**In vitro growth responses of the ‘Pyrodwarf’ pear rootstock to cytokinin types**

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**Abstract**

The present paper deals with the effect of different concentrations (1, 2, 5 and 15 μM) of 6-benzyladenine (BA) and thidiazuron (TDZ), combined with indole-3-butyric acid (IBA) at concentrations of 0, 0.5, 2.5 and 4.9 μM on in vitro multiplication of the pear rootstock ‘Pyrodwarf’. Murashige and Skoog (1962) (MS) was the basal medium used in all combinations. The multiplication index as well as the length of axial and lateral shoots were monitored. The highest multiplication index (2.89) was obtained on the medium supplemented with 5 μM BA and 0.5 μM IBA. Higher concentrations of TDZ (more than 2 μM TDZ), in combination with IBA, brought about fasciation of shoots, which was confirmed by SEM technique. On the rooting medium consisting in 1/2 MS supplemented with 5 μM IBA and 0.3 μM gibberellic acid (GA₃), the higher rooting percentage and other superior rooting parameters were also recorded in the case of the shoots originated from BA media. The obtained results suggest that to gain good multiplication parameters it is recommended to use good quality shoots for further subcultivation. In order to avoid the occurrence of fasciation it is required to use the cytokinin BA or to limit the concentration of TDZ in the multiplication phase of pear ‘Pyrodwarf’ rootstock.

**Key words:** cytokinins, fasciation, in vitro growth, multiplication, *Pyrus communis* L.

**Introduction**

A wide range of cytokinin types and concentrations are effective in vitro and the requirements among species are different (HUETTEMAN & PREECE [1]). It is well-known that cytokinins promote cell division and cell expansion in plant tissue culture and many studies have reported suitable cytokinin types and their concentrations for each species.

Cytokinins are divided into two major groups: synthetic phenylurea derivates, such as 1-phenyl-3-(1,2,3-thidiazol-5-yl)urea (Thidiazuron; TDZ), and adenine derivates, which may occur naturally, such as kinetin and 6-benzyladenine (BA) (KADOTA & NIIMI [2]).

Benzyladenine is widely used in micropropagation of fruit trees and has given good results so far when combined with different auxins (RUŽIĆ & al. [3]; RUŽIĆ & al. [4]; RUŽIĆ & VUJOVIĆ [5]). However, in vitro establishment and multiplication of species that are difficult to propagate can be greatly enhanced by the exposure to TDZ. Generally, extremely low concentrations of TDZ are needed to stimulate axillary shoot proliferation in many woody species (HUETTEMAN & PREECE [1]). Investigations of the effect of TDZ on fruit species reached their climax in the 90’s: adventitious shoots were regenerated from cotyledons of plum *Prunus domestica* L., sour cherry *Prunus cerasus* and peach *Prunus persica* L. (MANTE & al. [6]), *Rubus* sp. (FIOLA & al. [7]; COUSINEAU & DONNELLY [8]); stimulation of organogenesis from leaf in *Rubus* and *Malus domestica* L. (FASOLO & al. [9]; SWARTZ & al. [10]), *Pyrus* sp. (CHEVREAU & al. [11]). Similarly, the effect of
TDZ on some woody plants, such as sweet cherry cv. Lapins and on some herbaceous ones such as *Vigna radiata*, have been the subject of investigation in more recent time (RUŽIĆ & VUJOVIĆ [5]; AMUTHA & al. [12]).

Hence, the objective of this study was to improve the protocol for *in vitro* micropropagation of the promising ‘Pyrodwarf’ pear rootstock by using 2 cytokinins, BA and TDZ, combined with the auxin IBA, at different concentrations.

**Materials and methods**

**Plant material**

Low vigorous pear rootstock ‘Pyrodwarf’, belonging to *Pyrus communis* L., suitable for modern pear production (clone BU 5-18) was used as a plant source for the purpose of this study. It was obtained from the crossing of ‘Old Home’ x ‘Bonne Louise d’Avranches’ in Geisenheim Research Institute (Germany), among 800 seedlings. The patented name of this rootstock is ‘Rhenus 1’ (JACOB [13]).

