

# Effects of thidiazuron on *in vitro* morphogenic response of *Rhododendron sichotense* Pojark. and *Rhododendron catawbiense* cv. Grandiflorum leaf explants

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**Abstract** Thidiazuron (TDZ)-induced shoot organogenesis from *in vitro*-derived leaf explants of winter-hardy *Rhododendron sichotense* Pojark. and *Rhododendron catawbiense* cv. Grandiflorum was studied using two TDZ application methods: adding various concentrations to Anderson nutrient medium and a liquid pulse treatment. The highest frequency of shoot regeneration (93%) and maximum number of shoots per explant (24.6) from *R. sichotense* was obtained after a two-stage culture procedure that included exposure of leaf explants to 1.0  $\mu\text{M}$  TDZ followed by TDZ-free cultivation. TDZ at concentrations of 0.5 and 1.0  $\mu\text{M}$  was effective for leaf explants of *R. catawbiense* cv. Grandiflorum, inducing 60 and 85% regeneration frequencies, and 36.0 and 25.0 shoots per explant, respectively. The liquid-pulse treatment of leaf explants with 30  $\mu\text{M}$  TDZ for 4 h was effective only for *R. catawbiense* cv. Grandiflorum. Histological analysis revealed that epidermal cells of the adaxial side at the leaf-blade base were involved in *de novo* shoot regeneration in both genotypes. However, the adventitious shoot buds of *R. sichotense* developed from protuberances, whereas shoot organogenesis of *R. catawbiense* cv. Grandiflorum occurred through the formation of embryo-like structures. Optimum root formation for both genotypes was achieved using indole-3-butyric acid liquid-pulse treatment of microshoots followed by *ex vitro* rooting. An efficient system was developed for multiplication of *R. sichotense* and *R. catawbiense* cv. Grandiflorum, which

are cold-hardy genotypes promising for future genetic transformation.

**Keywords** *Rhododendron* · Thidiazuron · Leaf explant · Shoot regeneration · Histological analysis

## Introduction

*Rhododendrons* are highly ornamental plants that are widely used for landscaping around the world. However, these shrubs have been utilized rarely in urban plantings in the severe climate of Siberia, despite the existence of some well-known frost-resistant species and cultivars. One is *Rhododendron sichotense* Pojark. (subsection *Rhodorastrum* Maxim.), a wild species from the forest slopes of the Sikhote-Alin Mountains in the Russian Far East. The winter hardiness of this semi-evergreen shrub with corolla-color variability (pink, white, cream-colored, dark purple) and its early-spring blooming time make it one of the most promising species for use in breeding novel cultivars. Furthermore, this species is relatively tolerant of a wide range of soil pH, which is a rare quality among *Rhododendron* spp. Despite these characteristics, *R. sichotense* has not been previously included in breeding programs (Vrishch *et al.* 2010). Another interesting *Rhododendron* is *R. catawbiense* cv. Grandiflorum (subsection *Pontica*), which is native to the mountains of eastern North America. It is characterized as an important resource for the breeding of winter-hardy, sun-tolerant, evergreen rhododendrons (Van Veen 1969).

Clonal micropropagation, developed for many evergreen *Rhododendron* species, is the most effective method for large-scale production of valuable genotypes and varieties (McCown and Lloyd 1983; Preece and Imel 1991; Iapichino *et al.* 1991, 1992; Eeckhaut *et al.* 2010). However, there have

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been only a few attempts to apply these techniques to the propagation of Siberian and Far Eastern wild species, and the protocols used in these attempts were not adapted for commercial production. The culture of isolated leaf explants of *Rhododendron* spp. is a promising approach for mass propagation (Tomsone and Gertner 2003; Pavingerova 2009). Furthermore, leaf cultures serve as a model for studying *in vitro* morphogenesis, because the lack of apical meristems in leaves provides the opportunity to induce a broad range of morphogenic responses (Lo *et al.* 1997; Woo and Wetzstein 2008). Another application of *Rhododendron* leaf culture is genetic transformation for obtaining improved genotypes (Preece and Imel 1991).

One of the most effective known triggers of morphogenesis in differentiated cells of woody plants is thidiazuron (TDZ), a substituted phenyl-urea (Huetteman and Preece 1993; Murthy *et al.* 1998; Guo *et al.* 2011). The high activity of TDZ is associated with its ability to influence the level of endogenous plant growth regulators (PGRs) (Murch and Saxena 2001).

