

## **The effect of ethyl 5'-(4-methoxybenzoyl)-5',7'-dihydrospiro[cyclopentane-1,6'-[1,2,3]triazolo[5,1-b][1,3,4]thiadiazin]-3'-carboxylate on *Pinus sylvestris* L. seed germination**

T.A. Kalinina<sup>1</sup>, O.A. Vysokova<sup>1</sup>, L.A. Khamidullina<sup>1,2</sup>, A.A. Kochubei<sup>3</sup>,  
O.E. Cherepanova<sup>3</sup> and T.V. Glukhareva<sup>1,\*</sup>

<sup>1</sup>Ural Federal University named after the first President of Russia B.N. Yeltsin, Mira street 19, RU620002 Ekaterinburg, Russia

<sup>2</sup>Postovsky Institute of Organic Synthesis UB RAS, Sofia Kovalevskaya street 22, RU620990 Ekaterinburg, Russia

<sup>3</sup>Botanical Garden UB RAS, 8 Marta street 202a, RU620144 Ekaterinburg, Russia

\*Correspondence: [taniaglukhareva@yandex.ru](mailto:taniaglukhareva@yandex.ru)

**Abstract.** Plant growth stimulators are capable of enhancing both agricultural output and the rate of plant maturation, consequently improving the total crop yield and increasing their resistance to disease and adverse environmental conditions. This is why such compounds are used in the cultivation of both agricultural and ornamental plants. The aim of this work was to study the effect of ethyl 5'-(4-methoxybenzoyl)-5',7'-dihydrospiro[cyclopentane-1,6'-[1,2,3]triazolo[5,1-b][1,3,4]thiadiazin]-3'-carboxylate on *Pinus sylvestris* L. seed germination. The article describes the synthesis of ethyl 5'-(4-methoxybenzoyl)-5',7'-dihydrospiro[cyclopentane-1,6'-[1,2,3]triazolo[5,1-b][1,3,4]thiadiazin]-3'-carboxylate and the data from spectral and X-ray crystal analysis. The results of the experimental stimulation of *Pinus sylvestris* L. seed germination using spiro-1,2,3-triazolo[5,1-b]1,3,4-thiadiazine are given compared to the commercially available phytohormones thidiazuron and 6-benzylaminopurine. The estimation of germination, vitality, healthy seed ratio and cotyledon length indicated that the tested compound at a concentration of 0.5 mg L<sup>-1</sup> had an effect similar to 6-benzylaminopurine: indeed, the speed of germination and fungal invasion rate exceeded the effect of 6-benzylaminopurine.

**Key words:** *Pinus sylvestris* L., seed germination, phytohormones, plant growth regulators, 6-benzylaminopurine, thidiazuron, 1,2,3-triazoles.

### **INTRODUCTION**

Seed germination is one of the main stages of plant ontogenesis. The dormancy and germination of seeds might be controlled by affecting various exogenous factors, namely temperature (Giolo et al., 2017), electromagnetic and electrostatic fields (Palov et al., 2012), treatment with natural and synthetic growth regulators (Miransari & Smith, 2014) and others.

Recently, the application of phytohormones and their synthetic analogues has become widespread in agriculture and horticulture (Miransari & Smith, 2014; Shu et al., 2016; Zwanenburg et al., 2016), which is conditioned by the reduction of acreage, poorly predicted climate changes, the reduction of resource bases in separate regions of the Earth and increased food resource consumption (Anderson et al., 2010). For the aforementioned reasons, the search for new plant growth regulators is of great interest.

Using seeds is convenient for testing the plant hormone activity of new compounds. During seedling formation, the plant quickly goes through several steps, from mass cell division and the beginning of photosynthesis to the appearance of the first true organs (first leaf). In view of this, several parameters can be evaluated during treatment (germinating force, germinating capacity, seedling length, radicle length, development of cotyledons and first true leaves) regardless of the external factors (temperature, humidity, illumination, climatic seasonality etc.) (Kumar et al., 2014; Han et al., 2015; Takeuchi et al., 2015).

Previously (Kalinina et al., 2015) we demonstrated the effect of 5'H,7'H-spiro[1,2,3]triazolo[5,1-b][1,3,4]thiadiazines on the proliferative activity of animal cells; the compounds which could selectively stimulate or suppress their growth were determined. The influence of these compounds upon plant cells was not studied.

