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# Influence of thidiazuron (TDZ) pretreatment of shoot tips on shoot multiplication and ex vitro acclimatization of *Harpagophytum procumbens*

Renata Grąbkowska · Przemysław Sitarek · Halina Wysokińska

**Abstract** The effects of thidiazuron (TDZ) pretreatment of shoot tips on *Harpagophytum procumbens* shoot proliferation and successive stages of micropropagation, i.e. rooting of regenerated shoots and acclimatization of plantlets to ex vitro conditions, were described in the present study. The best response in terms of shoot proliferation (about seven shoots/explant) and shoot length ($3.2 \pm 0.4$ cm) was obtained when explants pretreated with 25 µmol $L^{-1}$ TDZ for 6 h were cultured on Schenk and Hildebrandt medium containing indole-3-acetic acid (IAA) (0.57 µmol $L^{-1}$) and 6-benzylaminopurine (BAP) (8 µmol $L^{-1}$). Under these conditions, a 330 % increase in shoot multiplication over TDZ non-pretreatment culture was achieved and TDZ pretreatment shoots were longer compared to those in control culture ($2.6 \pm 0.3$ cm). The TDZ pretreatment did not affect the percentage of rooted shoots, length of roots and number of roots formed per shoot. The rooted plantlets were transplanted from in vitro to pots with soil and grown during 1 year in the greenhouse. The hardening process was difficult and time-consuming. We found that the plants developed from the TDZ pretreated culture were superior to plants from non-pretreated culture in terms of survival rate and morphological features, such as shoot length, leaf size, flowering and earlier root tuberisation. Random amplified polymorphic DNA and inter-simple sequence repeat analyses of pretreatment with TDZ plants showed genetic similarity to non-pretreatment plants. We conclude that applying the strategy of initial explant pretreatment with TDZ may be valuable for the improvement in *H. procumbens* in vitro propagation.

**Keywords** Acclimatization · *Harpagophytum procumbens* · Molecular methods · Shoot proliferation · TDZ pretreatment · Thidiazuron

## Introduction

*Harpagophytum procumbens* (Burch.) DC. ex Meisn. (known also as grapple plant, wood spider and devil's claw) is an endangered, medicinally important perennial herb belonging to the family Pedaliaceae. The plant grows in the Kalahari savannas of South Africa, Botswana and Namibia (Hachfeld and Schippmann 2000; Wichtl 2004). The tuberous roots of *H. procumbens* are used for medicinal purposes. In South African folk medicine they have been used as an analgesic, a remedy for fevers, allergies and for digestive disorders as a better tonic (Wichtl 2004). The ESCOP Monographs (2003) and the European Pharmacopoeia (2010) recommend *Harpagophyti radix* as a diuretic and sedative drug and for treatment of degenerative painful rheumatic disorders. The major chemical constituents in *H. radix* are iridoids (e.g. harpagoside, harpagide, pagoside, procumbide) and phenylethanoid glycosides such as verbascoside and isoverbascoside (Burger et al. 1987; Boje et al. 2003; Qi et al. 2006).

*H. procumbens* is propagated through seeds. However, the cultivation of the plant is difficult because seeds display high level of dormancy (germination rate is less than 20 %) and the survival of seedlings is low (Raimondo and Donaldson 2002). Furthermore, over exploitation of the medicinal plant has led to its rapid depletion from the wild

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(Hachfeld and Schippmann 2000). In vitro culture techniques can help overcome the problems. In literature there are some reports on the micropropagation of *H. procumbens* from nodal explants (Shushu 2001; Levieille and Wilson 2002; Kaliamoorthy et al. 2008; Bairu et al. 2009; Jain et al. 2009) and shoot tips (Shushu 2001; Grąbkowska and Wysokińska 2009). The effects of different cytokinins (6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin, kinetin, *meta-*, *ortho-*, *para*-topolin and topolin riboside) on shoot multiplication in these studies have been also investigated. For example, Kaliamoorthy et al. (2008) have reported that the maximum number of shoots (7–8 per explant) was formed from the nodal explants of *H. procumbens* after 4 weeks of culture on MS (Murashige and Skoog 1962) medium containing 0.57 μmol L$^{-1}$ indole-3-acetic acid (IAA) and 9 μmol L$^{-1}$ zeatin or 23 μmol L$^{-1}$ kinetin. When shoot tips were used as the explants, 1.5–2.2 shoots were formed on MS medium supplemented with α-napthaleneacetic acid (NAA) (5.38 μmol L$^{-1}$) and BAP (0.44–2.22 μmol L$^{-1}$) (Shushu 2001). Adventitious shoot development from the nodal explant-derived callus cultured on MS medium in the presence of BAP (4.4 μmol L$^{-1}$) and IAA (5.4 μmol L$^{-1}$) has been reported by Jain et al. (2009). A mean number of five shoots was regenerated from the callus, but shoot tips of these shoots died. In our earlier study, an average number of seven buds and shoots per shoot tip of *H. procumbens* was obtained on SH (Schenk and Hildebrandt 1972) medium supplemented with 0.57 μmol L$^{-1}$ IAA and 8 μmol L$^{-1}$ BAP (Grąbkowska and Wysokińska 2009). However, we observed that the number of shoots was reduced in successive subcultures and in the 3-year-old culture there were 2–3 shoots/explant. In the present work attempts were made to improve the *H. procumbens* shoot regeneration by TDZ pretreatment of shoot tips excised from 3-year-old aseptically growing shoots. The response of the explants in terms of a number of regenerated shoots and their length to different concentrations and duration of TDZ exposure was determined. TDZ (a synthetic phenylurea derivative) is efficient for the formation of adventitious shoots and axillary shoot proliferation, and the technique of explant pretreatment with the growth regulator has been successfully used by several authors for the improvement of in vitro plant micropropagation (Singh and Syamal 2001; Prathanturarug et al. 2003, 2005; Siddique and Anis 2007; Jahan et al. 2011). For example, Jahan et al. (2011) used it to micropropagate *Nyctanthes arbor-tristis*. Pretreatment of explants with TDZ also employed Siddique and Anis (2007) to in vitro propagate *Ocimum basilicum*. All these studies, however, focus on regeneration potential of the plant species under the influence of TDZ and do not investigate the effect of the cytokinin on the quality of obtained regenerants. Therefore, in another set of

experiments we studied the possible influence of explant pretreatment with TDZ on further stages of *H. procumbens* micropropagation, i.e. shoot rooting, acclimatization to ex vitro conditions and growth of regenerated plants. In addition, we performed RAPD and ISSR analyses to assess the genetic fidelity of the regenerants obtained after preculture with TDZ.