TDZ-induced *in vitro* morphogenesis in *Rhododendron* leaf culture may occur through direct (Samyn *et al.* 2002; Tomsone and Gertner 2003) or indirect organogenesis (Pavingerova 2009; Hebert *et al.* 2010), and somatic embryogenesis (Vejsadová and Petrova 2003). Therefore, the creation of regeneration systems for *Rhododendron* leaf cultures should be accompanied by histological analysis, in order to clarify the type of morphogenesis. While the effects on the morphogenic potential of *R. catawbiense* cv. Grandiflorum leaf explants of TDZ in combination with other PGRs have been studied to some extent (Tomsone and Gertner 2003; Pavingerova 2009), no known *in vitro* studies have been conducted on *R. sichotense*.

Accordingly, the objectives of the present study were (a) to analyze the effects of different concentrations and treatment types with TDZ, including liquid pulse on regeneration potential of leaf explants, (b) to determine the morphogenic responses of the studied genotypes using histological analysis, and (c) to develop efficient protocols for propagation of *R. sichotense* and *R. catawbiense* cv. Grandiflorum.

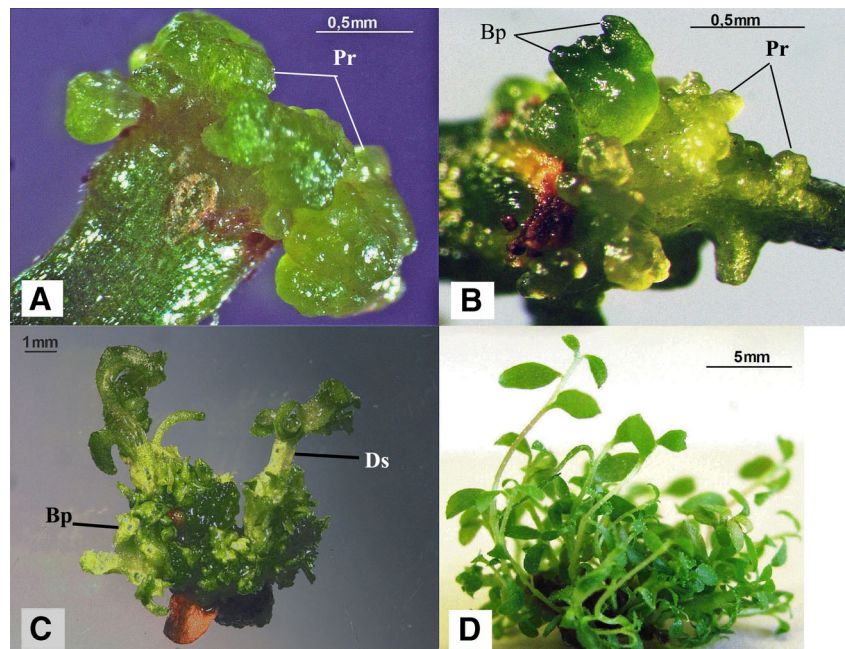
## Materials and Methods

**Plant material** Microclones of *R. catawbiense* cv. Grandiflorum and *R. sichotense* were maintained in a collection of the Laboratory of Biotechnology (CSBG RAS, Novosibirsk, Russia) on Anderson's medium (AM) (Anderson 1984) containing 0.6% Bacto® agar (PanReac®, Barcelona, Spain), 3% sucrose (Shostka Chemical Reagent Factory, Shostka, Ukraine), 24.5  $\mu\text{M}$  2-isopentenyladenine, and 5.7  $\mu\text{M}$  indole-3-acetic acid (all from ICN Biomedicals, Aurora, Ohio) during four passages of 6 wk each. Micro-cuttings with three nodes were then transferred to agar-solidified, hormone-free AM (AM0) and cultivated for two passages (4 wk each). The pH of the medium was adjusted to 5.0 before autoclaving (121°C, 1.05 kg cm<sup>-2</sup>), and all PGRs were added to the medium post-autoclaving. The cultures were maintained in culture jars (15 mL medium per vessel) at 23 ± 2°C under cool white fluorescent light (Philips, Pila, Poland) at an intensity of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with a 16-h photoperiod.

### Effect of TDZ on shoot regeneration from leaf explants

The first pair of young leaves with petioles from the *in vitro* microclones of *R. catawbiense* cv. Grandiflorum and

**Figure 1** Effects of 1.0  $\mu\text{M}$  TDZ on shoot morphogenesis in leaf culture of *R. sichotense*: (A) Protuberances formed at 14 d. (B) Protuberance development and bud primordia formation at 21 d. (C) Cluster of stunted abnormal shoots after 15 wk of culture. (D) Elongated shoot cluster after 8 wk on AM0. Bp bud primordium, Ds differentiated shoot, Pr protuberance.



