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## ***In vitro* establishment and TDZ-induced regeneration from shoot and leaf explants of *Rhododendron sichotense* Pojark.**

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**Keywords:** adventitious shoot regeneration, axillary shoot proliferation, *Rhododendron sichotense*, thidiazuron.

**Summary.** *Rhododendron sichotense* Pojark. (Ericaceae) is a winter-hardy species native to the Russian Far East, highly valued as an attractive ornamental woody plant. In order to establish of *in vitro* culture without the problem with exudation of polyphenols and to induce multiplication from leaf and shoot explants of field grown plants, the procedure including the pre-cultivation of explants on hormone free Anderson Medium (AM0) for 3 days followed by cultivation on AM supplemented with varying thidiazuron (TDZ) concentrations (0.1, 0.5, 1.0 µM) was developed. The elongation on hormone-free AM0 allowed obtaining the healthy shoots without any anomalies. The best response in terms of the regeneration frequency (50 %), the shoot number per explant ( $41.60 \pm 5.25$ ) and the shoot length ( $12.20 \pm 1.04$  mm) was achieved under 0.5 µM of TDZ from leaf explants.

## **Введение в культуру *in vitro* и ТДЗ-индуцированная регенерация *Rhododendron sichotense* Pojark. из листовых и побеговых эксплантов**

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**Ключевые слова:** пролиферация пазушных побегов, регенерация адвентивных побегов, тидиазурон, *Rhododendron sichotense*.

**Аннотация.** *Rhododendron sichotense* Pojark. (Ericaceae) является высокоценным декоративным древесным зимостойким видом природной флоры российского Дальнего Востока. Разработана методика введения в культуру *in vitro*, позволяющая предотвратить негативный эффект фенольного окисления. Экспланты, листья и части побегов изолировали от растений открытого грунта и предкультивировали на безгормональной среде Андерсона (AM0) в течение трех дней, с последующим переносом на среду того же минерального состава, дополненную тидиазуоном в различных концентрациях (0,1; 0,5; 1,0 мкМ). Элонгация на AM0 позволила получить здоровые побеги без аномалий развития. Наилучший ответ по частоте регенерации (50 %), количеству побегов на эксплант ( $41,60 \pm 5,25$ ) и длине побегов ( $12,20 \pm 1,04$  мм) получен из листовых эксплантов под действием 0,5 мкМ ТДЗ.

## Introduction

*Rhododendron sichotense* Pojark. is a highly ornamental, winter hardy, semi-evergreen shrub native to the Sikhote-Alin Mountains of the Russian Far East. This species possesses a corolla-color variability (pink, white, cream-colored, dark purple) and different terms of blooming (Vrishch et al., 2010), which make *R. sichotense* one of the most promising wild-growing species for breeding and landscaping. Moreover, the wide range of phenolic compounds containing in aboveground organs of *R. sichotense* provides it with medicinal value (Karpova, Karakulov, 2013). Due to existing difficulties to propagate rhododendrons using conventional methods, the biotechnological approaches are the most often applied for developing the efficient systems for the large-scale plant production (Eeckhaut et al., 2010). A key to success in the establishment of tissue culture protocol is the choice of plant growth regulators. Up to date, there have been many reports on the clonal micropropagation of evergreen rhododendrons using 2-isopentenyladenine (2-iP) as cytokinin (Eeckhaut et al., 2010), whereas for some species, the application of zeatin is preferable (Almeida et al., 2005; Zaytseva et al., 2018). However, none of these studies is devoted to *in vitro* establishment and micropropagation of *R. sichotense* from the natural population using leave and shoot explants.