A comprehensive study of the effect of a new compound on plant cells should be conducted using the seeds of those species for which the processes of germination, growth and consequent first true seedling formation are well known. *Pinus sylvestris* L. is one such species (Ivanov et al., 2011). The comprehensive collected data regarding the physiology of seed germination and consequent seedling formation allows us to use the seeds of the aforementioned species as a model for evaluating both the positive and negative influence of new synthetic compounds.

The aim of the work was the synthesis of 1,2,3-triazolo[5,1-b]1,3,4-thiadiazine and an investigation of its growth-regulating activity on the seeds of *Pinus sylvestris* L. has compared to 6-BAP and TDZ. It was expected that the compound would affect the growth and development of plants, since its influence on animal cells has previously been detected.

## MATERIALS AND METHODS

<sup>1</sup>H and <sup>13</sup>C NMR spectra were acquired on a Bruker Avance II spectrometer (400 and 100 MHz, respectively) in DMSO-*d*<sub>6</sub> with TMS as the internal standard. Mass spectra were recorded on a GCMS-QP2010 Plus gas chromat-mass spectrometer (EI ionization at 70 eV). Elemental analysis was performed on a Perkin Elmer PE 2400 CHNS-analyser. Melting points were determined on a Stuart SMP3 apparatus.

The tested compound (STT) was produced according to the previously described method (Kalinina et al., 2017). The spectroscopic characteristics of the compound are in accordance with the previously reported data (Kalinina et al., 2017).

**Ethyl ester 5'-(4-methoxybenzoyl)-5',7'-dihydrospiro[cyclopentane-1,6'-[1,2,3]triazolo[5,1-b][1,3,4]thiadiazin]-3'-carboxylic acid (STT).** Reaction yield is 82%, light-lilac powder, melting temperature 192–193 °C. Found, %: C 56.53; H 5.60; N 13.78. C<sub>19</sub>H<sub>22</sub>N<sub>4</sub>O<sub>4</sub>S. Calculated, %: C 56.70; H 5.51; N 13.92.

**X-ray crystal analysis of the compound (STT).** Single crystals (colourless plates) suitable for X-ray diffraction studies were obtained by slow evaporation of a dimethyl sulfoxide solution of the title compound at room temperature. Single-crystal X-ray diffraction was performed on an Oxford Diffraction Xcalibur-3 diffractometer with Mo K $\alpha$  radiation,  $\lambda = 0.7107 \text{ \AA}$ . Absorption correction was not applied. The structure was calculated and corrected using the Olex2 software (Dolomanov et al., 2009). The structure was solved by direct methods using the program SHELXS97 and refined on  $F^2$  by full-matrix least-squares procedures using the program SHELXL97 (Sheldrick et al., 2008). The non-hydrogen atoms were refined in the anisotropic approximation: hydrogen atoms were included in the refinement isotropically in the riding model.

Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Center as the supplementary publication No. CCDC 1576559. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB21EZ, UK (deposited@ccdc.cam.ac.uk).

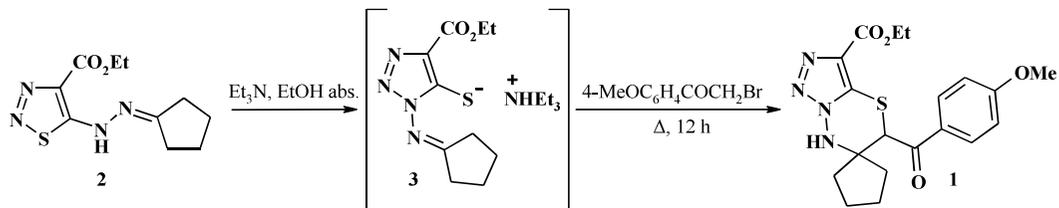
### **Biological study**

The experimental research on compound impact was conducted in the Binder growth chamber in order to create temperature, humidity and illumination levels close to the natural conditions for seed germination (20 °C, humidity level 55–60%, 8 daylight hours) and to regulate them throughout the entire experiment. It allowed us to eliminate the influence of extraneous factors on the formation of pine seedlings. The seeds with a laboratory germination of 98%, taken from a pine forest in the II productivity class (the sowing quality of seeds corresponded to GOST 14161-86), were used to estimate the effect of the compound upon the germination process. The seeds were couched in lots of 100 pieces (GOST 13056.6-97) in sterile Petri dishes covered with two layers of filter paper, previously disinfected by ultraviolet germicidal irradiation. The experimental material included 3,000 seeds (three repetitions for each concentration) in total. Three milliliters of aqueous solutions of compounds were added into the Petri dishes at the beginning of the experiment. Furthermore, the dishes were weighed each alternate day to ensure the preset humidity level. The experiment lasted 21 days; the main parameters (vitality and germinating force) were evaluated on the 7<sup>th</sup> and 15<sup>th</sup> day according to GOST 12038-84. The major results were obtained on the 21<sup>st</sup> day of the experiment.

