Establishment of a Rapid and Efficient Micropropagation System for Succulent Plant Haworthia turgida Haw.

Boling Liu, Hongzhou Fang, Chaorong Meng, Ming Chen, and Qingdong Chai
College of Life Sciences, Qufu Normal University, 273165, Qufu, Shandong, China

Kai Zhang
Culaishan Forest Farm of Tai’an City, 271000, Tai’an, Shandong, China

Shijuan Liu¹
College of Life Sciences, Qufu Normal University, 273165, Qufu, Shandong, China

Additional index words. callus, plant growth regulators, propagation, root, shoot

Abstract. In the present study, the effect of plant growth regulators (PGRs) on callus regeneration, adventitious shoot differentiation, and root formation of Haworthia turgida Haw. was investigated. The greatest callus induction percentage (95.6%) was achieved with leaf explants inoculated on Murashige and Skoog (MS) medium with 1.0 mg·L⁻¹ 6-benzyladenine (BA) and 0.1 mg·L⁻¹ 1-naphthaleneacetic acid (NAA), and this callus induction medium supplemented with 2.5 mg·L⁻¹ thidazuron (TDZ) was optimal for callus proliferation. The maximum number of shoots (25.7) was obtained when the callus was cultured on MS medium supplemented with 1.0 mg·L⁻¹ BA and 0.2 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D). The highest number of roots per shoot (6.2) and highest rooting frequency (82.0%) were obtained when adventitious shoots were inoculated on MS medium with 0.05 mg·L⁻¹ NAA. Regenerated plantlets were transferred to a mixture of vermiculite and soil and acclimated in a greenhouse. The survival rate of the transplanted plantlets was about 91.6%. The rate of ex vitro rooting was 83.3%, indicating that this technique is effective for root induction in H. turgida. This study has established a rapid and efficient micropropagation system that can be beneficial for commercial cultivation and germplasm conservation of H. turgida.

The succulent plant genus Haworthia, belonging to the Liliaceae family, is native to South Africa, Namibia, Swaziland, and Mozambique (Beyl and Sharma, 1983; Kaul and Sabharwal, 1972). Haworthia exhibits highly variable yet distinct plant and leaf morphology, thus sparking the interest of both amateur botanists and succulent plant collectors. Haworthia has been used in pot culture and landscaping of open-air rockeries (Barker, 1929). Its species are well adapted to shady environmental conditions, require minimal maintenance, and have been popularly used in various countries as ornamental plant. Some Haworthia species have also been used in miniature gardens, office, and balconies (van Jaarsveld, 1999). Most leaf succulents possess translucent epidermis that protects its underlying transparent water storage parenchymatous tissue layer. The translucent regions of the plant can take the shape of scattered dots, lines, and wide streaks. Based on this feature, these have been called window-leaved plants and are mainly observed in the Liliaceae, Compositae, and Mesembryanthemaceae (Krulik, 1980). Haworthia turgida has radial leaves with a flat cross-section at the leaf apexes and contains several lines forming the special window structure (Egbert and Martin, 2002), and is valued as an important ornamental plant in Chinese flower markets. Haworthia is self-incompatible and generally propagated by leaf cutting. However, this relatively slow process is caused by the low number of offshoots produced by the parent plants (Mycock et al., 1997; Rogers, 1993a). Consequently, micropropagation has been considered as an attractive way of propagating Haworthia genus plants. This method facilitates the propagation of plants without seasonal and environment limitations and in a large scale, which are important factors in the breeding plants for ornamental and landscaping purposes (Kitamura et al., 2002; Kumari et al., 2016; Prei et al., 1988). Plantlets have been successfully induced in several Haworthia species using explants from inflorescences (Kaul and Sabharwal, 1972; Majumdar, 1970a; Ogihara, 1979; Ogihara and Tsunewaki, 1978), ovary walls (Majumdar, 1970b), and leaves (Beyl and Sharma, 1983; Rogers, 1993b). Growth characteristics in response to various auxins and cytokinins, such as NAA, 2,4-D, and BA (Kaul and Sabharwal, 1972; Ogihara, 1979; Ogihara and Tsunewaki, 1978), have been examined in some Haworthia species. However, none of these studies have used TDZ in vitro propagation, although it is an efficient PGR and is widely used for rapid propagation of plant tissues (Fatima and Anis, 2011; Kitamura et al., 2002; Kumari et al., 2016; Murthy et al., 1998). To the best of our propagation knowledge, there has also been no published study on in vitro propagation of H. turgida through the leaf explants either. Therefore, the current study aimed to establish a rapid and efficient micropropagation protocol for H. turgida using the TDZ as a PGR and the leaves as explants.