**Table 1** Effect of different concentrations and treatments of TDZ on morphogenic response of *R. sichotense* and *R. catawbiense* cv. Grandiflorum leaf explants after 15 wk of culture

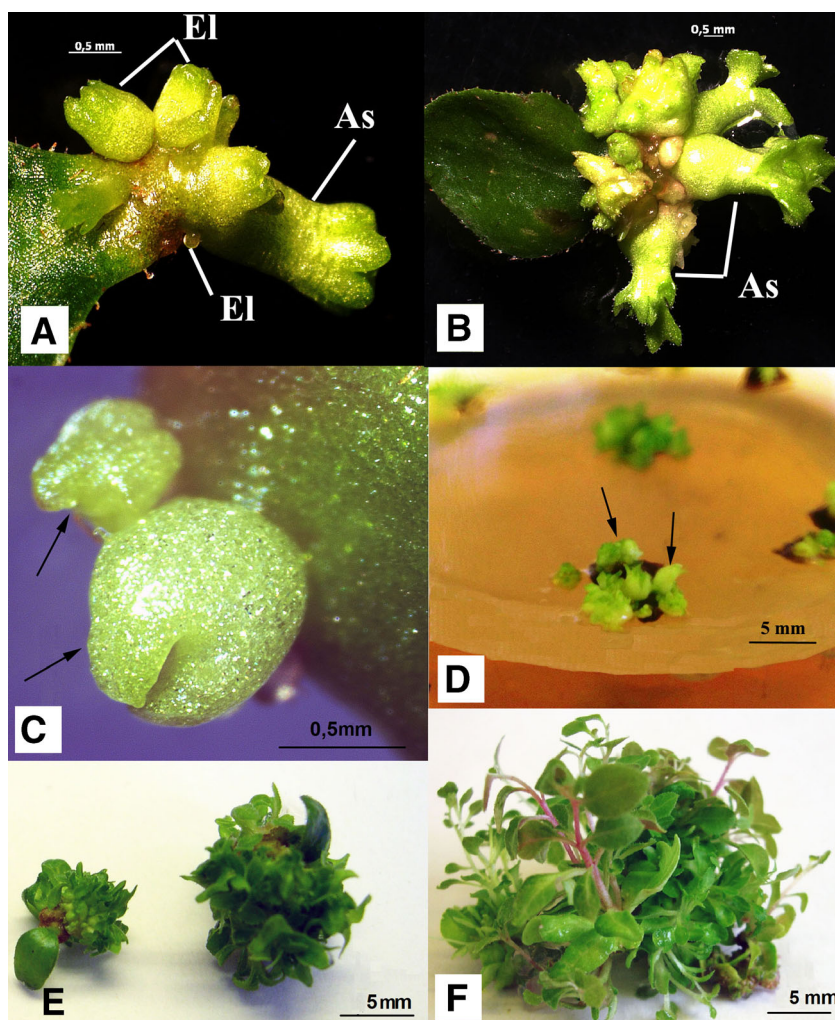
TDZ ( $\mu\text{M}$ )	<i>R. catawbiense</i> cv. Grandiflorum			<i>R. sichotense</i>		
	% response	% bud clusters	% shoot clusters	% response	% bud clusters	% shoot clusters
0.1	35	0	35	73	28	45
0.5	60	0	60	75	9	66
1.0	85	0	85	93	23	70
5.0	47	47	0	15 <sup>a</sup>	0	0
10.0	0	0	0	0	0	0
pulse treatment with 30 $\mu\text{M}$	89	0	89	6	6	0

<sup>a</sup> Callus formed

*R. sichotense* cultivated on AM0 was excised and used as explants for this study. To enable exclusion of residual axillary meristems on the leaf petiole, the removal of leaf explants was carried out under a stereoscopic microscope (Lomo, MSP-1 var.1, St. Petersburg, Russia). Leaf explants were placed adaxial side up on the surface of agar-solidified AM. To study the effects of TDZ (plant cell culture tested, BioReagent,

Sigma-Aldrich®) on regenerative capacity and morphogenesis, two types of TDZ treatment were used: (a) direct explant cultivation on AM supplemented with various concentrations of TDZ (0.1, 0.5, 1.0, 5.0, or 10.0  $\mu\text{M}$ ) and (b) pulse treatment of leaf explants in water solution of 30.0  $\mu\text{M}$  TDZ for 4 h followed by cultivation on AM0. The pH of the medium was adjusted to 5.0 before autoclaving (121°C; 1.05 kg cm<sup>-2</sup>), and

**Figure 2** Morphogenesis of *R. catawbiense* cv. Grandiflorum *in vitro* from leaf explants. (A) Formation of embryo-like structures and stunted abnormal shoots at the base of leaf after 35 d on AM supplemented with 0.5  $\mu\text{M}$  TDZ. (B) Clusters of short shoots at 45 d of culture on AM0 after pulse treatment with 30  $\mu\text{M}$  TDZ. (C) Embryo-like structures (arrow) at 35 d on AM supplemented with 5.0  $\mu\text{M}$  TDZ. (D) Embryo-like structures (arrow) on brown leaf explant after 15 wk of culture with 5.0  $\mu\text{M}$  TDZ. (E) Shoot clusters after 15 wk of culture on AM with 0.5  $\mu\text{M}$  TDZ. (F) Elongated shoot clusters after 8-wk culture on AM0. *As* abnormal shoot, *El* embryo-like structure.