Two well-known phytohormones (6-BAP and TDZ) and one synthetic compound (STT) at three concentrations (0.5, 1 and 5 mg L<sup>-1</sup>) were chosen as the objects of the study. Each line was provided a control tube with distilled water. The seedling quality was evaluated based on the following parameters: total seedling length, cotyledon length and thickness of the stem at the bottom of the cotyledons (because it is precisely these parameters that indicate the seedling vitality and quality of formed palisade apparatus). The experimental results were processed using the Statistica 6.0 software (ANOVA). All the attributes used in statistical analysis are normally distributed. Levene's test was used to check that variances are equal for all the samples.

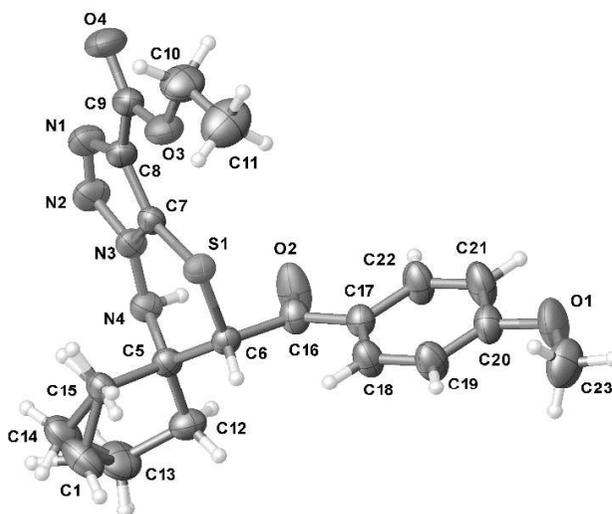
## RESULTS AND DISCUSSION

The spiro-1,2,3-triazolo-1,3,4-thiadiazine STT (see Scheme 1), which turned out to be one of the most active compounds during the tests conducted with animal cells, was chosen to study the effect of the compounds on *Pinus sylvestris* L. seed germination. The STT was obtained following the previously developed method (Kalinina et al., 2017): through the Dimroth rearrangement of starting 1,2,3-thiadiazolylyhydrazone **1** under the effect of triethylamine and interaction of formed 1,2,3-triazolyliolat **2** with  $\alpha$ -bromo-4-methoxyacetophenone (Scheme 1).



**Scheme 1.** Synthesis of STT.

The structure and purity of the obtained compound were confirmed using NMR spectroscopy data  $^1\text{H}$  and  $^{13}\text{C}$ , mass spectrometry and elemental analysis. Single-crystal X-ray diffraction analysis of the compound was performed for the first time (Fig. 1). The main parameters of structural experiments for the STT are given in Table 1.

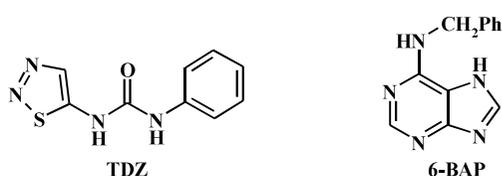


**Figure 1.** ORTEP view of the molecular conformation of the STT with the atom-labelling scheme. The displacement ellipsoids are drawn at the 50% probability level.