**Multiplication**

The shoots were in multiplication phase on MS medium (MURASHIGE & SKOOG [14]) supplemented with 4.4 μM BA, 0.5 μM IBA and 0.3 μM GA₃ from a previous experiment (RUŽIĆ & al. [15]). Upon multiplication of sufficient number of shoots they were placed on the MS medium supplemented with BA or TDZ, combined with the auxin IBA at different concentrations (Table 1). Prior to placement on the appropriate media, shoots were subcultured once on them to avoid the effect of residues from the previous medium. Therefore all samples for measurements were taken from the second subculture. Stock solution of TDZ was obtained by dissolving it with DMSO and was sterilized by autoclaving. Prior to autoclaving, the pH value of all media was adjusted to 5.75 with 0.1 N KOH. The media were sterilized in an autoclave for 20 min at 120 ºC. All the media contained agar and sucrose at concentrations of 7 g l⁻¹ and 20 g l⁻¹, respectively.

Multiplication parameters were determined by standard morphometry. Shoots smaller than 0.5 cm were not taken into consideration. The multiplication index and the length of axial and lateral shoots were monitored. Some specific issues, such as appearance of fasciation, leaf and callus color and size, incidence of chlorosis or necrosis were also monitored.

**Fasciation**

To confirm the appearance of undesirable fasciation, i.e. to distinguish from the similar signs of hyperhydricity, especially pronounced on media supplemented with higher concentrations of TDZ, shoots taken from these media and shoots originated from BA media as a control, were subjected to scanning electron microscopy (SEM). For SEM studies, the specimens were prepared without any treatment. Plant material was mounted on aluminum stubs covered with double-faced transparent tape and were coated with a gold layer of 20 nm thick in a sputter coater (BAL-TEC SCD 005). The samples were observed at an accelerating voltage of 10 kV with a scanning electron microscopy (JEOL JSM-6390LV, Japan). Photographs of plant materials were taken at a magnification of 22 x.

**Rooting and acclimatization**

Shoots grown on media with BA and TDZ (longer than 0.5 cm) were placed directly on the rooting medium (MS medium having the mineral salts reduced to 1/2, unchanged organic compounds according to MS, IBA 5 μM and GA₃ 0.3 μM). The shoots were maintained in the growth room for 28 days. The following rooting parameters were monitored: rooting percentage, number of roots, root length and height of rooted plants. Rooted shoots were removed from culture vessels, washed carefully with water to remove
adhering medium, transferred to plastic pots containing sterile soil substratum (Steckmedium-Klasmann) and acclimatized on a ‘mist’ bench in greenhouse for two weeks.

**Cultural conditions**
The cultures were grown in growth room under a 16 h photoperiod, with a light intensity of 41 mol m⁻² s⁻¹ under culture surface provided by cool white fluorescent tubes 40 W, 6,500 K in strength. The temperature was 25 ± 1°C.

**Data analysis**
The multiplication experiments included six culture vessels x 3 uniform shoots x 40 treatments (40 media/combinations) x 3 replications (total 2,160 shoots). Rooting trials comprised five culture vessels x 5 uniform shoots x 2 replications. The data were analyzed by ANOVA and F-test, followed by Duncan's Multiple Range Test for means separation.

**Results**

**Multiplication parameters and appearance of fasciation**
On the medium with 5 µM BA and 0.5 µM IBA, which gave the highest multiplication index (2.89), the shoots were vigorous, with dark green leaves and with firm nodular calli developed at the base (Table 1; Fig. 1a). These shoots exhibited good quality for further subculturing.

The highest shoot multiplication index on media containing TDZ (2.28) was obtained on media supplemented with 1 µM TDZ. However, the multiple shoots were short, having numerous tiny bud rudiments not only at the base of the shoot, but also along its entire length (Fig. 1b).