Materials and Methods

Plant materials. Haworthia turgida plants were donated by the Institute of Botany, Chinese Academy of Sciences. Plants were transplanted in a greenhouse at College of Life Sciences, Qufu Normal University, China in July 2014. Fresh leaves were used as explants, which were washed in running tap water mixed with a few drops of liquid detergent for 30 min. Then, the leaves were decontaminated with 70% ethanol for 30 s followed by two to three washes with sterile water. After decontamination, the explants were sterilized in 0.1% HgCl₂, supplemented with 0.05% Tween-20 for 5 min, then washed with sterilized water for 3–4 times and dried with disinfected filter paper. The sterilized leaves were cut into 0.5–1.0 cm pieces for subsequent experiments after removing injured leaf bases using a sterilized blade. Culture medium and culture conditions. The culture medium consisted of Murashige and Skoog (1962) basal salts and vitamins as base medium, mixed with 3% (w/v) sucrose and solidified with 0.7% (w/v) plant agar. The pH of the medium was adjusted to 5.8 using 0.1 m NaOH or 0.1 M HCl, and the medium was autoclaved at 121 °C for 20 min. The MS medium was supplemented with PGRs before pH adjustment and sterilization. All cultures were placed at 24 ± 1 °C under a 14-h photoperiod with 40 μmol·m⁻²·s⁻¹ photosynthetic photon density quipped with cool-white fluorescent lamps (Philips 40 W tubes). Callus induction and propagation. The explants were inoculated on a basal MS.
medium with 0, 1.0, 2.0, or 3.0 mg L\(^{-1}\) BA in combination with 0, 0.1, 0.2, or 0.5 mg L\(^{-1}\) NAA (Table 1) based on previous reports (Ogihara, 1979; Ogihara and Tsunewaki, 1978) on \(H. \) setata and \(H. \) aristata for induction of callus generation. Then callus was cut into \(1-2 \text{cm}^2\) pieces and transferred to another medium supplemented with 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mg L\(^{-1}\) TDZ (Table 2) for callus proliferation. In each treatment, 15 explants were used, and each experiment was repeated three times. Fresh callus weight of each treatment was recorded after 30 d. Callus growth rate was calculated as the following equation: 

\[
\text{Growth rate} = \frac{w_t - w_0}{w_0} \times 100
\]

where \(w_t\) and \(w_0\) are the final and initial fresh weight of each callus, respectively. The term “growth” is used to represent callus development as evaluated by fresh weight (Ogihara and Tsunewaki, 1978), and callus induction ratio was calculated as follows:

\[
\text{Callus induction ratio} = \frac{\text{Total number of callus}}{\text{Total number of explanted leaves}} \times 100
\]

Adventitious shoot differentiation. Each callus was cut into small pieces of around \(2 \text{cm}^2\) and transferred to the MS medium containing 0, 1.0, 2.0, or 3.0 mg L\(^{-1}\) BA in combination with 0, 0.1, 0.2, or 0.5 mg L\(^{-1}\) 2,4-D for adventitious shoot induction (Table 3). The number of callus-forming adventitious shoots and the average number of shoots per callus were recorded after 30 d. In each treatment, 24 explants were used, and each experiment was repeated thrice.

Rooting of shoots and acclimatization. The elongated shoots (2–3 cm in length) were inoculated on the MS medium with 0, 0.05, 0.10, 0.15, or 0.20 mg L\(^{-1}\) NAA for root formation (Table 4). After 30 d, the frequency of root induction, number of roots per plantlet, and length of the roots were assessed. In each treatment, 24 shoots were used, and each experiment was repeated thrice. Meanwhile, 60 elongated shoots (2–3 cm in length) were wounded gently at the bottom of the stem, and then transplanted to a mixture of vermiculite and soil (Flower Nutrition Soil, Deli, Fuzhou, China; 1:1, v/v), and established in the greenhouse for simultaneous ex vitro rooting. The rooting percentage was calculated as follows:

\[
\text{Rooting percentage} = \frac{\text{Number of shoots with roots}}{\text{Total number of shoots planted}} \times 100
\]

Well-rooted plantlets were taken out from culture vessels, washed in running tap water to remove attached agar, transplanted in sterilized vials in the dark, then moved into culture boxes (7×7 cm) with a mixture of vermiculite and flower nutrient soil (1:1, v/v), and placed in the greenhouse under a natural photoperiod condition and 25±2 °C and 70% relative humidity. Plantlet survival percentage was calculated after 30 d based on the following:

\[
\text{Plantlet survival percentage} = \frac{\text{Total number of shoots}}{\text{Total number of shoots}} \times 100
\]

Statistical analysis. All percentage data were transformed using arcsine square root. The equation \(P' = \text{ASIN}([\text{SQRTP}])\) was used to normalize error distribution before variance analysis (Compton, 1994), then the percentage data were employed to express the original units. All data were analyzed statistically using a statistical software SPSS (version 19.0; IBM, Armonk, NY). Significant differences among the treatments were determined using the Duncan’s multiple range test at \(P < 0.05\). The results were expressed as the mean ± SE of repeated experiments.