**Table 1.** Crystal data and structure refinement for the STT

Molecular formula	C <sub>19</sub> H <sub>22</sub> N <sub>4</sub> O <sub>4</sub> S
Molecular weight, gmole <sup>-1</sup>	402.47
Temperature, K	295
Crystal system	Triclinic
Space group	P <sub>1</sub>
a, Å	9.1304 (5)
b, Å	9.9986 (6)
c, Å	11.8551 (6)
α, degree	107.458 (5)
β, degree	99.719 (4)
γ, degree	99.414 (5)
V, Å <sup>3</sup>	990.84 (9)
Z	2
D <sub>calc.</sub> , gm <sup>-3</sup>	1.349
μ, mm <sup>-1</sup>	0.20
F(000)	424
Crystal size, mm	0.3 × 0.25 × 0.2
Range θ for data collection, degree	2.6 ≤ θ ≤ 30.7
Subscript ranges	-12 ≤ h ≤ 12 -12 ≤ k ≤ 14 -16 ≤ l ≤ 16
Measured / independent reflections	9553/5400
Reflections with c I > 2σ(I)	4047
Corrected parameters	272
R-factor based on F <sup>2</sup> > σ(F <sup>2</sup> )	0.047
wR <sub>2</sub> for all reflections	0.156
Residual electron density (min/max), eÅ <sup>-3</sup>	-0.21/0.34

Commercially available synthetic heterocyclic phytohormones (applied for plant growth regulation and development), thiazuron (TDZ) and 6-benzylaminopurine (6-BAP) (Victor et al., 1999; Abad et al., 2004; Kong et al., 2016; Zhang et al., 2016) were used as positive controls (Scheme 2) during the investigation of the effect of STT on *Pinus sylvestris* L. seed germination.

**Scheme 2.** The structure of phytohormones thiazuron (TDZ) and 6-benzylaminopurine (6-BAP).

The seed germinating force was estimated on the 7<sup>th</sup> day of the experiment and varied from 21.6% to 54.3% in the studied lines. *Pinus sylvestris* L. seeds treated with STT (54.33 ± 3.48%) with concentration 0.5 mg L<sup>-1</sup> had the maximum germinating force, while seeds treated with TDZ (21.60 ± 2.73%; 5 mg L<sup>-1</sup>) had the minimum germinating force. The germinating force of the control lot was 43.30 ± 10.49%. In the case of the STT line with concentration 5 mg L<sup>-1</sup>, we noticed almost a decrease in the germinating force almost double to that of the control lot – 21.60 ± 2.70%. The 6-BAP line with concentrations 0.5 and 1 mg L<sup>-1</sup> had the same seed germinating force as the control lot; in the line with concentration 5 mg L<sup>-1</sup>, the force was close to the maximum (51.00 ± 1.52%).

The linear parameters of the pine seedlings were estimated on the 14<sup>th</sup> day. The compound STT with concentration 0.5 mg L<sup>-1</sup> demonstrated the best results in growth stimulation (Table 2). This line had the maximum total seedling length (5.91 ± 0.25 cm) and cotyledon length (1.21 ± 0.08 cm) increase compared to the control lot. The lines treated with the same concentration showed the minimum seedling stem thickness (0.63 ± 0.02 mm), which is probably connected with fast cell growth by stretching (see Table 2). Lines treated with TDZ demonstrated the slowest seedling growth, especially with concentration 5 mg L<sup>-1</sup>.

**Table 2.** The morphological parameters of *Pinus sylvestris* L. seedlings on the 15<sup>th</sup> and 21<sup>st</sup> experimental day

Concentration, mg L <sup>-1</sup>	15 <sup>th</sup> day (M ± m)*			21 <sup>st</sup> day (M ± m)*		
	Total length, cm	Cotyledons length, cm	Stem thickness, mm	Total length, cm	Cotyledons length, cm	Stem thickness, mm
<b>STT</b>						
0.5	5.91 ± 0.25	1.21 ± 0.08	0.63 ± 0.02	6.40 ± 0.11	1.76 ± 0.04	0.74 ± 0.01
1	4.94 ± 0.22	1.20 ± 0.27	0.67 ± 0.02	5.68 ± 0.12	1.36 ± 0.03	0.72 ± 0.01
5	5.80 ± 0.18	1.08 ± 0.07	0.68 ± 0.02	6.33 ± 0.11	1.52 ± 0.04	0.70 ± 0.01
<b>TDZ</b>						
0.5	4.20 ± 0.21	0.97 ± 0.07	0.71 ± 0.02	5.35 ± 0.11	1.51 ± 0.04	0.67 ± 0.01
1	3.85 ± 0.14	0.95 ± 0.06	0.66 ± 0.02	4.83 ± 0.11	1.33 ± 0.05	0.72 ± 0.02
5	3.11 ± 0.19	0.71 ± 0.07	0.78 ± 0.03	3.13 ± 0.12	0.65 ± 0.03	0.99 ± 0.03
<b>6-BAP</b>						
0.5	5.19 ± 0.36	1.01 ± 0.08	0.83 ± 0.02	4.55 ± 0.26	1.23 ± 0.06	0.48 ± 0.03
1	5.36 ± 0.28	1.10 ± 0.10	0.83 ± 0.01	6.29 ± 0.11	1.59 ± 0.04	0.62 ± 0.02
5	4.73 ± 0.24	1.08 ± 0.07	0.86 ± 0.02	3.82 ± 0.13	1.08 ± 0.05	0.43 ± 0.02
<b>Control lot</b>						
–	5.31 ± 0.23	1.07 ± 0.08	0.84 ± 0.02	5.72 ± 0.33	1.35 ± 0.13	0.81 ± 0.02