## Materials and methods

### Plant material and culture conditions

Shoot tips (5 mm length) excised from 5-week-old aseptically grown seedlings (derived from seeds obtained from Groenvlei Farm, belonging to Grassroots Group, Gouda, South Africa) were used to initiate shoot culture of *H. procumbens*. The shoot culture was maintained on agar (0.7 %) solidified SH (Schenk and Hildebrandt 1972) medium supplemented with sucrose (3 %), 0.57 μmol L$^{-1}$ indole-3-acetic acid (IAA) and 8 μmol L$^{-1}$ 6-benzylaminopurine (BAP) and subcultured every 5 weeks. The culture was kept in a growth chamber at $26 \pm 2$ °C with a 16/8 h (light/dark) photoperiod under a PPFD of 40 μM m$^{-2}$ s$^{-1}$ using cool-white fluorescent lamps. The shoot tips (5–10 mm long containing two leaf primordia) excised from shoots cultured for 3 years under the above mentioned conditions were used as explants in the experiments described in the work.

### Explant pretreatment with TDZ and shoot proliferation

Shoot tips excised from aseptically grown shoots were transferred into 100 mL Erlenmeyer flasks containing 20 mL water solution of TDZ at different concentrations: 0 (control), 15, 25 and 50 μmol L$^{-1}$. The flasks were maintained on a rotary shaker (100 rpm) for three different time periods (6, 24 and 48 h). A total of 8–15 explants were cultured in each flask.

After the initial treatment, explants were transferred into a shoot multiplication medium. Three different SH media: SH without growth regulators (designated as SH-0), SH supplemented with IAA (0.57 μmol L$^{-1}$) and BAP (8 μmol L$^{-1}$) (SH-BAP) and SH with IAA (0.57 μmol L$^{-1}$) and TDZ (6 μmol L$^{-1}$) (SH-TDZ) were tested. The choice of SH-BAP and SH-TDZ media was based on the earlier experiment in which these types and concentrations of growth regulators were effective for shoot multiplication in the 1-year-old culture of *H. procumbens* (Grąbkowska and Wysokińska 2009). Control explants (i.e. non-pretreated with TDZ) were also cultured on the same media (SH-0, SH-BAP, SH-TDZ). A schematic procedure of *H. procumbens* shoot multiplication
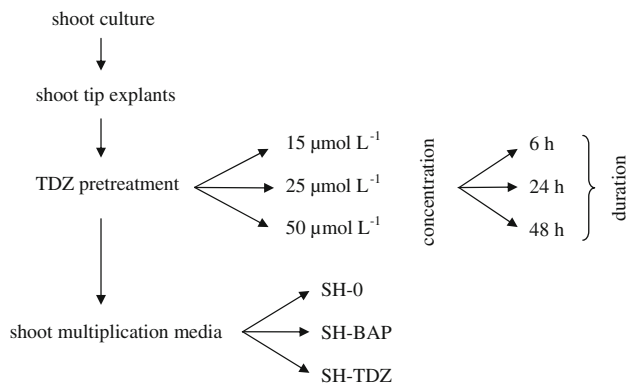
**Fig. 1** Schema of *H. procumbens* shoot multiplication from TDZ pretreatment shoot tips

from TDZ pretreatment shoot tips is shown in Fig. 1. The number of shoots per explant and the length of regenerated shoots were determined 8 weeks after the placement on the multiplication medium. 28 to 35 explants were used for each treatment (concentration of TDZ, duration of exposure, type of multiplication medium) and the experiments were repeated three times.

### Shoot rooting

For rooting, axillary shoots 1–2 cm in length regenerated from explants pretreated with 25 $\mu$mol L$^{-1}$ TDZ for 6 h and from explants non-pretreated with TDZ (control) cultured on SH-BAP multiplication medium were used. The shoots were transferred individually into agar-solidified MS (Murashige and Skoog 1962) medium without growth regulators. The percentage of rooted shoots, length of roots, the number of roots per shoot and the length of rooted shoots were calculated at 7-day intervals within 4 weeks. Each result was based on three replicates, each of which consisted of 15 shoots.

### Statistical analysis

All results were calculated as mean $\pm$ SE. The Kruskal–Wallis test was used to test the statistical significance. The significance differences of means were assessed using a multiple comparisons of average ranks. The results were regarded as statistically significant at $p \leq 0.05$. Statistical analyses were performed using the STATISTICA version 10 software (STATSoft).

### Plantlet acclimatization

The rooted shoots were transferred into pots (10 cm in diameter) containing a sterilized mixture of sand, soil, peat and perlite (4:2:2:4 v/v/v/v) and maintained in the greenhouse. The acclimatizing plantlets were initially covered with glass beakers to maintain a condition of high humidity and watered (every week) with sterile distilled water for 1 month. 28 plantlets regenerated from explants pretreated with TDZ (25 $\mu$mol L$^{-1}$ TDZ, for 6 h) (otherwise called TDZ pretreatment plants) and 30 control plantlets obtained from explants non-pretreated with TDZ (otherwise called TDZ non-pretreatment plants) were used in the experiment. The survival percentage (number of surviving plants/the total number of plantlets transferred into soil $\times 100$) and morphological features (an average length of the plantlets, the number of nodes per shoot, the length and width of leaves as well as the percentage of plants forming root tubers and the percentage of flowering of the plants) were recorded periodically within 48 weeks (about 1-year) after the transplantation to the ex vitro environment. Data for the morphological characteristics were calculated as mean values of the tested growth parameters of a minimum number of ten different plants.

### DNA extraction and RAPD and ISSR analyses

Genomic DNA was isolated from fresh leaves of 40 randomly selected 4-week-old plantlets grown in MS medium without growth regulators, 20 of which were derived from TDZ pretreated explants (25 $\mu$M for 6 h) (group 1) while the remaining 20 from non-pretreated explants (group 2). Genomic DNA from leaves of twenty 3-week-old seed-derived plants was also isolated to constitute the control (group 3). Additionally, we isolated DNA from explant-donor shoot culture line subcultured for a period of 3 years on SH-BAP medium (SH medium with 0.57 $\mu$M IAA and 8 $\mu$M BAP) (group 4). The plant DNA was isolated using a commercial DNA-extraction kit (NucleoSpin$^{\circledR}$ Plant Core Kit Macherey–Nagel GmbH & Co. KG, Germany). The plant materials (200 mg) were powdered in liquid nitrogen and stored at $-80$ °C until used for extraction. Three replicate DNA extractions from leaves were used to assess the consistency of the band profiles.

RAPD-PCR amplifications were performed in a volume of 20 $\mu$l containing 10 ng DNA, 1$\times$ PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 11 mM MgCl$_2$, 0.1 % gelatin), 1.5 mM MgCl$_2$, 50 mM dNTPs, 250 nM of random decamer primer obtained from Blirt (Poland), 1.25 U of TaqNova DNA polymerase (Blirt, Poland) and MilliQ water to make up the volume. A total of 8 RAPD primers were tested using plant DNA (Table 1). Amplification was performed in a thermal cycler MJ Mini (BioRad, UK) programmed as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 32–36 °C (depending on the primer type) (Table 1) for 45 s and 2 min extension at 72 °C with a final extension at 72 °C for 7 min.