### Results and Discussion

Effect of NAA and BA on induction of callus from the leaf explants. PGRs such as auxins and cytokinins are the main regulators of plant de- and redifferentiation (Frello et al., 2002; Kordi et al., 2013; Liu et al., 2016; Peeters et al., 1991). Jako et al. (1993) used BA and NAA for callus induction of sugar beet (Beta vulgaris). In this study, various types and concentration of PGRs were selected based on previous studies with some modifications (Kaul and Sabharwal, 1972; Kitamura et al., 2002; Ogihara, 1979; Ogihara and Tsunewaki, 1978). The leaf explants of \(H. \) turgida formed calli on all MS media supplemented with various concentrations of NAA and BA (Table 1). The explants started to differentiate at 20 d after the inoculation and formed reddish-colored calli (Fig. 1A). After 30 d, yellow-green tumor-like calli started to form at the cutting edges (Fig. 1B). After 40 d, green and compact callus formed in most of the explanted leaves and some globular shoot primordia were also observed (Fig. 1C). The greatest percentage of callus induction (95.6%) was achieved when the leaf explants were inoculated on medium that contained 1.0 mg L\(^{-1}\) BA and 0.1 mg L\(^{-1}\) NAA (Table 1). Similar results were also reported in Kalanchoe blossfeldiana (Kordi et al., 2013).

Table 1. Effects of 1-naphthaleneacetic acid and 6-benzyladenine concentrations on callus induction from the leaf explants of \(H. \) turgida. Data recorded after 40 d.

| NAA (mg L\(^{-1}\)) | BA (mg L\(^{-1}\)) | Induction (%) | Notes |
|---------------------|------------------|---------------|-------|
| 0.00                | 0.00             | 10.9 ± 2.20 d | Rare individual explants developed into calli |
| 0.10                | 1.00             | 95.6 ± 3.84 a | The explants developed yellowish green calli with compact texture and several globular structures |
| 0.20                | 2.00             | 62.4 ± 5.88 b | The explants developed yellowish green calli with a few yellow-green globular structures |
| 0.30                | 3.00             | 37.7 ± 2.23 c | The explants developed a few calli |
| 0.40                | 4.00             | 60.1 ± 3.87 b | The explants developed yellowish green calli with several yellow-green globular structures |
| 0.50                | 5.00             | 57.8 ± 2.23 b | The explants developed yellowish green calli with a few yellow-green globular structures |
| 0.60                | 6.00             | 31.1 ± 2.20 c | The explants developed a few calli |
| 0.70                | 7.00             | 39.9 ± 3.87 c | The explants developed a few calli |
| 0.80                | 8.00             | 28.6 ± 5.88 c | The explants developed a few calli |
| 0.90                | 9.00             | 24.4 ± 2.23 c | Few individual explants developed into calli |

*Means followed by the same letter(s) within a column are not significant at \(P < 0.05\). Values represent the mean ± SE of three replications each with five explants. BA = 6-benzyladenine; NAA = 1-naphthaleneacetic acid.

Table 2. Effects of thidiazuron plus 1.0 mg L\(^{-1}\) 6-benzyladenine and 0.1 mg L\(^{-1}\) 1-naphthaleneacetic acid on the growth rate of callus of \(H. \) turgida. Data recorded after 30 d.

| TDZ (mg L\(^{-1}\)) | Growth rate (fold) | Notes |
|---------------------|-------------------|-------|
| 0.0                 | 1.15 ± 0.04 d     | Callus formation was slowly induced |
| 0.5                 | 1.95 ± 0.05 c     | Callus formation was slowly induced and showed compact texture |
| 1.0                 | 2.29 ± 0.04 c     | Callus formation was induced and showed a compact texture |
| 1.5                 | 2.37 ± 0.08 c     | Callus formation was induced and showed a compact texture |
| 2.0                 | 2.90 ± 0.13 b     | Callus formation was induced and showed a fragile globular morphology |
| 2.5                 | 4.07 ± 0.36 a     | Callus formation with the highest induction rate and development of a fragile globular morphology |
| 3.0                 | 3.68 ± 0.08 a     | Callus formation was rapidly induced and development of a fragile globular morphology |