The most powerful trigger for morphogenesis in tissue culture of woody plants is a synthetic cytokinin thidiazuron (TDZ), a substituted phenylurea compound, which, depending on the concentration and the type of explant, can initiate a wide range of morphogenic reactions (Murthy et al., 1998; Novikova, Zaytseva, 2018). TDZ is known to induce the higher regeneration rates in the comparison to purine-based cytokinins and also demonstrates the ability of fulfilling both the cytokinin and auxin requirements of regeneration responses in a number of woody plants (Jones et al., 2007). However, unfavorable side effects of TDZ have been reported including morphological abnormalities (i. e., fasciated shoots, hyperhydricity, inhibited shoot elongation) and difficulties in rooting and *ex vitro* acclimatization. The undesirable effects associated with applying TDZ can be overcome by reducing TDZ concentrations and exposure time (Dewir et al., 2018; Novikova, Zaytseva, 2018). At the same time, the establishment of *in vitro* culture of wild-growing rhododendrons is difficult due to the high content of phenolic compounds in explants (Vasilieva, 2009). The accumulation and subsequent oxidation

of phenolic compounds result in reduced growth, lower rates of regeneration or recalcitrance and can ultimately lead to explant necrosis (Uchendu et al., 2011). Therefore, the establishment of rhododendron explants from field conditions requires the special procedures to escape the problem associated with exudation of polyphenol compounds from cut surface.

The present study aimed to establish an efficient protocol of *in vitro* multiplication for *R. sichotense* from shoot tips and young leaves using TDZ to induce direct shoot regeneration without developmental abnormalities.

## Materials

### Objects

1. Fresh first pairs of *R. sichotense* leaves with petioles (from field plants).
2. Fresh juvenile shoots consisted of 3 nodes with apical buds (from field plants).

### Reagents

1. Ethanol (70 % [v/v] in distilled water).
2. 0.05 % Sodium hypochlorite (20 % Domestos Unilever, Russia [v/v] in distilled water).
3. Macro- and microelements as well as adenine sulfate and vitamins according to Anderson's medium prescription (Anderson, 1984).
4. Bacto agar (PanReac, Barcelona, Spain).
5. Sucrose (Shostka Chemical Reagent Factory, Shostka, Ukraine).
6. Thidiazuron (BioReagent, SigmaAldrich, USA).

### Equipment

1. Micropipetters, 10-, 100-, 1000- µl with corresponding tips.
2. Culture jars of 100 mL.
3. Cool white fluorescent light (Philips, Pila, Poland).
4. Orbital shaker (Elmi, S-3-02L, Latvia).

## Procedures

### Culture Media and Conditions

1. 0.6 % (w/v) agar-solidified Anderson's medium (AM) supplemented with 3 % sucrose (w/v) was used in all experiments.
2. The pH of the medium was adjusted to 5.0 before autoclaving (121 °C; 1.05 kg cm<sup>-2</sup>).
3. Plant growth regulators (PGRs) were added post-autoclaving.

4. Cultures were maintained under cool white fluorescent light (Philips, Poland) at the intensity of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  with a 16-h photoperiod and  $23 \pm 2 \text{ }^\circ\text{C}$ .

#### *Sterilization*

1. Leaves and shoots were soaked in 70 % ethanol for 2 min.

2. Leaves and shoots were transferred to 0.05 % sodium hypochlorite solution and placed on an orbital shaker at 90 revolutions per min. for 20 min.

3. A sterilized plant material was washed in sterile distilled water three times.

#### *Explant preparation*

1. To obtain shoots explants, all leaves were removed from the sterilized shoots.

2. To obtain leaf explants, the leaves with petioles were cut into two parts near the middle of lamina across the main vein. To induce morphogenesis, the parts of leaves with petioles were used.

#### *Shoot induction from shoot explants*

1. Shoot explants were inoculated horizontally on hormone-free AM (AM0) for 3 days (pre-cultivation step).

2. After pre-cultivation the explants were transferred to AM supplemented with 0.1, 0.5, 1.0  $\mu\text{M}$  of TDZ for 8 weeks (multiplication step).

3. After 8 weeks of cultivation, the frequency and the type of morphogenic response, the number of axillary shoots per explant (with length more than 5 mm) and the shoot length were examined (Table, Fig.1).