**Table 1** List of RAPD primers used for assessment of the in vitro regenerants, the explant-donor shoot culture and the seed-derived plants of *Harpagophytum procumbens*

| Primer | Sequence (5′–3′) | Annealing temperature (°C) | Total bands | Approximate fragment size range (bp) |
|---|---|---|---|---|
| OPA1 | CAGGCCCTTC | 32 | 8 | 200–1.500 |
| OPA3 | GGGTAACGCC | 34 | 9 | 350–2.000 |
| OPA4 | AATCGGGCTG | 34 | 3 | 350–650 |
| OPA8 | GTGACGTAGG | 32 | 4 | 500–1.400 |
| OPA9 | GGGTAACGCC | 34 | 8 | 400–1.500 |
| OPA10 | GTGATCGCAG | 34 | 1 | 1.000 |
| OPA13 | CAGCACCCAC | 36 | 7 | 300–1.700 |
| OPA17 | AGCCAGGCAA | 35 | 5 | 550–1.500 |

**Table 2** List of ISSR primers used for assessment of the in vitro regenerants, the explants-donor shoot culture and the seed-derived plants of *Harpagophytum procumbens*

| Primer | Sequence (5′–3′) | Annealing temperature (°C) | Total bands | Approximate fragment size range (bp) |
|---|---|---|---|---|
| UBC807 | AGA GAG AGA GAG AGA GT | 52 | 6 | 350–1.600 |
| UBC811 | GAG AGA GAG AGA GAG AA | 52 | 5 | 400–1.200 |
| UBC812 | GAG AGA GAG AGA GAG AC | 55 | 5 | 400–1.000 |
| UBC813 | CTC TCT CTC TCT CTC TT | 50 | 7 | 450–1.400 |
| UBC830 | TGT GTG TGT GTG TGT GG | 52 | 8 | 450–1.500 |
| UBC834 | AGA GAG AGA GAG AGA GCT | 54 | 2 | 300–500 |
| UBC835 | AGA GAG AGA GAG AGA GTC | 55 | 7 | 200–1.200 |
| UBC836 | AGA GAG AGA GAG AGA GCA | 53 | 6 | 200–1.500 |

In case of ISSR primers, optimal annealing temperature was found to vary according to the base composition of the primers. Eight ISSR primers (UBC primer, Blirt, Poland) were used in the study (Table 2). Amplification was carried out in 20 µl reaction volume containing 10 ng genomic DNA as template, 1.5 mM $MgCl_2$, 50 mM dNTP, $1\times$ PCR buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, 0.1 % gelatin), 250 nM ISSR oligodeoxynucleotide primer, 1.25 U TaqNova DNA polymerase (Blirt, Poland) and MilliQ water to make up the volume. The amplification reaction consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 30 s denaturation at 94 °C, 1 min at the annealing temperature (depending on

the primer) (Table 2), extension at 72 °C for 2 min and a final extension at 72 °C for 10 min.

In the genetic analyses, PCR mixture without DNA was used as a negative control (Rewers et al. 2012). RAPD and ISSR amplifications were repeated three times and only the reproducible PCR products were scored. The amplification RAPD-PCR and ISSR-PCR products were resolved by electrophoresis on 2.0 % agarose gel (Bioline, UK), stained with ethidium bromide, measured with a 100 bp and 3 kb ladder (Fermentas, Lithuania) as the band size standard and photographed using DNR Bio-Imaging System MiniBIS Pro (Israel).

## Results and discussion

### Shoot proliferation

The effects of TDZ pretreatment of *H. procumbens* explants taken from the 3-year-old shoot culture on shoot regeneration capacity by comparing three different concentrations of TDZ (15, 25 and 50 µmol $L^{-1}$), three different durations of exposure (6, 24 and 48 h) and the use of three different multiplication media (SH-0, SH-BAP, SH-TDZ) were investigated. Two parameters were determined: the mean number of shoots per explant (Fig. 2) and the mean shoot length (Fig. 3). We found that SH-0 medium containing no growth regulators was the least effective for shoot proliferation. On this medium shoot tips pretreated with TDZ regenerated maximally 2.2 shoots per explants (Fig. 2). The choice of this medium for shoot induction was based on the observations of other authors indicating that a continuous exposure of explants to TDZ was not needed since cultures after the exposure to TDZ showed cytokinin autonomy (Malik and Saxena 1992; Cruz de Carvalho et al. 2000).

The number of shoots per explant was enhanced two-threefold over non-pretreatment control cultures, when shoot tips pretreated with TDZ were transferred into multiplication media containing growth regulators: IAA (0.57 µmol $L^{-1}$) and BAP (8 µmol $L^{-1}$) (SH-BAP) or IAA (0.57 µmol $L^{-1}$) and TDZ (6 µmol $L^{-1}$) (SH-TDZ). When three different durations of TDZ exposure were compared, it was found that 6 h pretreatment induced the highest number of shoots (up to seven shoots/explant after 8 weeks of culture on SH-BAP or SH-TDZ medium). At the length of TDZ exposure (6 h) the optimum response was observed with explants pretreated with a higher concentration of TDZ, i.e. 25 µmol $L^{-1}$ when SH-BAP medium was used for shoot multiplication and 50 µmol $L^{-1}$ for SH-TDZ medium. Here, control explants (non-pretreated with TDZ) proliferated on average $2.1 \pm 0.3$ and $3.9 \pm 0.4$ shoots per explant on SH-BAP and SH-TDZ medium,

