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Original Research Paper

Efficient *in vitro* plantlets regeneration from leaf explant of *Haworthia retusa*, an important ornamental succulent

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ABSTRACT

This study was conducted to establish an efficient *in vitro* plantlet regeneration protocol using the *ex vitro* leaves as explants for *Haworthia retusa*. Leaf tissues were cultured on liquid full-strength Murashige and Skoog (MS) medium supplemented with 2.0 mg/L indole 3-butyric acids (IBA) for callus induction, followed by sub-cultured to solid medium for callus proliferation. Callus was then transferred to a fresh medium supplemented with 6-benzyl amino adenine (BA) for shoot development. The result showed that the maximum rate of shoot regeneration (100%), number of shoots per explant (43), and shoot height (9.4 mm) were recorded on the solid MS medium supplemented with 1.0 mg/L BA and 30 g/L sucrose. IBA improved rooting, whereas, NAA (naphthaleneacetic acid) causes calli to form at the base of the shoots. The half-strength MS medium supplemented with 0.5 mg/L IBA provided the best rooting response for the shoot. This medium formulation resulted in the highest rooting rate (100%) and the highest mean root number (5 roots/explant). The result of the present study would be helpful for the mass propagation of commercially important *H. retusa*.

Keywords : 6-benzyl amino adenine, *Haworthia retusa,* indole 3-butyric acid, leaf explants, micropropagation, plantlet regeneration

INTRODUCTION

The genus *Haworthia* includes succulent plants belonging to the Asphodelaceae family and is cultivated commercially as ornamentals. Some rare species are quite valuable but grow slowly and are difficult to propagate (Bayer, 1982). *Haworthia* species are small and have beautiful shapes and colors. The leaves are often arranged in the shape of flowers and used for decorating offices, apartments, restaurants, and hotels. They are also used as a meaningful gift because of their appealing shape, color, and ability to adapt to low moisture conditions. Traditionally, *Haworthia* species can propagate from seed, leaf-cutting, and offsets division with low multiplication efficiency. Currently, micropropagation is a potential technique to enhance multiplication efficiency in many succulents such as *Kalanchoe pinnata* (Jaiswal and Sawhney, 2006), *Aloe polyphylla* (Bairu *et al.*, 2007), *Pelecyphora aselliformis* (Badalamenti *et al.*, 2016), *Cotyledon orbiculata* (Kumari *et al.*, 2016), and *Aloe adigratana* Reynolds (Niguse *et al.*, 2020). Limited information is available on *Haworthia* micropropagation (Lizumi and Amaki, 2011; Liu *et al.*, 2017; Kim *et al.*, 2017; Kim *et al.*, 2019; Chen *et al.*, 2019). Different explants such as inflorescences, flower buds, and leaves of *Haworthia* species are used for regeneration, however, the leaf would be a more desirable explant for micropropagation due to its availability. Adventitious shoots can be regenerated directly from leaf tissue (Kim *et al.*, 2017) or indirectly through leaf-derived callus (Liu *et al.*, 2017; Chen *et al.*, 2019). In addition, somatic embryogenesis is a suitable method for *in vitro* plantlet regeneration in *Haworthia* species (Kim *et al.*, 2019). *Haworthia retusa* is a popular succulent plant with a short rosette of thick, triangular leaves. Propagation of *H. retusa* is possible by collecting offsets or leaf or stem cuttings. However, conventional propagation methods are difficult for large-scale plant production in slow-growing succulents. Therefore, an efficient alternative method for the propagation of *H. retusa* is required. In the present study, we establish an efficient micropropagation protocol using the leaf explants through organogenesis and embryogenesis in *H. retusa* to meet the growing market demand for this species.

MATERIALS AND METHODS

Plant material

H. retusa plants were grown in a greenhouse at the Institute of Tropical Biology, VAST, Vietnam. The leaves of *H. retusa* were excised from healthy plants and were surface sterilized first with 70% ethanol for 30 seconds and then with 0.1% $HgCl_2$ for 10 min, followed by washing with sterilized distilled water four times. The surface-sterilized leaves were cut into 2.0 x 3.0 mm pieces for subsequent experiments after removing the injured leaf.