Table

Effect of TDZ on shoot regeneration from *Rhododendron sichotense* shoot and leaf explants. Means followed by the same letter are not significantly different according to Duncan test ( $P > 0.05$ ). Means  $\pm$  SE

Type of explant	TDZ concentration, $\mu\text{M}$	Regeneration frequency, %	Shoot number per explant	Shoot length, mm
Shoot	0.1	100	$9.80 \pm 1.83$ a	$8.54 \pm 0.48$ a
	0.5	100	$7.80 \pm 0.58$ a	$6.72 \pm 0.41$ b
	1.0	60	$7.00 \pm 0.97$ a	$6.13 \pm 0.61$ b
Leaf	0.1	0	–	–
	0.5	50	$41.60 \pm 5.25$ a	$12.20 \pm 1.04$ a
	1.0	20	$32.50 \pm 8.30$ a	$9.50 \pm 1.65$ a



Fig. 1. Axillary shoot proliferation from sterile fresh juvenile shoot explants of *Rhododendron sichotense* induced by 0.1 (a), 0.5 (b) and 1.0 (c)  $\mu\text{M}$  TDZ. Bar – 10 mm.

#### *Shoot induction from leaf explants*

1. Leaf explants were placed adaxial side up on the surface of AM0 for 3 days (pre-cultivation step)

2. Explants were transferred to AM supplemented with 0.1, 0.5, 1.0  $\mu\text{M}$  of TDZ for 8 weeks (multiplication step).

3. After 8 weeks of cultivation, the frequency and the type of morphogenic response were analyzed.

4. To overcome TDZ-induced abnormalities, the regenerants from leaf explants were cultivated on AM0 for 8 weeks (elongation step).

5. After elongation, the number of adventitious shoots per explant (with length more than 5 mm) and the shoot length were determined (Table, Fig. 2).