\* M – mean value; m – standard deviation.

The growth tendencies on the 21<sup>st</sup> day were the same. Following the overall estimation of all parameters, the compound STT ensured the best growth stimulation on the 21<sup>st</sup> day. The seeds treated with this compound in minimal (0.5 mg L<sup>-1</sup>) and maximal (5 mg L<sup>-1</sup>) concentrations had the best parameters or were close to them compared to the results shown by the other experimental compounds (see Table 2). In the case of a medium concentration (1 mg L<sup>-1</sup>), the total seedling length and cotyledon length parameters were slightly less than that of the seedlings treated with 6-BAP with concentration 1 mg L<sup>-1</sup>, but they were better than in the other lines.

The total quantity of seedlings in the TDZ line with concentration 0.5 mg L<sup>-1</sup> on the 21<sup>st</sup> day was maximal – 93.3 ± 0.6%; the quantity of healthy seedlings in this line was 1.16 times higher (51.1 ± 0.6%) than in the control lot (44.0 ± 0.5%) (Table 3). The STT lines with all concentrations were characterised with the total minimal quantity of germinated seeds compared to the control lot: 0.5 mg L<sup>-1</sup> – 83.6 ± 0.2%, 1 mg L<sup>-1</sup> – 86.1 ± 0.6%, 5 mg L<sup>-1</sup> – 81.3 ± 0.3%. The aforementioned STT line with concentration 0.5 mg L<sup>-1</sup> had the maximum quantity of healthy seedlings – 52.3 ± 0.2%. The 6-BAP lines with all concentrations had a stable high total quantity of seedlings (90%), but the

percentage of fungal-affected seedlings in these lines was higher than in all the other lines and the control one.

**Table 3.** The quantity of healthy, suppressed and underdeveloped *Pinus sylvestris* L. seedlings on the 21<sup>st</sup> day

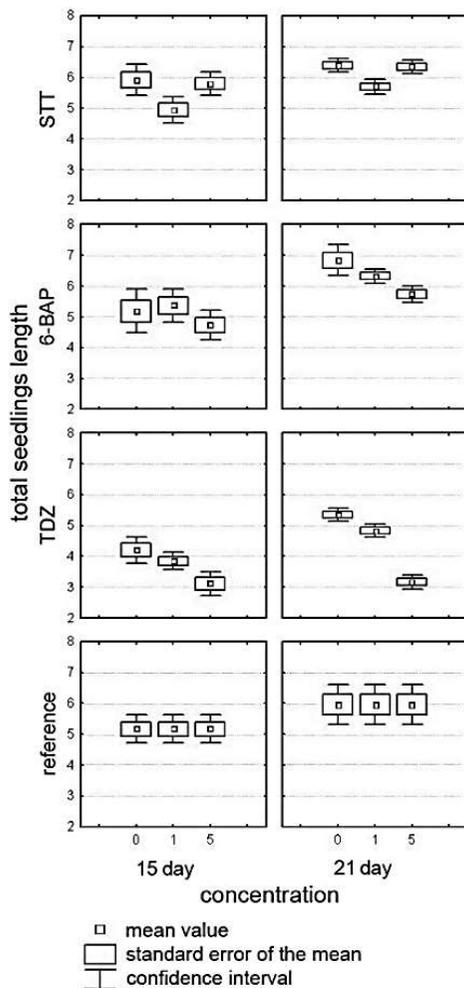
Concentration, mg L <sup>-1</sup>	Healthy, %	Suppressed, %	Underdeveloped, %
STT (M ± m)*			
0.5	52.34 ± 0.22	26.63 ± 0.84	8.63 ± 1.46
1	22.36 ± 0.64	57.95 ± 0.87	5.69 ± 0.68
5	49.35 ± 0.31	6.91 ± 0.71	25.04 ± 0.49
TDZ (M ± m)*			
0.5	51.11 ± 0.61	20.37 ± 7.12	21.82 ± 0.58
1	37.41 ± 0.5	17.04 ± 0.4	39.56 ± 1.07
5	14.35 ± 0.32	19.31 ± 0.21	60.34 ± 0.63
6-BAP (M ± m)*			
0.5	19.26 ± 0.68	42.22 ± 4.79	28.52 ± 4.28
1	29.63 ± 0.49	47.41 ± 1.56	12.96 ± 1.25
5	35.56 ± 0.4	45.18 ± 1.27	9.26 ± 0.55
Control lot (M ± m)*			
–	44.00 ± 0.5	38.89 ± 0.8	16.11 ± 0.56