Fig. 2 Effect of explant
pretreatment in the presence of
different concentrations (15, 25
and 50 μmol L$^{-1}$) of TDZ and
the duration of pretreatment (6,
24 and 48 h) on *H. procumbens*
shoot multiplication. Explants
were cultured on three
multiplication media (☐ SH-0,
■ SH-BAP, ☐ SH-TDZ) for
8 weeks. Results are
mean ± SE from three
independent experiments;
means marked by the *same
letter* are not significantly
different from each other at a
5 % level according to the
Kruskal–Wallis test and
multiple comparisons of
average ranks; composition of
SH-0, SH-BAP, SH-TDZ media
is described in "Materials and
methods"; the control is bar
with 0 μmol L$^{-1}$ concentration
of TDZ (explants without
pretreatment with TDZ)
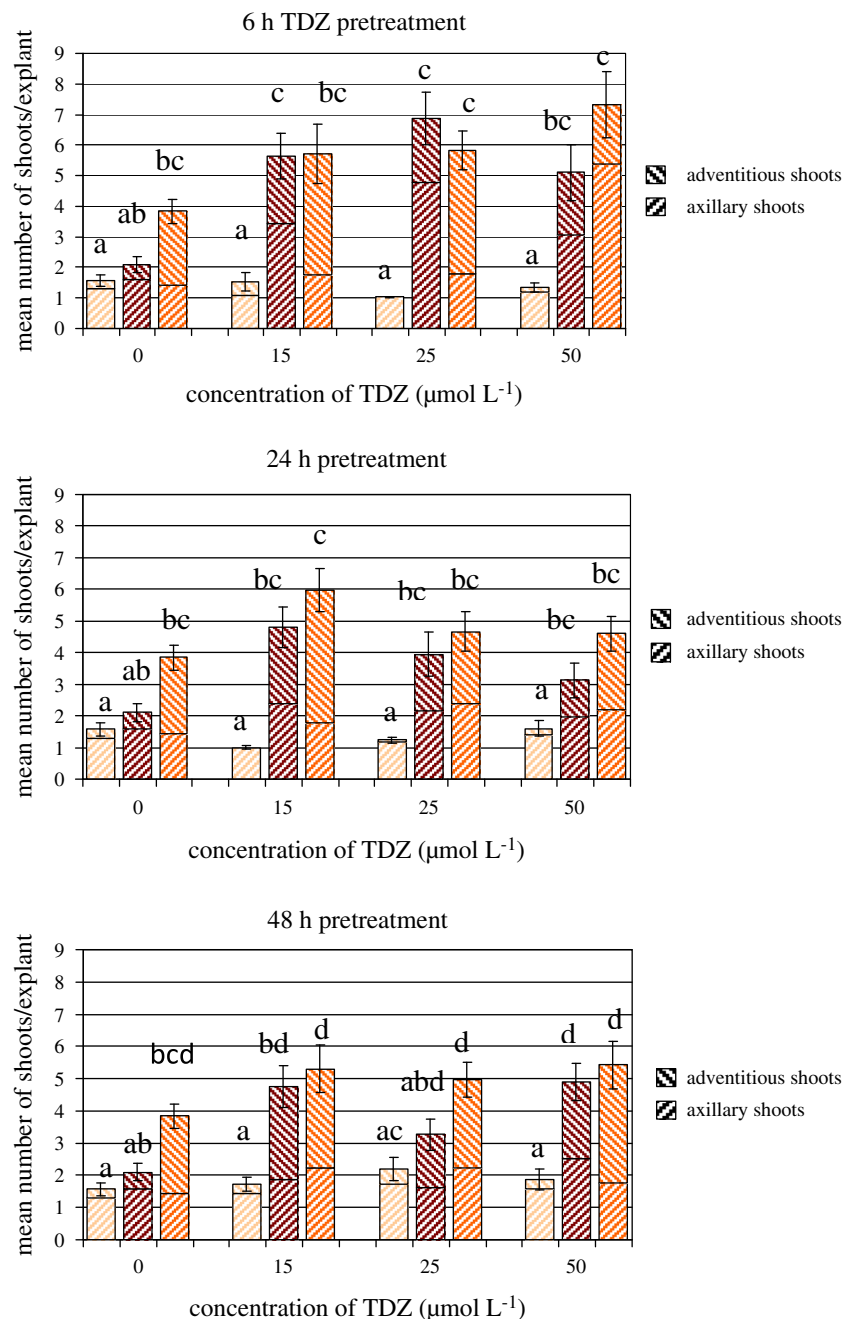


respectively. Shoot multiplication was achieved through axillary bud development from preexisting meristems and adventitious shoots, which were sometimes initiated from a callus forming at the base of some explants. However, the adventitious shoots were not used in further stages of micropropagation. The results suggest that a short (6 h) exposure of explants to 25 or 50 μmol L$^{-1}$ TDZ, prior to the transfer to SH multiplication medium with growth regulators, led to increased shoot proliferation of *H. procumbens*. Similar stimulating effects of TDZ pretreatment on multiple shoot formation have been previously

reported for some other plant species, such as *Curcuma longa* (Prathanturarug et al. 2003, 2005), *Prunus cerasus* (Song and Sink 2005), *Ocimum basilicum* (Siddique and Anis 2007), *Rosa* spp. (Kucharska and Orlikowska 2009) and *Nyctanthes abor-tristis* (Jahan et al. 2011).

In the present study the number of *H. procumbens* regenerated shoots (about seven from one explant) for TDZ pretreatment explants taken from the 3-year-old culture was similar to that reported in our previous publication for the 1-year-old shoot culture of *H. procumbens* (Grąbkowska and Wysokińska 2009). There was no

**Fig. 3** Effect of explant pretreatment in the presence of different concentrations (15, 25 and 50 µmol L$^{-1}$) of TDZ and the duration of pretreatment (6, 24 and 48 h) on *H. procumbens* axillary shoot length. Explants were cultured on three multiplication media (SH-0, SH-BAP, SH-TDZ) for 8 weeks Results are mean ± SE from three independent experiments; means marked by the *same letter* are not significantly different from each other at a 5 % level according to the Kruskal–Wallis test and multiple comparisons of average ranks; composition of SH-0, SH-BAP, SH-TDZ media is described in "Materials and methods"; the control is bar with 0 µmol L$^{-1}$ concentration of TDZ (explants without pretreatment with TDZ)
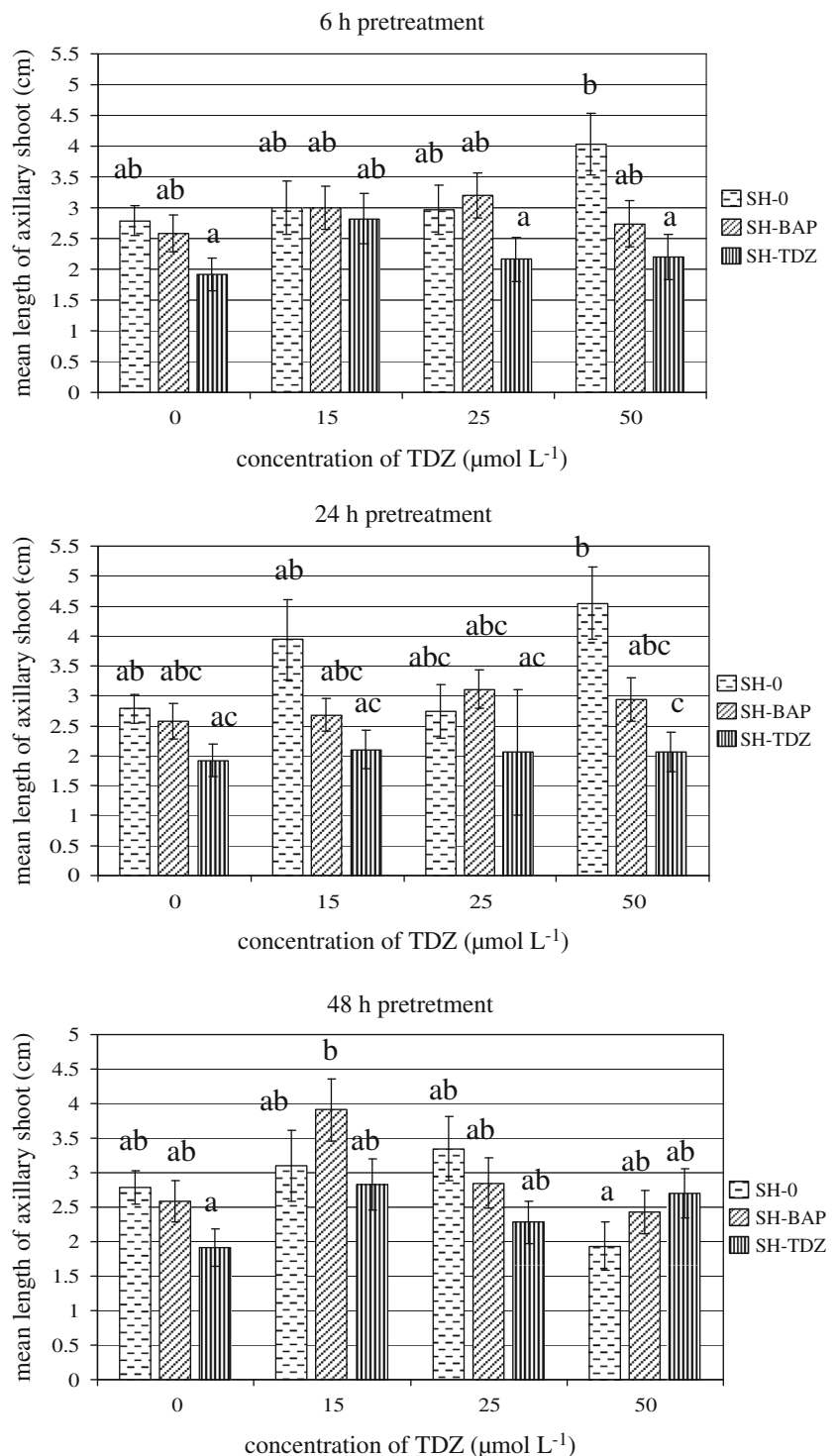