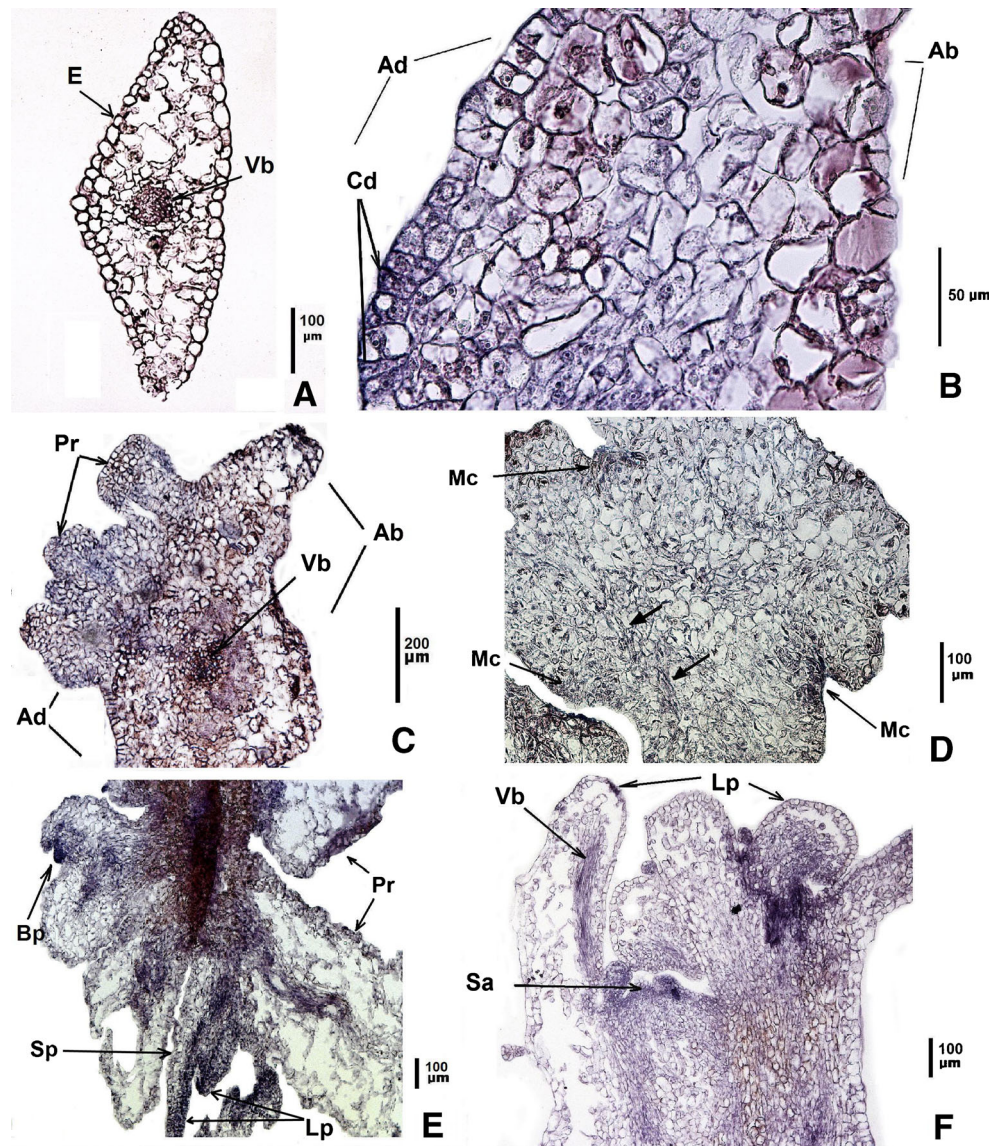
the TDZ was added to the medium post-autoclaving. The cultures were maintained on 15-mL medium in 100-mL culture glass jars (Sigma-Aldrich®, St. Louis, MO) at  $23 \pm 2^\circ\text{C}$  under cool white fluorescent light (Philips) at an intensity of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  with 16-h photoperiod. The cultivation time was 15 wk. Each treatment consisted of ten explants with three replicates. The morphology of regenerants was studied with the help of Stereo Discovery V12 microscope and AxioCam HRc camera, (all from Carl Zeiss, Gottingen, Germany).