\* M – mean value; m – standard deviation.

The obtained linear parameters, germination data, percentage of fungal-affected and healthy seedlings were collected in an overall data matrix with two time points (15<sup>th</sup> and 21<sup>st</sup> day) and processed with the help of software package Statistica 6.0 (ANOVA) (Fig. 2). According to the analysis results on the 15<sup>th</sup> and 21<sup>st</sup> days, the TDZ lines with concentration 5 mg L<sup>-1</sup> significantly differed from the STT line (5 mg L<sup>-1</sup>) – 0.000025 (21<sup>st</sup> day), 6-BAP line (5 mg L<sup>-1</sup>) – 0.000017 (21<sup>st</sup> day) and control lot 0.000017 (21<sup>st</sup> day). In turn, the 6-BAP lines and STT lines did not show significant differences either with respect to each other or to the control, which evidenced for normal pine seedling growth in these lines throughout the experiment.

The TDZ line was characterised by a strong growth suppression with the increasing levels of concentration (see Table 2). With maximal concentration, the cotyledons did not develop at all or were very small (compared to the control lot and other lines); the root system was also underdeveloped. Petri dishes treated with this compound were less fungal-affected, which probably demonstrates some retardation of the floccus in the presence of the aforementioned compound. The average linear parameters in TDZ line with all concentrations were much lower than in the control lot. These peculiarities of TDZ influence on *Pinus sylvestris* L. seed germination have been reported before (Kalinina et al., 2016). The STT line with a concentration of 0.5 mg L<sup>-1</sup> had the best parameters of total seedling length and cotyledon length and exceeded the control lot parameters. The seedling thickness was minimal compared to the control lot and all the studied compounds with 0.5 mg L<sup>-1</sup> concentration. The sharp decrease of stem thickness can be attributed to the cell growth stimulation by stretching (Gonzalez et al., 2012; Tsukaya et al., 2006). The percentage of fungal-affected seeds was minimal in concentrations of 0.5 mg L<sup>-1</sup> and 1 mg L<sup>-1</sup>. This parameter was best for all the studied compounds and control lot. In case of 0.5 mg L<sup>-1</sup> concentration, the percentage of healthy

seedlings was maximal (about 60%) compared to all the studied compounds and control lot.



**Figure 2.** Total seedling length in the studied lines on the 15<sup>th</sup> and 21<sup>st</sup> days of the experiment.

## CONCLUSIONS

Following the obtained experimental results, we can conclude that the growth regulating properties of the compound STT are close to that of 6-BAP and their impact is similar but for some parameters, such as cotyledon length and healthy seed percentage, STT exceeds the 6-BAP. No adverse effect of the synthesized compound on a seed germination of *Pinus sylvestris* which have good natural germination, was observed. The seedlings obtained by day 21 were found to have increased viability, longer cotyledon length and less fungal invasion with respect to the 6-BAP and TDZ lines. Consequently, it is advisable to check the effect of this compound on another plant species that has difficulties in germination and the formation of healthy, viable seedlings. The practical application of the compounds with the aforementioned properties lies in the stimulation

of root formation and sprout formation in case of micropropagation and *in vitro* propagation. Thus, the study of the effect of 1,2,3-triazolo[5,1-*b*]-1,3,4-thiadiazines on plants is of current interest as they can be applied in agriculture and plant biotechnology.

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