Callus induction and proliferation

The surface-sterilized leaf explants (0.1 g fresh weight) were inoculated in flasks containing 50 mL of liquid MS (Murashige and Skoog, 1962) medium supplemented with 30 g/L sucrose. To induce callus formation, IBA was added to the medium at different concentrations of 1.0, 2.0, and 3.0 mg/L. These flasks were incubated on the shaker at 100 rpm. The IBA concentration at which the explant obtained the highest dry weight will be used to induce callus formation and proliferation in subsequent experiments. For the callus proliferation, the response explants in liquid shaking medium were transferred to solid MS medium supplemented with 30 g/L sucrose and IBA at the same concentration appropriate for callus induction. Calli were then used for shoot regeneration.

Shoot formation from callus derived from leaf explants

The callus (0.1 g FW) derived from the leaf of *H. retusa* were cultured on the MS medium supplemented with BA or KIN at different concentrations of 0.5, 1.0, 1.5, and 2.0 mg/L for shoot formation. After 6 weeks of cultured, the rate of shoot formation (%), the number of shoots/explants, and the shoot length (mm) were observed.

Root induction and plantlets formation

For root induction, six weeks old *in vitro* shootlets (1.5 cm) were cultured on different media such as full-strength MS, half-strength MS (½MS), full-strength SH (Schenk and Hildebrant, 1972), and half-strength SH (½SH) supplemented with 30 g/L sucrose. After four weeks of culturing, the rate of root formation (%), number of roots/explant, and root length (mm) were observed.

In addition to enhancing root formation, exogenous auxins such as IBA and NAA were added to the culture medium. The medium was used here as the previously determined optimal medium, and exogenous auxins were added to the medium before being autoclaved at different concentrations of 0.5, 1.0, 1.5, and 2.0 mg/L. Plantlets were collected after six weeks of culture.

Medium and culture conditions

All media were adjusted to pH 5.8 with NaOH 1N or HCl 1N, and plant growth regulators (PGRs) were added to the media before autoclaving (Hirayama, Japan) at 121°C and 101 kPa for 15 min. The chemicals and PGRs were analytical grades (Duchefa Biochemie, the Netherlands). The cultures were maintained in light at an intensity of 45 µmol m^{-2} s^{-1} under a 12h photoperiod at a room temperature of 24 ± 2°C.

Statistical analysis

The experiments were arranged in a completely randomized design (RCD) with three replications, 10 flasks per treatment. All data were analyzed statistically using the Statgraphics software (version 18.0) and the graph was drawn by Microsoft Office Excel 2010 Software. Significant differences among the treatments were determined using LSD's multiple range test at $p<0.05$. The results were expressed as the mean±SE of the repeated experiments.

RESULT AND DISCUSSION

Callus induction and proliferation

IBA is a synthetic growth regulator of the auxin group that plays a vital role in stimulating callus or root formation. This has been demonstrated in the present experiment, the leaf explants induced to form callus and adventitious roots in the culture medium supplemented with IBA at concentrations of 1.0 - 3.0 mg/L, and they did not trigger morphogenesis in the IBA-free MS medium. The highest total dry weight of the explants obtained in each culture flask was 0.52 g/flask at the concentration of 2.0 mg/L IBA after four weeks of cultured (Fig. 1, Fig. 2a). In the shaking liquid medium, the explants were directly exposed to the medium with a large area because they were immersed in the medium. Therefore, leaf fragments cultured in a liquid shaking medium absorbed nutrients better than in a solid medium, resulting in explants that

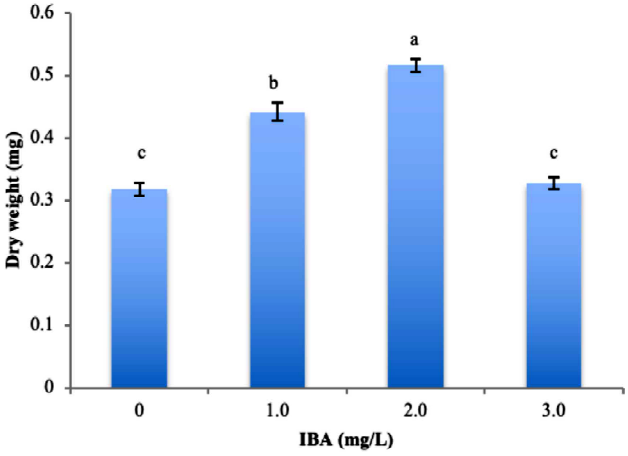

Fig. 1 : The total dry weight of the explants was obtained in each flask at different concentrations of IBA after 4 weeks of culture. Bars with different letters are significantly different at $p < 0.05$. Data are means ± SE.