significant difference between two multiplication media (SH-BAP and SH-TDZ) in the number of shoots regenerated from explants pretreated with TDZ (Fig. 2). However, the type of cytokinin in multiplication medium had effect on the quality of regenerated shoots in terms of their length and morphology (Fig. 4a, b). When explants were pretreated with TDZ for 6 h and cultured on SH-

TDZ medium containing 6 µmol L$^{-1}$ TDZ, the length of adventitious shoots did not exceed 1.0 cm after 8 weeks, and this parameter was practically not changed by a dose of TDZ (15, 25 or 50 µmol L$^{-1}$) used for the explant pretreatment. Moreover, more than half of the shoots showed symptoms of hyperhydricity (data not shown). It has been well known that TDZ, especially at higher
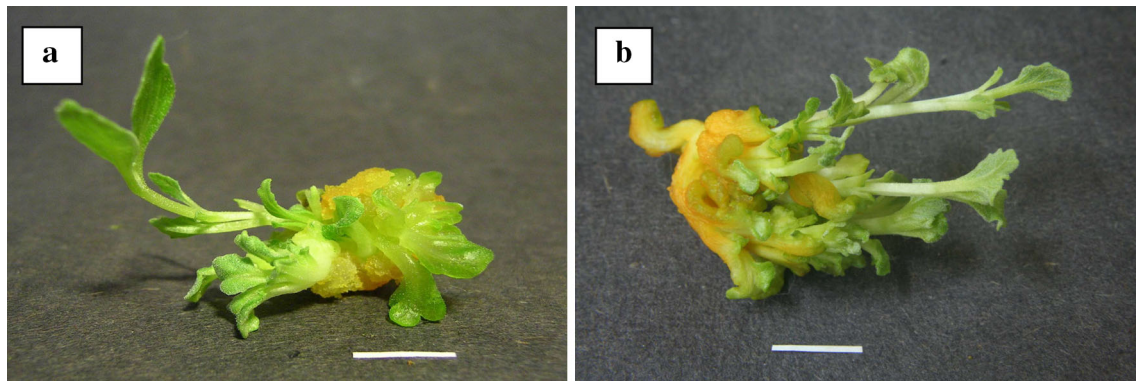
**Fig. 4** *H. procumbens* shoots produced from shoot tip explants pretreated with TDZ (25 μmol $L^{-1}$ for 6 h) after 8 weeks of culture on SH medium supplemented with 0.57 μmol $L^{-1}$ IAA and 6 μmol $L^{-1}$ TDZ (**a**) or 8 μmol $L^{-1}$ BAP (**b**). *Bar* 1 cm

concentrations, can be responsible for reduction in shoot growth, their hyperhydricity and fasciation (Huetteman and Preece 1993). Shoots of *H. procumbens* were longer and of better quality (lower frequency of hyperhydricity) when the explants pretreated with TDZ were cultured on medium containing BAP (SH-BAP). On SH-BAP medium the average axillary shoot length of pretreated cultures (6 h exposure to TDZ at a dose 15, 25 or 50 μmol $L^{-1}$) was 2.7–3.2 cm as against 2.6 cm of the shoot cultured without any pretreatment on the same medium composition (no significant difference at $p \leq 0.05$) (Fig. 3). The results were similar to studies reported by Thomas (2007) who found that shoots of *Curculigo orchioides* derived from cultures pretreated with TDZ or BAP were longer than those in untreated cultures. The length of shoots is important for their capacity to form roots. Previously, we found that *H. procumbens* shoots about 1 cm in length showed high frequency of rooting on MS medium supplemented with 0.57 μmol $L^{-1}$ IAA (Grąbkowska and Wysokińska 2009). Taken together both parameters (i.e. the number of shoots per explant and the shoot length), the combination of 6 h pretreatment of explants with 25 μmol $L^{-1}$ TDZ and SH-BAP medium was chosen as optimal for induction of multiple shoots in the 3-year-old culture of *H. procumbens*. Therefore, the shoots were used in all subsequent experiments.

Rooting of shoots

We also made an attempt to determine whether the use of explant pretreatment with TDZ has effect on the rooting response of regenerated shoots or not. The axillary shoots derived from the TDZ pretreated culture (6 h exposure to TDZ at a dose of 25 μmol $L^{-1}$) and those from the non-pretreated culture were transferred to MS medium without growth regulators. The highest rate of root initiation occurred approximately 2 weeks after the placement on the

rooting medium, although some new root initiation on shoots was still observed in 4th week of the culture (Table 3). No significant differences in the percentage of shoot rooting, the number of roots per shoot and the root length between cultures pretreated with TDZ and non-pretreated cultures were detected. Only one little difference in respect to the time required for the initiation of rooting was observed. Roots appeared after 7 days of the culture when shoots were originated from shoot tip explants pretreated with TDZ and after 14 days from shoots derived on non-pretreated explants. However, the complete development of roots, which was suitable for hardening, took 4 weeks in both cases. The results suggest that the pretreatment of *H. procumbens* explants with TDZ according to the protocol described in the present work had no evident effects on rooting of *H. procumbens* shoots. In some other observations, the difficulty in rooting of shoots associated with the presence of TDZ in the shoot multiplication medium has been reported (Cruz de Carvalho et al. 2000; Singh et al. 2001; Kozak 2010). On the other hand, Ramanayake et al. (2006) found that rooting in a species of *Bambusa vulgaris* was increased if shoots were pretreated with TDZ before transferring to the rooting medium.