The highest length of axial shoots was obtained on medium with 5 µM BA (2.62 cm). However, the highest length of lateral shoots were recorded on media supplemented with 2 and 5 µM of BA in combination with 0.5, 2.5 and 4.9 µM IBA (Fig. 1a).

**Table 1.** The *in vitro* multiplication parameters of ‘Pyrodwarf’ rootstock on culture media with different hormonal supplements.
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<td>15</td>
<td>-</td>
<td>0.5</td>
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<td>B19</td>
<td>15</td>
<td>-</td>
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<td>B20</td>
<td>15</td>
<td>-</td>
<td>4.9</td>
<td>2.17 cd</td>
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<td>T01</td>
<td>-</td>
<td>1</td>
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*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan’s Multiple Range Test.*
In vitro growth responses of the ‘Pyrodwarf’ pear rootstock to cytokinin types

Fig. 1. The effect of hormonal composition of media on multiplication and rooting of ‘Pyrodwarf’ rootstock shoots. Shoots grown on medium with 5.0 μM BA and 0.5 μM IBA (a); 1 μM TDZ (b); 1 μM TDZ and 2.5 μM IBA (c); 15 μM TDZ and 0.5 μM IBA (d). Rooted shoots of ‘Pyrodwarf’ originated from the medium with BA (e); TDZ (f) and acclimatized plants in greenhouse (g).

Shoots revealed a different morphology already on media supplemented with 1μM TDZ combined with IBA. These differences were reflected in thick axial shoot, short internodes, small leaves and high callus mass (Fig. 1c). Higher concentrations of TDZ (more than 2 μM) resulted in manifestation of fasciation, i.e. axial shoot was short, thick, and exceptionally pronounced bud rudiments were observed along the entire stem. Stems of axial shoots were fused with axillary shoots (Fig. 1d).

Rooting parameters and acclimatization

Higher rooting rate, 96% and quality of rooted plants were observed in shoots maintained previously on BA media (Table 2). Plants were well developed, with long stems and wide dark green leaves. Roots were long, white and brittle, without secondary roots (Fig. 1e).

Long axillary shoots taken directly from the medium supplemented with TDZ had high callus mass at the shoot base and gave short and thick, radial spread roots (Figure 1f).

Rooted shoots exhibited high ability to acclimatize (90.9% of acclimatization survival) under the ‘mist’ system in greenhouse (Fig. 1g).

Table 2. Rooting parameters of ‘Pyrodwarf’ rootstock.

<table>
<thead>
<tr>
<th>Origin of shoots</th>
<th>% of rooting</th>
<th>No of roots</th>
<th>Length of roots (cm)</th>
<th>Height of rooted plants (cm)</th>
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<td>From BA medium</td>
<td>96 a*</td>
<td>4.88 a</td>
<td>1.83 a</td>
<td>1.35 a</td>
</tr>
<tr>
<td>From TDZ medium</td>
<td>80 b</td>
<td>3.92 b</td>
<td>0.30 b</td>
<td>0.68 b</td>
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*Means followed by the same letter within columns are not significantly different at the 5% level of significance using Duncan’s Multiple Range Test.
Scanning electron microscopy

Using SEM technique we have proved that fasciation (several stem were fused) has occurred in the presence of TDZ (Fig. 2).

**Fig. 2.** SEM micrograph showing: fused shoots of ‘Pyrodwarf’ on medium with 10 µM TDZ and 5.0 µM IBA (left); shoots with normal morphology – 3 separated multiplied shoots from the same shoot base on control BA medium (right). Photographs of plant materials were taken at a magnification of 22x.

