia divaricata* (Turcz. ex Ledeb.) Schischk from leaf explants: formation of primary callus on leaf petioles after 30 d of cultivation (*A*), development of callus after 4 passages (*B*), and its globular structure (*C*) when cultivated in darkness on Murashige and Skoog (MS) medium supplemented with 10.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D); asynchronous development of somatic embryos from globular callus (*D*), morphological (*E*) and histological (*F*) structure of somatic embryo when cultivated under light on hormone-free MS medium for 30 d; development of regenerated plants from somatic embryos after two (*G*) and three (*H*) passages when cultivated under light on hormone-free MS medium.

the successful formation of callus cultures from the leaves of aseptic *S. divaricata* seedlings has also been observed on MS medium supplemented with 1 mg L^{-1} kinetin and 0.5 mg L^{-1} 2,4-D (Milentyeva *et al*. 2021).

Callus growth was insignificant in both cases. Cultivation on hormone-free media did not induce any morphogenetic responses, with chlorosis and subsequent necrosis of explants. Periodic subculturing resulted in further callus

development only on media containing 2,4-D, with a minimal increase in biomass (approximately 5 mm in diameter) (Fig. 2*B*). The callus had a globular structure (Fig. 2*C*). The callus was then grown in the light on BA medium or on hormone-free medium. With medium containing BA, the globular structures acquired a green color and increased in size during subculturing, but no further development occurred. On hormone-free MS media, the formation of bipolar structures (Fig. 2*D*, *E*, *F*) with asynchronous development was observed, which were easily detached from the explant. Morphological (Fig. 2*E*) and histological (Fig. 2*F*) analyses revealed the presence of apical and root meristems and a primary vascular system, indicating the formation of somatic embryoids. Subsequently, when cultivated on hormone-free MS medium under light, the somatic embryoids developed into complete regenerated microshoots with shoot apices, primary leaves, and roots (Fig. 2*G*, *H*).

According to Qiao *et al*. (2009), the optimal medium for somatic embryo differentiation was MS supplemented with 1.74 µM naphthaleneacetic acid (NAA), 4.44 µM BA, and 1.90 µM abscisic acid (ABA). In order to reduce the frequency of abnormal embryo formation during prolonged subculturing, Ma *et al*. (2005) recommended the replacement of 2,4-D with NAA and the supplementation of the culture medium with ABA and 4 to 5% sucrose during the later stages of embryogenesis. The number of regenerated plants per explant was 5.5 to 14 times higher than when leaf explants were cultivated on cytokinin-containing media (Table 1).

***In vitro* rooting** Following the cultivation of both cotyledonary nodes and true leaves obtained on media with PGRs, the plant material was transferred to hormone-free MS medium for the elongation of the formed structures. For microshoots derived from leaves, two passages on hormone-free medium were required for elongation, while for those derived from cotyledonary nodes, one passage was sufficient. In addition to elongation, spontaneous rooting of regenerated shoots occurred, with the efficiency of this process dependent on both the type of explant and the type and concentration of PGRs used for preliminary cultivation. For instance, rhizogenesis was observed in 14% of microshoots derived from cotyledonary nodes and cultivated under low concentrations of BA (Table 1), although increasing its concentration led to the cessation of rhizogenesis. The occurrence of rhizogenesis was not observed in microshoots derived from leaves following incubation with BA at any of the investigated concentrations.

The inhibitory effect of BA pretreatment on rhizogenesis has also been observed during *in vitro* regeneration of *Lens culinaris* Medik. (Polanco and Ruiz 1997), which is consistent with our findings. Following the cultivation of explants on media containing TDZ, rhizogenesis was also inhibited. The highest percentage of root formation (11 to 21%) was observed with 0.5 µM TDZ for both types of explants. According to other studies, pretreatment with TDZ has shown contradictory effects on shoot rooting. In some cases, rooting was inhibited, similar to our results (Singh *et al*. 2001; Kozak 2010). However, there have also been reports of stimulated rooting in *Bambusa vulgaris* (Ramanayake *et al*. 2006) and no effect on rooting in *Harpagophytum procumbens* (Grąbkowska *et al*. 2014).

Pre-cultivation on media with *m*-T resulted in a significantly higher proportion of rooted regenerated shoots compared to the other two cytokinins. As the concentration of *m*-T increased, the proportion of rooted regenerated plants increased, ranging from 44 to 69% for microshoots derived from cotyledonary nodes, and from 66 to 97% for those derived from leaves. The favorable effect of *m*-T on rooting of regenerated plants and their adaptation to *ex vitro* conditions is well known (Aremu *et al*. 2012). The frequency of rooting of somatic embryoids obtained on medium with 2,4-D was 96%.

Conclusions

An aseptic *in vitro* culture of *S. divaricata* was obtained from seeds. Removal of the pericarp resulted in accelerated germination and increased seed viability *in vitro* by eliminating exogenous dormancy and significantly reducing contamination levels. It has been demonstrated that the morphogenesis of *S. divaricata* followed a direct pathway when influenced by various cytokinins (BA, *m*-T, TDZ). Both cotyledonary nodes and first leaves gave rise to adventitious microshoots, the number of which depended on the type and concentration of PGR. The greatest number of microshoots for both types of explants was formed when using TDZ, while the highest rhizogenesis was observed with pre-cultivation using *m*-T. The first true leaves also exhibited regeneration *via* an indirect pathway of morphogenesis under the influence of an auxin (2,4-D). Furthermore, the formation of somatic embryoids has been observed, which subsequently developed into regenerated plants with a high frequency of rooting.

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Data availability The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by the authors.

Conflict of interest The authors declare no competing interests.

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