*Means followed by the same letter(s) within a column are not significant at \(P < 0.05\). Values represent the mean ± SE of three replications each with five explants. TDZ = thidiazuron.
### Table 3. Effects of 6-benzyladenine and 2,4-dichlorophenoxyacetic acid on adventitious shoot induction from callus of *H. turgida*. Data recorded after 30 d.

| BA (mg·L⁻¹) | 2,4-D (mg·L⁻¹) | Induction (%) | No. of shoots/callus | Description of growing status |
|------------|----------------|---------------|----------------------|-------------------------------|
| 0.0        | 0.0            | 8.0 ± 2.40 d  | 4.3 ± 0.33 b         | Few shoots were obtained      |
| 1.0        | 0.1            | 45.8 ± 2.40 b | 22.0 ± 0.58 b        | Adventitious shoots were induced; shoots were strong and in green color |
| 1.0        | 0.2            | 76.6 ± 3.67 a | 25.7 ± 0.67 a        | Cluster shoot formation was induced; shoots were thick and strong; fast growing; with grass green color |
| 1.0        | 0.5            | 38.8 ± 3.67 bc| 18.0 ± 0.58 c        | Adventitious shoot formation was induced; slow growing; light-green in color |
| 2.0        | 0.1            | 23.4 ± 3.67 c | 12.7 ± 0.67 e        | Adventitious shoot formation was induced; yellow-green in color and slender in shape |
| 2.0        | 0.2            | 29.1 ± 2.40 c | 17.3 ± 0.33 d       | Adventitious shoot formation was induced; light-green in color |
| 2.0        | 0.5            | 26.2 ± 3.67 c | 14.7 ± 0.33 d       | Adventitious shoot formation was induced; yellow-green in color and slender in shape |
| 3.0        | 0.1            | 27.8 ± 1.38 c | 15.3 ± 0.33 d       | Adventitious shoot formation was induced; yellow-green in color and slender in shape |
| 3.0        | 0.2            | 11.0 ± 1.39 d | 6.3 ± 0.37 f        | Few shoots were induced       |
| 3.0        | 0.5            | 9.6 ± 1.39 d  | 6.0 ± 0.58 g        | Few shoots were obtained      |

*Means followed by the same letter(s) within a column are not significant at P < 0.05. Values represent the mean ± SE of three replications each with eight explants.

### Table 4. Effect of 1-naphthaleneacetic acid on root induction in micropropagated plantlets of *H. turgida*. Data recorded after 30 d.

| NAA (mg·L⁻¹) | Root induction (%) | Avg no. of roots induced per shoot | Avg root length (cm) |
|--------------|--------------------|-----------------------------------|----------------------|
| 0.0          | 76.4 ± 1.39 a      | 4.9 ± 0.38 b                      | 3.2 ± 0.19 a         |
| 0.05         | 82.0 ± 1.39 a      | 6.2 ± 0.42 a                      | 2.6 ± 0.29 ab        |
| 0.10         | 62.5 ± 2.40 b      | 3.5 ± 0.34 c                      | 2.0 ± 0.27 bc        |
| 0.15         | 44.4 ± 2.70 c      | 2.8 ± 0.25 cd                     | 1.7 ± 0.26 cd        |
| 0.20         | 33.3 ± 2.40 d      | 1.9 ± 0.23 d                      | 1.3 ± 0.16 d         |

*Means followed by the same letter(s) within a column are not significant at P < 0.05. Values represent the mean ± SE of three replications each with eight explants.

BA = 6-benzyladenine; 2,4-D = 2,4-dichlorophenoxyacetic acid.

Fig. 1. Callus formation and adventitious shoot induction in *Haworthia turgida*. (A) reddish callus developed from the leaf explants on the Murashige and Skoog (MS) medium supplemented with 1.0 mg·L⁻¹ 6-benzyladenine (BA) and 0.1 mg·L⁻¹ 1-naphthaleneacetic acid, (B) the color of callus changed from red to green, (C) Callus with green shoot primordia (arrows), (D) induction of adventitious shoots on the MS medium supplemented with 1.0 mg·L⁻¹ BA and 0.2 mg·L⁻¹ 2,4-dichlorophenoxyacetic acid, and (E and F) proliferation of adventitious shoots. Scale bars = 0.5 cm.