**Histological analysis** Leaf explants cultured on AM supplemented with  $1.0 \mu\text{M}$  TDZ were collected at 0 d, 7 d, 10 d, 12 d, 14 d, 21 d, 35 d, and 8 wk from the start of the experiment and prepared for examination by light microscopy. Explants were fixed in glacial acetic acid (99.9%), formalin (40%), and ethyl alcohol (96%) in the proportions 7:7:100

(v/v/v). The samples were dehydrated and embedded in Paraplast® (Sigma-Aldrich®) according to Pausheva (1988) and sectioned at  $7 \mu\text{m}$ , using a microtome (HM-325 Microm, Walldorf, Germany). Sections were stained with Ehrlich's hematoxylin for 15 min and 0.1% aniline blue (all dyes from Sigma-Aldrich®) for 3 min (Pausheva 1988). Histological analysis was conducted using a microscope equipped with Axioplan 2 imaging, Axioskop-40, camera AxioCam MRc5 and AxioVision 4.8 software (all from Carl Zeiss).

**Shoot elongation, rooting, and acclimatization** Clumps of adventitious shoots were transferred to AM0 for elongation. The numbers of shoots (length  $\geq 5$  mm) per explant were counted after 8 wk. To stimulate root formation, a 4-h pretreatment with a sterile aqueous solution of  $148.0 \mu\text{M}$  IBA (ICN Biomedicals) was tested. For rooting, pretreated regenerants

**Figure 3** Histological observations of adventitious buds formation from *R. sichotense* leaf explants under  $1.0\text{-}\mu\text{M}$  TDZ exposure. (A) Cross section through leaf explant at 0 d. (B) First cell divisions in adaxial epidermis of leaf explant after 10 d of culture. (C) Protuberance formation at the adaxial side of leaf explant at 14 d. (D) Formation of meristematic center and vascular system at the protuberance (arrow). (E) Longitudinal section of differentiated bud with leaf primordia at 35 d. (F) Formation of multiple leaf primordia at 8 wk of culture. Ab abaxial surface, Ad adaxial surface, Bp bud primordium, Cd cell divisions, E epidermis, Lp leaf primordium, Mc meristematic center, Pr protuberance, Sa shoot apex, Sp shoot primordium, Vb vascular bundle.



were placed either *in vitro* on AM0 or *ex vitro* in a mixture of peat (pH 4.0–5.0) and sand (1:1; v/v). The pH of the *in vitro* rooting medium was adjusted to 5.0 before autoclaving. The rooting frequency, number and length of roots, the percentage of plants with secondary roots, and shoot lengths were counted after 6 wk. Thirty regenerants in three replicates were used for each treatment. Acclimatization of *in vitro* rooted plants was carried out in the mixture of peat and sand (1:1; v/v) under highly humid conditions. Plants were maintained at  $23 \pm 2^\circ\text{C}$  under cool white fluorescent light (Philips) at an intensity of  $27 \mu\text{mol m}^{-2} \text{s}^{-1}$  with 16-h photoperiod. Plants acclimatized were transplanted to 10-cm diameter pots with soil (pH 5.5–6.5) and transferred to the greenhouse.

**Statistical analysis** All data were analyzed by one-way ANOVA to assess treatment differences and interactions using STATISTICA 8 (StatSoft Inc., Tulsa, OK). Significance between means was tested by Duncan's test ( $P=0.05$ ). Data are presented as means  $\pm$  SE.