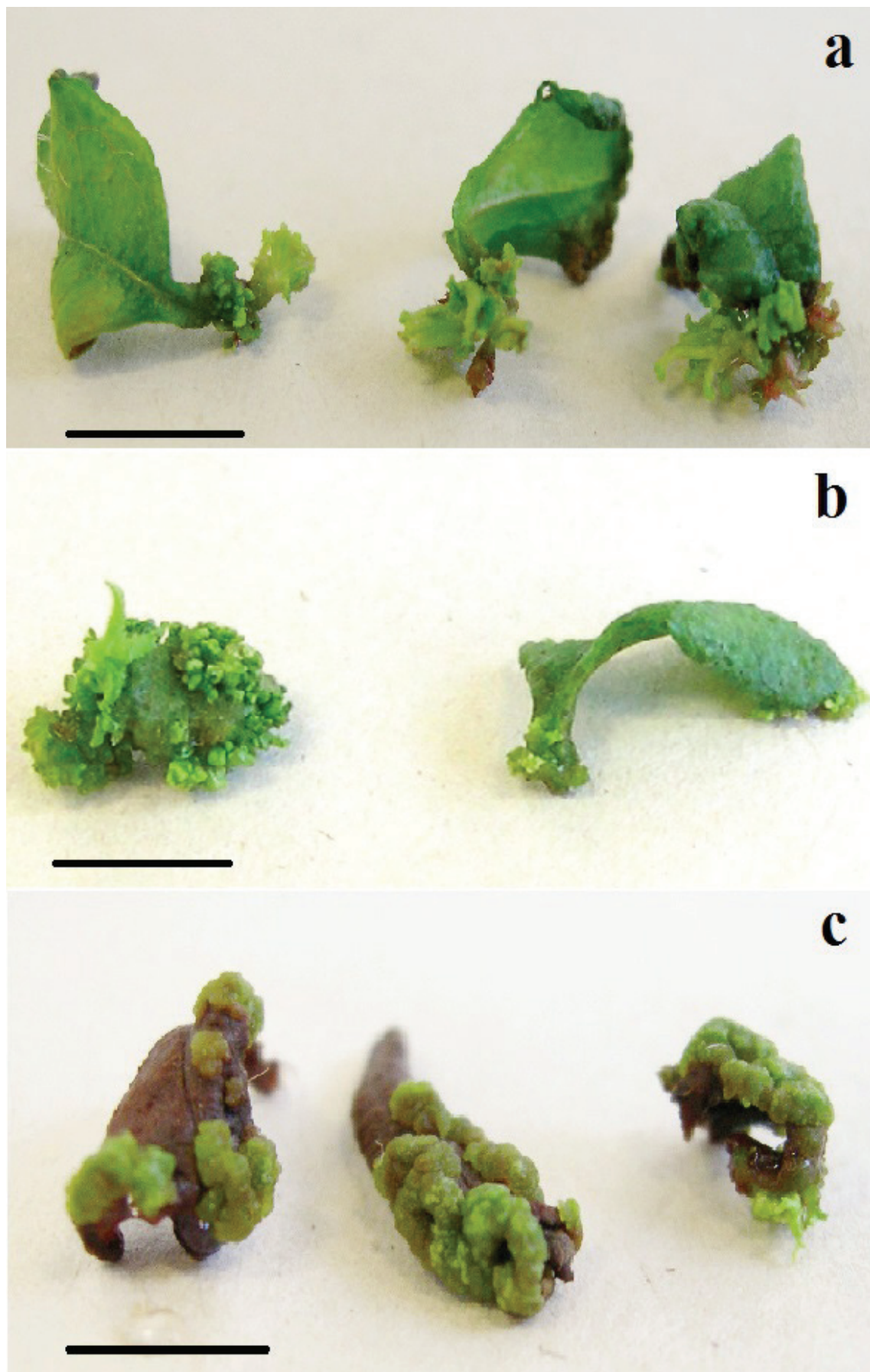


Fig. 2. Adventitious shoot proliferation from sterile fresh leaf explants of *Rhododendron sichotense* induced by 0.1 (a), 0.5 (b) and 1.0 (c)  $\mu\text{M}$  TDZ. Bar – 10 mm.

*Statistical analysis*

1. Fifteen explants were used per treatment in each experiment replicated three times.

2. Data was analyzed by one-way ANOVA using STATISTICA 8 (StatSoft Inc., OK) and presented as means  $\pm$  SE (standard error).

3. Significance between means was tested by Duncan's test ( $P = 0.05$ ).

**Conclusion**

*In vitro* establishment and initiation of regeneration are the most crucial stages in clonal micropropagation of rhododendrons. Here, we demonstrate an effective protocol for *in vitro* establishment and micropropagation of *R. sichotense* through activation the axillary bud proliferation from shoot explants as well as *de novo* direct adventitious shoot regeneration from leaf explants of field plants. To *in vitro* establish, all tested explants were pre-cultivated for 3 days on AM0 to minimize the negative effects of cutting due to phenolic exudation and drawback of TDZ treatment. The pre-cultivation step enhances the capability for regeneration due to opening the "window of competence" to morphogenesis in plant tissues (Woo, Wetzstein, 2008). This approach has been successfully used for inducing direct shoot regeneration from floral explants of *R. sichotense* (Zaytseva, Novikova, 2017). Depending on the con-

centration, TDZ is known to induce the wide range of morphogenic pathways from callus formation to the shoot production and somatic embryogenesis (Murthy et al., 1998). In the present study, the same TDZ concentrations were found to be useful for initiating both the axillary and adventitious shoot production. The elongation on hormone-free AM0 allowed obtaining the healthy shoots without any anomalies. The shoot explants demonstrated the highest regenerate frequency (60–100 %), however the leaf explants produced the maximum shoot number per explant (up to  $41.60 \pm 5.25$ ) and the shoot length ( $12.20 \pm 1.04$  sm) (Table). Consequently, the regeneration system of *R. sichotense* based on direct morphogenesis from leaf explants is found to be more effective for the large-scale plant production.

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