**Effect of TDZ on callus redifferentiation.**

TDZ facilitates callus formation in various plant culture systems. It induces high cell proliferation rates compared with other PGRs (Jain and Rashid, 2001; Murthy et al., 1998). In some recalcitrant woody plants, TDZ stimulates callus formation at concentrations higher than 1 μM (Huetteman and Preece, 1993). In *Withania somnifera* tissue cultures, high concentrations of TDZ (8–20 μM) efficiently induce the formation of both callus and adventitious shoots (Sharma et al., 2014). Zhang et al. (2004) indicated that TDZ is more effective than BA in sugar beet regeneration. The present study showed that calli elicit different growth rate responses to various TDZ concentrations with 1.0 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA in MS medium. We observed that the growth rate increases with higher TDZ concentration, reaching the highest rate of proliferation (4.07 ± 0.36 fold), followed by a decrease. The highest callus growth rate was observed on MS medium containing 2.5 mg·L⁻¹ TDZ, 1.0 mg·L⁻¹ BA, and 0.1 mg·L⁻¹ NAA, which may possibly be due to the optimal cytokinin activity of TDZ (Sivanesan et al., 2011). Calli appeared as transparent green particles or lumps with a compact or fragile organization, some of them containing shoot primordia (data not shown).

**Effect of BA and 2,4-D on adventitious shoot regeneration from callus.** Adventitious shoot proliferation in many succulent plants relies on the crosstalk between auxins and cytokinins (Amoo et al., 2009; Kitamura et al., 2002; Solis et al., 2013; Zhao et al., 2013). The leaf explants of *Cotyledon orbiculata* form adventitious shoots from calli on the medium supplemented with combinations of PGRs, among which BA with NAA, and TDZ with NAA were the optimal combinations (Kumari et al., 2016). In the present study, multiple adventitious shoots were induced from calli on MS medium using various concentrations of BA and 2,4-D (Table 3). Shoots initiation from green compact calli was detected at about 12 d of culture. After 3 weeks, adventitious shoots were observed with normal green leaves (Fig. 1D). Among the PGRs tested, the greatest induction ratio (76.6%) of shoot regeneration and number of shoots (25.7 ± 0.67) were observed on MS medium containing 1.0 mg·L⁻¹ BA and 0.2 mg·L⁻¹ 2,4-D (Fig. 1E and F). This result is in line with the report that BA together with 2,4-D can induce shoot primordia from the surface of the callus of *Curcuma longa* (Salvi et al., 2001). This study showed that the combination of BA and 2,4-D significantly improves shoot formation. However, it could not induce multiple shoots when the callus was placed on the MS medium with a combination of BA and NAA (data not shown). We also observed that subculturing adventitious shoots of *H. turgida* allows these to continue producing more shoots. Because adventitious shoot regeneration is a relatively long process, the use of this method can save time and resources.

**Effect of NAA on rooting and acclimatization.** Generally, auxins such as NAA, IBA, and IAA that are added to the medium induce rooting (Sivanesan et al., 2011). Previous
reports have shown that the largest number of roots in Aloe vera could be achieved by culturing in a medium supplemented with 1 mg L−1 NAA (Hashem and Kaviani, 2010). The positive effect of NAA on both root induction and root elongation was demonstrated at a concentration of 0.5 mg L−1 on Kalanchoe blossfeldiana (Kordi et al., 2013). In the present study, elongated shoots (2–3 cm in length) were inoculated on rooting medium with different concentrations of NAA in Table 4 (Fig. 2A). The greatest rooting percentage (82.0%), number of roots (6.2 ± 0.42), and length of root (2.6 ± 0.29) per shoot were achieved on MS medium with 0.05 mg L−1 NAA, although number of shoots (4.9 ± 0.38) and longer roots (3.2 ± 0.19 cm) were produced on MS medium without NAA supplementation (Table 4). Furthermore, roots showing abnormal morphology were observed when the concentration of NAA was increased to 0.15 or 0.2 mg L−1 (Fig. 2D). After 30 d, healthy and well-rooted plantlets from the rooting medium were successfully transplanted (average 91.6%) to a mixture of vermiculite and soil (1:1, v/v), and (E) ex vitro rooting in the mixture of vermiculite and soil (1:1, v/v), and (G) ex vitro rooting system. Scale bars = 0.5 cm.

**Fig. 2. In vitro and ex vitro rooting, and acclimatization of Haworthia turgida. (A) In vitro rooting on the Murashige and Skoog (MS) medium supplemented with 0.05 mg L−1 1-naphthaleneacetic acid (NAA), (B) in vitro root system, (C) transplantation of in vitro–rooted plantlets, (D) Abnormal roots induced from MS medium with 0.15 mg L−1 or 0.20 mg L−1 NAA, (E) shoots before ex vitro rooting, (F) ex vitro rooting in the mixture of vermiculite and soil (1:1, v/v), and (G) ex vitro rooting system. Scale bars = 0.5 cm.**

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