## Results and Discussion

### Effect of TDZ on shoot regeneration from leaf explants

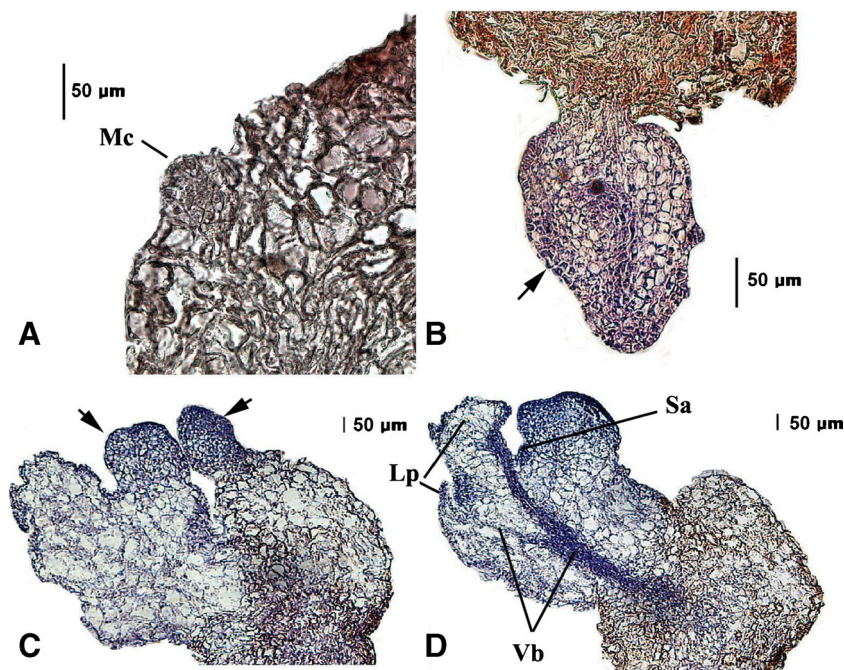
Despite a report of high effectiveness of TDZ on the regeneration of numerous woody plants *in vitro* (Murthy *et al.* 1998), there have been only a few studies of the effect of this synthetic cytokinin in combination with various PGRs on micropropagation of rhododendrons using leaf explants from microclones (Mertens *et al.* 1996; Tomsone and Gertner

2003). The present research is the first on plant regeneration from *in vitro* derived leaf explants of *R. sichotense* and *R. catawbiense* cv. Grandiflorum using TDZ treatments only.

After 14 d of culture on media containing 0.1, 0.5, or 1.0  $\mu\text{M}$  TDZ, visible protuberances formed on the adaxial side of *R. sichotense* leaf explants at the base of the leaf blade and petiole (Fig. 1A). Prolonged cultivation resulted in increased numbers of protuberances and bud primordia formation in this zone (Fig. 1B). However, clusters of adventitious buds consisting of light-green protuberances and vitrified shoot primordia formed only after 8 wk. By the 15th wk, shoots developed (Table 1). The regeneration processes in *R. sichotense* leaf culture were nonsynchronous: in the adventitious clusters, both the bud primordia at the early stages of development and fully formed, well-differentiated shoots with length  $\geq 5$  mm were found (Fig. 1C). The highest regeneration frequency (93%) and the number of clusters consisting of shoots were observed with the medium containing 1.0  $\mu\text{M}$  TDZ. Increasing TDZ concentrations up to 5.0  $\mu\text{M}$  reduced the regeneration rate and was accompanied by callus formation (Table 1). On the 10.0- $\mu\text{M}$  TDZ treatment, most leaf explants turned brown and died. A liquid-pulse treatment of 30  $\mu\text{M}$  TDZ for 4 h, followed by cultivation on AM0, was ineffective and did not induce shoot formation from *R. sichotense* leaf explants (Table 1).

The lower half of petioles of *R. catawbiense* cv. Grandiflorum leaf explants turned red after a 10–12-d exposure to AM when supplemented with 0.1, 0.5, or 1.0  $\mu\text{M}$  TDZ, and after TDZ pulse treatment. Further cultivation led to formation of embryo-like structures and stunted abnormal shoots from red areas (Fig. 2A, B). New buds appeared, and intense

**Figure 4** Light microscopy observations of *R. catawbiense* cv. Grandiflorum leaf culture under TDZ exposure. (A) Meristematic center in adaxial epidermis after 12-d exposure to 0.5  $\mu\text{M}$  TDZ. (B) Embryo-like structure formation (arrow) on AM supplemented with 0.5  $\mu\text{M}$  TDZ at 35 d of culture. (C) Embryo-like structure (arrows) developed under 5.0  $\mu\text{M}$  TDZ. (D) Bud development in tissues exposed to 5.0  $\mu\text{M}$  TDZ after 15 wk of culture. *Lp* leaf primordium, *Mc* meristematic center, *Sa* shoot apex, *Vb* vascular bundle.