It is important to note in the present study that during the rooting phase the shoots continued to grow and two- to fivefold increase in their length was observed within 4 weeks. These shoots coming from TDZ pretreatment explants were longer than shoots originating from control explants (significant difference at $p \leq 0.05$) (Table 3).

Acclimatization of plantlets ex vitro

In the continuation of the present study, the effect of in vitro TDZ pretreatment on the adaptation to ex vitro environment of *H. procumbens* micropropagated plantlets was established. For that purpose, plantlets regenerated

**Table 3** In vitro rooting of *H. procumbens* shoots derived from TDZ pretreatment (25 μmol L$^{-1}$ TDZ for 6 h) or TDZ non-pretreatment (control) shoot tip explants cultured on SH-BAP multiplication medium (SH medium supplemented with 0.57 μmol L$^{-1}$ IAA and 8 μmol L$^{-1}$ BAP)

| Source of shoots | Time of rooting (weeks) | Shoots of rooting ± SE (%)[A] | Mean number of roots/shoot ± SE [A] | Mean root length ± SE (cm)[A] | Mean shoot length ± SE (cm)[A] |
|---|---|---|---|---|---|
| Explants pretreated with TDZ | 1 | 23.3 ± 3.3[b] | 1.14 ± 0.14[b] | 0.28 ± 0.04[b] | 3.48 ± 0.16[c] |
| | 2 | 66.7 ± 6.7[c] | 2.40 ± 0.32[c] | 2.04 ± 0.20[c] | 4.38 ± 0.22[de] |
| | 3 | 73.3 ± 8,8[c] | 3.14 ± 0.33[cd] | 4.23 ± 0.25[d] | 4.83 ± 0.29[de] |
| | 4 | 76.7 ± 6.7[c] | 3.13 ± 0.34[cd] | 5.86 ± 0.31[e] | 5.25 ± 0.32[e] |
| Explants non-pretreated with TDZ (control) | 1 | 0.0[a] | 0.0[a] | 0.0[a] | 2.25 ± 0.14[a] |
| | 2 | 56.7 ± 6.7[c] | 2.47 ± 0.37[c] | 2.0 ± 0.18[c] | 2,90 ± 0.16[b] |
| | 3 | 83.3 ± 8.8[c] | 3.52 ± 0.34[d] | 3.70 ± 0.20[d] | 3.34 ± 0.21[bc] |
| | 4 | 86.7 ± 8.8[c] | 3.77 ± 0.33[d] | 5.91 ± 0.37[e] | 4.06 ± 0.26[cd] |

For rooting MS medium without growth regulators was used

[A] Results are mean ± SE from three independent experiments

Different letters within the column (the superscript a, b, c, d and e) indicate significant differences at a 5 % level according to the Kruscal-Wallis test and multiple comparisons of average ranks

after transferring shoots multiplied from TDZ pretreatment explants (TDZ pretreatment plants) (Fig. 5a) and plantlets developed after transferring shoots regenerated from non-pretreated with TDZ explants (TDZ non-pretreatment plants), serving as control, were transplanted into pots with the mixture of sand, soil, peat and perlite and cultivated for about 1 year in the greenhouse. Comparison of survival rate and growth performance of these plants during acclimatization (up to 24 weeks) and post-acclimatization (24–48 weeks) period is presented in Table 4. We found that TDZ pretreatment increased the percentage of plantlet survival and resulted in higher recovery of plants after transplantation to the greenhouse. After 8 weeks, the survival rate of the plants was over 90 %, whereas it was 63 % in the control plants. However, the survival began to decline rapidly with an increasing acclimatization period and 14 and 24 weeks after initial ex vitro transplanting, 80 and 43 % of TDZ pretreatment plants and 40 and 33 % of TDZ non-pretreatment plants survived, respectively. The survival did not change over the next 24 weeks (Table 4). Kaliamoorthy et al. (2008) have also reported that acclimatization of in vitro derived *H. procumbens* plants was difficult and only 40 % of them survived during hardening phase (4 weeks). The problem of low survival of micropropagated plants, when they were transferred into ex vitro conditions, was observed for many other plant species, such as *Delphinium* sp. (Pryce et al. 1993) or *Notocactus magnificus* (Medeiros et al. 2006). It is probably due to nonfunctional stomata, weak root system and poorly developed cuticules in the cultured plants (Estrada-Luna et al. 2001; Isutsa 2004). Therefore, the plants need a period of acclimatization to correct the abnormalities. Our experiments showed that the adaptation of *H. procumbens* micropropagated plants to ex vitro conditions lasted several months and was longer than generally accepted (5–6 weeks) (Levieille and Wilson 2002; Bairu et al. 2009).