**Discussion**

Although BA is the cytokinin of choice for micropropagation of many species and cultivars in the genus *Pyrus* (YEO & REED [16]; BELL & REED [17]), some investigations showed that TDZ is more effective than BA in adventitious shoot induction from cultured stem slices of *P. communis* cv. Bartlett (BOMMINENI & al. [18]). Thus, due to the low multiplication rate of pear rootstock ‘Pyrodwarf’ in our earlier investigations (RUŽIĆ & al. [15]; RUŽIĆ & al. [19]), we initiated a trial with TDZ, bearing in mind its high effect on cell division and expansion potential. On the one hand, TDZ increased shoot development in many woody plant species more efficiently than adenine derivatives, but on the other hand it was ineffective for the proliferation in some species as it occurred in the multiplication experiments of cherry cv. Lapins (RUŽIĆ & VUJOVIĆ [5]).

Our results agree with those reported for pear *Pyris pyrifolia* N. which suggest that BA displayed a more noticeable effect than TDZ, i.e. BA is more suitable for shoot multiplication of pear than phenylurea derivatives (KADOTA & NIIMI [2]). However, high concentrations of adenine type cytokinins are often necessary for growth and differentiation in tissue culture.

On the other hand, TDZ is one of several substituted ureas that has been investigated for cytokinin activity and the most effective concentrations are 10 to 1,000 times lower than with other plant growth regulators. Extremely low concentrations of TDZ are needed to stimulate axillary shoot proliferation (HUETTEMAN & PREECE [1]), which has also been confirmed in this paper. Until now the reason for high activity of low concentrations of TDZ in woody plant micropropagation has not been investigated at a molecular level (MOK & al. [20]). It is a known fact that TDZ stimulates endogenous biosynthesis of cytokinins, which brings about an increase in the level of naturally occurring cytokinins, and it is likely to have a common site of action with the naturally occurring cytokinins. More ribosomes and polysomes were observed in the cytoplasm of cells on media containing TDZ compared to media containing BA, indicating a more intensive protein synthesis and higher cell activity in the presence of TDZ (CHVOJKA & al. [21]).
We observed that TDZ inhibits shoot elongation and induces formation of shortened internodes, as it occurred also in apple cv. Gala (VAN NIEUWKERK & al. [22]), and abnormally short, callused and difficult-to-count shoots of three strains of ‘McIntosh’ apple (SARWAR & al. [23]). High concentrations of TDZ tend to stimulate callus formation in many woody species as it was also with ‘Pyrodwarf’ shoots. Similar responses have been reported for several woody species (THENGANE & al. [24]; PRUSKI & al. [25]).

Thidiazuron was also effective in multiplication of black walnut but most of the regenerated shoots were swollen or fasciated as regards the morphology (BOSELA & MICHLER [26]). Potential liabilities when using TDZ for organogenesis include problems with shoot fasciation and hyperhydric shoots (HUETTEMAN & PREECE [1]; AMUTHA & al. [12]; BOSELA & MICHLER [26] etc.). The occurrence of fasciation is certainly an undesirable phenomenon during shoot multiplication in vitro.

Fasciated shoots differ markedly from normal ones, appearing as several fused together shoots and the main stem appears somewhat squashed. In our experiment the occurrence of fasciation has been confirmed by SEM technique showing several shoots fused into one. It is therefore likely that the phenyl group of TDZ may be the cause of fasciation (HUETTEMAN & PREECE [1]). Conditions favoring rapid growth also encourage the development of fasciation but can be caused by a hormonal imbalance as well.

Rooting of excised microshoots may be difficult because of ‘carry over’ effect from cytokinins in the shoot multiplication medium, especially using a cytokinin as potent as TDZ. Despite this fact, in ‘Pyrodwarf’ shoots previously grown on media containing TDZ we obtained high frequency of rooting, nonetheless the roots were short and thick.

Conclusions

In comparison with BA which displayed a certain better effect, relatively low concentrations of TDZ have the capacity to induce multiplication of ‘Pyrodwarf’ shoots, but higher concentrations may result in huge callus mass and fasciated shoots which is an undesirable manifestation in vitro.

The obtained results undoubtedly suggest that the cytokinin type and concentration suitable for micropropagation of woody plants may depend on plant species, i.e. are probably genotype dependent and require further investigation.

Acknowledgments

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References