**Table 2** The number of regenerated shoots after elongation on AM0 for 8 wk of TDZ exposure

TDZ ( $\mu\text{M}$ )	<i>R. catawbiense</i> cv. Grandiflorum	<i>R. sichotense</i>
0.1	8.2 $\pm$ 1.6 c	12.3 $\pm$ 1.8 b
0.5	36.0 $\pm$ 8.3 a	23.5 $\pm$ 3.8 a
1.0	25.0 $\pm$ 4.8 ab	24.6 $\pm$ 2.9 a
5.0	23.2 $\pm$ 2.1 abc	0
10.0	–	–
Pulse treatment with 30.0 $\mu\text{M}$	13.2 $\pm$ 2.1 bc	0

Means followed by the same letter are not significantly different ( $P=0.05$ )

branching of *de novo* shoots led to the formation of spherical clusters of short shoots (Fig. 2E). Treatment with 5.0  $\mu\text{M}$  TDZ led to the direct regeneration of embryo-like structures which occurred on the adaxial side of the leaf lamina (Fig. 2C). After 15 wk of culture, the highest percentage of regeneration was obtained with the pulse treatment of 30.0- $\mu\text{M}$  TDZ and the 1.0- $\mu\text{M}$  TDZ continuous cultivation treatments (89 and 85%, respectively). The 5.0- $\mu\text{M}$  TDZ treatment resulted in reduction of regeneration frequency to 47% (Table 1) and only buds formed on the browned leaf explants (Fig. 2D).

These data demonstrate the effectiveness of low TDZ levels for promoting shoot regeneration of both rhododendron genotypes. Increasing the concentration to 5.0  $\mu\text{M}$  in AM and using liquid-pulse treatment revealed the genotypic variation in regeneration capacity between these two species. The pulse treatment was tested to minimize the negative effects on shoot morphology caused by continuous exposure to TDZ, which typically can result in hyperhydricity, stunting, fasciation, distortion, and fusion of shoot-like structures (Ahmad and Anis 2012). Pulse treatments reduce the exposure time to growth regulators (Aasim *et al.* 2010). According to recent reports, the treatment duration may vary from a few hours using a liquid pulse to a few days when PGRs were added to regeneration media (Pascual and Marin 2005; Shaik *et al.* 2009; Graner *et al.* 2013). Data presented here show that TDZ liquid-pulse treatment of *R. catawbiense* cv. Grandiflorum leaf explants induced a high regeneration rate, whereas this effect was not found in *R. sichotense*. At the same time, the pulse

treatment did not prevent the negative effects of TDZ on the shoot morphology of *R. catawbiense* cv. Grandiflorum. Further studies are needed to take advantage of TDZ pulse treatment on shoot regeneration of rhododendrons.

**Histological analysis** To better understand the morphogenetic pathways leading to *de novo* shoot organogenesis, histological studies were conducted. Light microscopy of transverse sections of *R. sichotense* leaf blades in the area close to the petiole at the time of explant isolation (0 d) demonstrated the single-layered epidermis, formed by oval cells, as well as the inner parenchyma with large intercellular air spaces and a vascular bundle (Fig. 3A). Incubation of explants for 10 d on induction medium with 1.0  $\mu\text{M}$  TDZ caused anticlinal and periclinal cell divisions in the adaxial epidermis of both the petiole and leaf base area (Fig. 3B). Further proliferation of epidermal cells led to protuberance development by day 14 (Fig. 3C). In the following few days, meristematic centers were observed in proximity to protuberance surfaces (Fig. 3D). After 35 d of culture, the division of these groups of cells gave rise to bud primordia, which further differentiated to form leaf and shoot primordia and procambial strands, connected with the vascular system of the explant (Fig. 3E). However, some of the protuberances did not develop into bud primordia. Histological analysis confirmed the asynchronous character of morphogenesis induced by TDZ: The initial stage of bud primordium formation and well-developed leaf primordia was observed in the same protuberances (Fig. 3E). Abnormalities of

**Table 3** Effect of *in vitro* and *ex vitro* conditions on percent rooting, number of roots, and root and shoot lengths of *R. sichotense* and *R. catawbiense* cv. Grandiflorum 6 wk after pulse treatment with an aqueous solution of 148.0  $\mu\text{M}$  IBA

Rooting condition	% rooting	Root number	Root length (cm)	% plants with secondary roots	Shoot length (cm)
<i>R. sichotense</i>					
<i>In vitro</i> on AM0	40	2.32 $\pm$ 0.17 b	1.02 $\pm$ 0.11 b	70	2.17 $\pm$ 0.09 ab
<i>Ex vitro</i> in mix of peat and sand (1:1)	84	6.33 $\pm$ 0.58 a	1.71 $\pm$ 0.15 a	100	2.64 $\pm$ 0.21 ab
<i>R. catawbiense</i> cv. Grandiflorum					
<i>In vitro</i> on AM0	37.5	1.33 $\pm$ 0.21 b	0.48 $\pm$ 0.03 b	0	1.37 $\pm$ 0.10 b
<i>Ex vitro</i> in mix of peat and sand (1:1)	77	4.00 $\pm$ 0.34 a	0.59 $\pm$ 0.04 a	85	1.73 $\pm$ 0.13 a

Means followed by the same letter are not significantly different ( $P=0.05$ )

structure and development in *de novo* buds were exhibited as evidenced by the formation of multiple leaf primordia (Fig. 3F).