Pretreatment with TDZ has also effect on the quality of obtained regenerants. The effect was demonstrated, e.g. in the shoot length and leaf size. After 48 weeks (about 1 year) of the growth in the greenhouse TDZ pretreatment plants reached 107.3 ± 8.9 cm of the mean length with approximately 45 nodes per shoot (Table 4; Fig. 5b). The data were quite similar to those obtained for seed-derived plants of *H. procumbens* growing in identical conditions (an average shoot length of 100.2 ± 10.4 cm, 60 nodes/shoot) (data not shown), but markedly higher compared to TDZ non-pretreatment micropropagated plants (the mean shoot length 60.0 ± 2.6 cm with 30 nodes per shoot) (Table 4). The average leaf length was 4.8 ± 0.4 and 3.6 ± 0.2 cm in TDZ pretreatment and non-pretreatment plants, respectively. The leaf width of TDZ pretreatment and non-pretreatment plants was 3.0 ± 0.3 and 2.2 ± 0.2 cm, respectively. These features concerned more vigorous plantlets and resulted in increasing their survival in ex vitro conditions as described above. Only, TDZ pretreatment plants (8 out of 12) developed lateral shoots and flowers which appeared in 25 % of them during the first year of cultivation in the greenhouse (Fig. 5c, d). TDZ may promote in vitro flowering (Singh et al. 2000; Lin et al. 2007), but its role in stimulation of flowering in ex vitro growing *H. procumbens* plants remains to be explained. After 10 weeks of being transplanted, TDZ pretreatment plants began to produce tuberised roots. The process was finished after 14 weeks (Fig. 5e). In the case of control plants, the time was extended by 4 weeks and occurred between 14 and 18 weeks of the growth in the greenhouse (Table 4). Also, an average weight of root tubers of TDZ pretreatment plants was increased: it was 12.3 ± 1.4 g compared to 9.8 ± 1.0 g of root tubers of
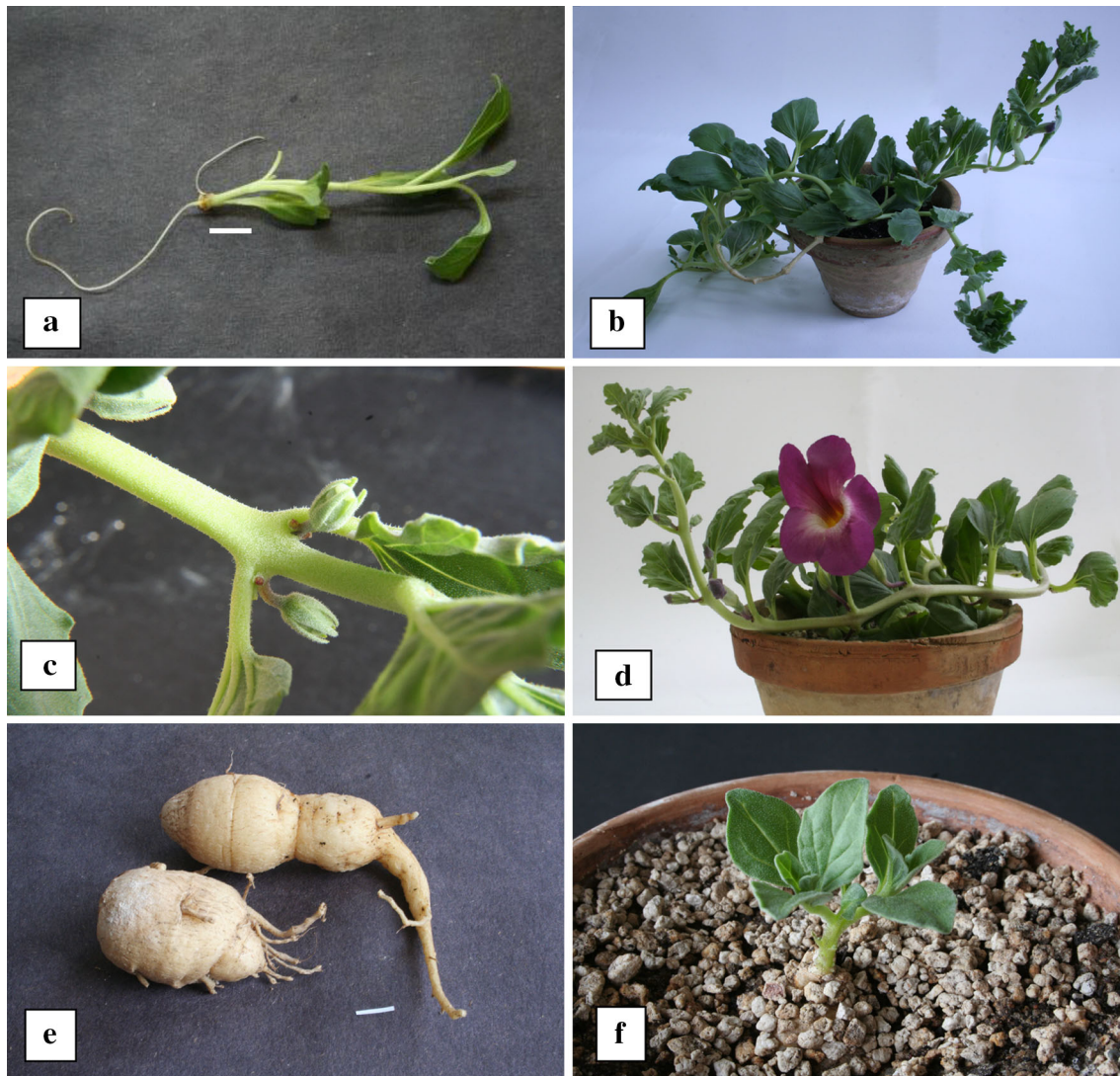
**Fig. 5** Plants of *H. procumbens* obtained from TDZ pretreated explants and growing in the greenhouse; **a** the plantlet ready for transfer to soil; **b** fully acclimatized plant (about 1 year after transfer to the greenhouse); **c** formation of floral buds; **d** flowering plant; **e** root tubers; **f** shoot buds initiated from root tubers left in pots for the next growing season. *Bar* 1 cm

TDZ non-pretreatment plants. It is important to note that TDZ pretreatment plants of *H. procumbens* underwent a period of dormancy after the first growing season. The next growing season in the greenhouse was initiated by the emergence of shoot buds from root tubers (Fig. 5f). The buds developed into shoots, which after 3 months reached about 15 cm length.

RAPD-PCR and ISSR-PCR analyses

Since there were differences in morphology between *H. procumbens* plants derived from TDZ pretreated explants (25 µM for 6 h) and plants from non-pretreated explants (Table 4) were observed in this study, the plants were tested in terms of genetic conformity using two PCR-based systems (RAPD and ISSR). In RAPD-PCR analysis, eight primers (Table 1) were selected for DNA amplification as they produce distinct reproducible scorable bands. All these RAPD-PCR primers resulted in the amplification of monomorphic bands. These eight primers produced a total of 53 bands and an average of 6.6 bands per primer. The number and size range of amplified scorable bands for each RAPD primer have been presented in Table 1. Each primer generated a unique set of amplification products that were monomorphic across all regenerants (Fig. 6).

During ISSR-PCR analysis, eight selected ISSR primers yielded 46 clear distinct bands, with an average of 5.8 bands per primer. Each ISSR primer generated a unique set of amplification products of size ranging from 200 to 1.650 bp (Table 2). The number of bands amplified in each

Table 4 Comparison of the survival percentage and morphological characteristics during 1 year of ex vitro transfer for *H. procumbens* plantlets raised with and without TDZ pretreatment

| Growth parameters | Ex vitro growth period (weeks) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 4 | 6 | 8 | 10 | 14 | 18 | 24 | 36 | 48 |
| TDZ pretreatment plants | | | | | | | | | |
| Survival of plants (%) | 93 | 93 | 93 | 86 | 79 | 68 | 43 | 43 | 43 |
| Mean shoot length (cm) | 9.77 ± 0.56 | 11.43 ± 0.75 | 12.70 ± 0.48 | 24.82 ± 0.72 | 35.12 ± 1.50 | 47.87 ± 1.50 | 60.48 ± 2.37 | 75.33 ± 6.0 | 107.34 ± 8.86 |
| Mean number of nodes/shoot | 4.77 ± 0.35 | 5.0 ± 0.67 | 8.51 ± 0.80 | 10.14 ± 0.59 | 13.45 ± 0.35 | 19.77 ± 0.56 | 30.50 ± 1.50 | 33.67 ± 2.86 | 45.0 ± 3.25 |
| Leaf length (cm) | 1.20 ± 0.12 | 1.90 ± 0.92 | 2.71 ± 0.65 | 3.11 ± 0.46 | 3.66 ± 0.72 | 3.83 ± 0.33 | 4.09 ± 0.86 | 4.56 ± 0.40 | 4.84 ± 0.42 |
| Leaf width (cm) | 0.40 ± 0.09 | 1.03 ± 0.11 | 1.47 ± 0.25 | 1.82 ± 0.12 | 2.05 ± 0.25 | 2.22 ± 0.22 | 2.60 ± 0.35 | 2.89 ± 0.44 | 3.04 ± 0.30 |
| Plants forming tubers (%) | – | – | – | 40 | 55 | 100 | 100 | 100 | 100 |
| TDZ non-pretreatment plants (control) | | | | | | | | | |
| Survival of plants (%) | 100 | 77 | 63 | 47 | 40 | 37 | 33 | 33 | 33 |
| Mean shoot length (cm) | 9.17 ± 0.56 | 11.60 ± 0.21 | 13.77 ± 0.59 | 16.01 ± 0.40 | 19.77 ± 0.56 | 23.47 ± 0.35 | 26.0 ± 0.37 | 38.92 ± 1.33 | 60.0 ± 2.57 |
| Mean number of nodes/shoot | 5.36 ± 0.35 | 5.50 ± 0.31 | 7.60 ± 0.59 | 8.14 ± 0.57 | 11.77 ± 0.56 | 12.50 ± 0.33 | 16.86 ± 0.31 | 18.92 ± 0.50 | 29.80 ± 1.50 |
| Leaf length (cm) | 1.14 ± 0.23 | 1.88 ± 0.54 | 2.47 ± 0.23 | 2.89 ± 0.68 | 3.05 ± 0.44 | 3.19 ± 0.76 | 3.34 ± 0.21 | 3.42 ± 0.30 | 3.56 ± 0.25 |
| Leaf width (cm) | 0.38 ± 0.11 | 0.86 ± 0.20 | 1.25 ± 0.16 | 1.50 ± 0.33 | 1.82 ± 0.60 | 1.96 ± 0.58 | 2.08 ± 0.36 | 2.20 ± 0.11 | 2.23 ± 0.20 |
| Plants forming tubers (%) | – | – | – | – | 42 | 67 | 100 | 100 | 100 |

Values are mean ± SE from three independent experiments

Plants were induced from shoot tip explants. Explants were pretreated or not (control) with TDZ (25 μmol $L^{-1}$ for 6 h) and cultured on agar SH medium containing 0.57 μmol $L^{-1}$ IAA and 8 μmol $L^{-1}$ BAP for 8 weeks. Shoots were rooted on MS medium without growth regulators for 4 weeks. Plants were grown in pots containing a sterilized mixture of sand, soil, peat and perlite (4:2:2:4 v/v/v/v) in the greenhouse
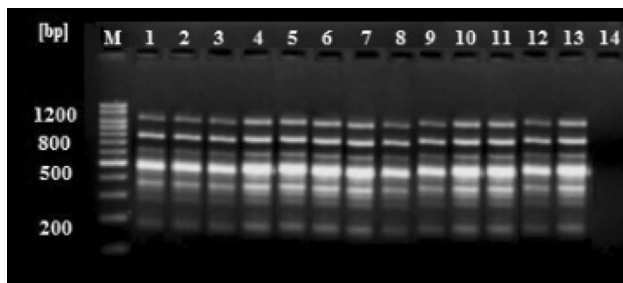
**Fig. 6** Gel electrophoresis of RAPD fragments obtained with primer OPA1. Lane M—DNA marker 100–3,000 bp DNA ladder, *lane 1*—DNA of *H. procumbens* seed-derived plant, *lane 2*—DNA of explant-donor shoot culture, *lines 3–8* DNA randomly selected TDZ pretreatment plantlets, *lanes 9–13* DNA randomly selected TDZ non-pretreatment plantlets, *lane 14*—negative control
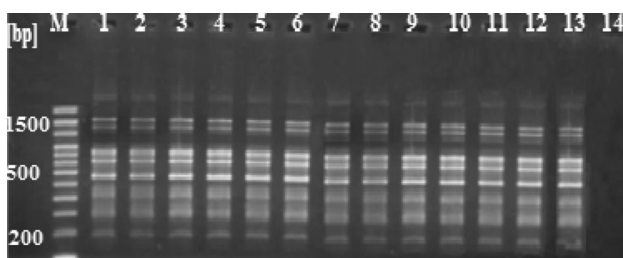


**Fig. 7** Gel electrophoresis of ISSR fragments obtained with primer UBC830. *Lane M* DNA marker 100–3,000 bp DNA ladder, *lane 1* DNA of *H. procumbens* seed-derived plant, *lane 2* DNA of explant-donor shoot culture, *lines 3–8* DNA randomly selected TDZ pretreatment plantlets, *lanes 9–13* DNA randomly selected TDZ non-pretreatment plantlets, *lane 14* negative control

ISSR primer ranged from 2 to 8. As like ISSR banding patterns of in vitro regenerated plants were also monomorphic (Fig. 7).

The genetic profiles obtained in RAPD and ISSR assays suggest that there are no genetic changes between regenerants derived from explants pretreated with TDZ (25 μM, 6 h) and plants regenerated from explants non-pretreated with TDZ. Also, the use of RAPD and ISSR analyses revealed that no genetic changes were present in explant-donor shoot culture and seed-derived plant (Figs. 6, 7). This suggests that the in vitro conditions in this study provide relatively high genetic stability of *H. procumbens* on direct regeneration in vitro from long-term shoot culture.

## Conclusion

We conclude that pretreatment of *H. procumbens* shoot tips with TDZ (25 μmol $L^{-1}$ for 6 h) can be valuable for the improvement in in vitro micropropagation by enhancing shoot proliferation as well as by having a positive effect on

the subsequent growth and quality of the plants when they are transferred to ex vitro conditions. Currently, no reports exist on long-term effects of explant exposure to TDZ and further studies are needed to explain the mechanism by which TDZ improves growth parameters of *H. procumbens* plants during their transfer to ex vitro conditions and increases their survival rate. The RAPD and ISSR techniques demonstrated genetic conformity of micropropagated plants of *H. procumbents*, which were pretreated or non-pretreated with TDZ.

**Author contribution** R. Grąbkowska designed and carried out the experiment for in vitro regeneration of *H. procumbens* plants, isolation of genomic DNA from TDZ pretreatment and TDZ non-pretreatment plantlets and seed-derived plants. Moreover she performed statistical analyses and wrote the first draft of the manuscript. P. Sitarek preparated DNA, performed RAPD and ISSR analyses. H. Wysokińska was responsible for verification of the paper.

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