The initial stages of *R. catawbiense* cv. Grandiflorum morphogenesis were similar to *R. sichotense*. However, *R. catawbiense* cv. Grandiflorum shoot organogenesis occurred through the formation of embryo-like protrusions of epidermal origin (Fig. 4A, B). Histological analysis revealed the connection of these *de novo* structures to the parent tissue through the vascular system (Fig. 4D). No root poles were formed in embryo-like structures.

An outstanding property of TDZ is it potentially can simultaneously induce organogenesis and somatic embryogenesis in the same explant type, depending on the concentration (Li *et al.* 2002; Mithila *et al.* 2003; Wojtania *et al.* 2004). In the present experiment, this dual response to TDZ treatments in rhododendron leaf explant culture was not observed. Histological examination provided detailed analysis of the differences in regeneration pathways of the genotypes tested. The adventitious shoot buds of *R. sichotense* developed from protuberances that were formed from the adaxial epidermis of petiole and leaf base when treated with 0.1 to 1.0  $\mu\text{M}$  TDZ. The structures formed under TDZ exposure of *R. catawbiense* cv. Grandiflorum leaf explants were epidermal-derived, embryo-like protrusions. Although these structures looked similar to somatic embryos under the stereo microscope (Fig. 2c, d), histological examination revealed their connection with explant tissue and the lack of a root apex. These results confirm shoot regeneration via direct organogenesis. The development of shoots through embryo-like structure formation under TDZ treatment was also found in Georgia plume, *Elliottia racemosa* (Woo and Wetzstein 2008), sugarcane, *Saccharum officinarum* (Wamaitha *et al.* 2010), and *Pelargonium*  $\times$  *hortorum* (Madden *et al.* 2005).

**Shoot elongation, rooting, and acclimatization** To overcome the adverse effects of exposure to TDZ, cultures of *R. sichotense* and *R. catawbiense* cv. Grandiflorum were transferred to AM0 for shoot elongation. The number of regenerated shoots in a cluster after elongation for 8 wk depended on TDZ concentration in the induction medium (Table 2). Elongated shoots of *R. sichotense* on AM0 were obtained only after cultivation at low concentrations of TDZ (0.1–1.0  $\mu\text{M}$ ) (Fig 1D). Elongated shoots of *R. catawbiense* cv. Grandiflorum were obtained on AM0 after the pulse treatment with 30.0  $\mu\text{M}$  TDZ as well as after cultivation on media supplemented with 0.1, 0.5, 1.0, or 5.0  $\mu\text{M}$  TDZ (Table 2; Fig 2F). The highest number of shoots per explant ( $36.0 \pm 8.3$ ) was obtained with the 0.5- $\mu\text{M}$  TDZ treatment (Table 2). These results agree with the suggestion that a two-stage culture procedure consisting of a TDZ-treatment of explants followed by TDZ-free cultivation is preferable for shoot organogenesis (Guo *et al.* 2011).

Rooting of *R. sichotense* and *R. catawbiense* cv. Grandiflorum shoots (length  $\geq 5$  mm) was carried out *in vitro* and *ex vitro* after 4-h pulse treatment with 148.0  $\mu\text{M}$  IBA. The best rooting for both genotypes was obtained *ex vitro* in a mixture of peat and sand (Table 3). The plants rooted in this way had a well-developed root system with secondary roots and were successfully acclimatized. The percentage of microclones rooted *in vitro* was significantly lower than in the *ex vitro* treatment, and the plants needed subsequent acclimatization.

The present study showed the effectiveness of IBA liquid pulse treatment, which reduced rooting time twofold (Table 3). Furthermore, *ex vitro* rooting resulted in better root quality, greater shoot length, and shorter acclimatization time, compared to *in vitro* rooting, a result that is similar to the results of many authors (Leva 2012; Benmahioul *et al.* 2012; Phulwaria and Shekhawat 2013). This approach, consisting of IBA liquid-pulse treatment of microshoots followed by *ex vitro* rooting, considerably reduces the expense of rhododendron micropropagation.

## Conclusions

A rapid and efficient regeneration system for *R. sichotense* and *R. catawbiense* cv. Grandiflorum from *in vitro* derived leaf explants was developed based on the high activity of TDZ in low concentrations. This study has revealed the differences in morphogenic responses and in regeneration pathways of both genotypes in response to TDZ. The use of winter-hardy rhododendrons, especially wild *R. sichotense*, expands the germplasm resource base for the creation of new cultivars with increased ornamental value, suitable for cold environments, through both conventional breeding and genetic transformation.

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