respond early to the medium. In some previous studies, callus induction from the inflorescence explants of *H. obtuse* x *comptoniana* 'Sansenjyu' (Chen *et al.,* 2019); *H. splendens* and five commercial *Haworthia* cultivars which included *Haworthia* 'Natalie', 'Musin', 'Tiffany x Fertenon B Com', 'Baeckbong', 'White Wolf' (Reshma *et al.,* 2020), or the stem segments of *H. cymbiformis* (Haw.) (Lizumi and Amaki, 2011), or the leaf explant of *H. retusa* (Kim *et al.,* 2017, 2019) were performed in solid medium. In this study, leaf explants have cultured on a solid medium; however, this explant did not induce callus formation after 4 weeks of culture. Therefore, a liquid shaking culture system was used for the callus-induced culture.

After that, the response explants in this liquid shaking medium were transferred to the solid MS medium supplemented with 30 g/L sucrose and 2 mg/L IBA for callus formation (Fig. 2b). The callus was then used to induce shoot regeneration in the next experiments.

Shoot formation from callus derived from leaf explants

After six weeks of culture, all explants in the medium culture added BA enhanced shoot formation, with the proportion of shoot induction being 100%. The number of shoots per explant and shoot height increased with increasing concentration of BA from 0.5 to 1.0 mg/L, then they gradually decreased as the concentration of BA increased to higher concentrations (1.5 and 2.0 mg/L). The highest number of shoots, shoot height and the rate of shoot regeneration are recorded in the experiment using MS medium supplemented with 1.0 mg/L BA (43 shoots per explant, 9.4 mm, and 100%, respectively) (Table 1). In contrast, callus did not trigger shoot development in the MS medium without BA. According to Malik *et al.* (2005), the increase in BA concentration beyond the optimal level led to a decrease in shoot height and number of shoots. This was probably because exposure of explants to higher BA concentrations during the induction phase may have led to the accumulation of cytokinins, which inhibited further shoot growth. Liu *et al.* (2017) showed that multiplication adventitious shoots of *H. turgida* were induced from calli on MS medium using BA. The

Table 1 : Effect of BA and KIN on the shoot regeneration from callus derived from the leaf of *H. retusa* after 6 weeks of culture

BA (mg/L)	KIN (mg/L)	Rate of shoot regeneration (%)	Number of shoots/explant	Shoot height (mm)
0.5	-	100 ± 0.0^{a}	31.7 ± 1.8^{b}	8.3 ± 0.3^{b}
1.0	-	100 ± 0.0^{a}	43.0 ± 1.2^{a}	9.4 ± 0.4^{a}
1.5	-	100 ± 0.0^{a}	25.1 ± 1.8^{c}	7.3 ± 0.3^{c}
2.0	-	100 ± 0.0^{a}	22.2 ± 1.4^{d}	7.2 ± 0.5^{c}
-	0.5	44.3 ± 2.5^{e}	5.4 ± 0.7^{f}	5.1 ± 0.3^{d}
-	1.0	64.3 ± 2.5^{d}	7.1 ± 0.5^{ef}	5.2 ± 0.4^{d}
-	1.5	75.0 ± 1.7^{c}	8.3 ± 0.3^{e}	5.3 ± 0.2^{d}
-	2.0	78.7 ± 3.1^{b}	8.2 ± 0.4^{e}	5.2 ± 0.4^{d}
P	*	*	*	

*Means in the same column that is followed by different letters are significantly different ($p \leq 0.05$) using LSD's multiple range test

greatest induction ratio of shoot regeneration and several shoots was 76.6%, and 25.7, respectively. In the study of Lizumi and Amaki (2011), *H. cymbiformis* (Haw.) was propagated via thin cell layer (TCL) culture. The result showed that only stem transverse-TCL explants induced adventitious shoots. The maximum number of regenerated shoots was 24.0 per explant, and the percentage of shoot formation was 28.6% on the MS medium supplemented with 0.1 mg/L BA. Chen *et al.* (2019) established a shoot regeneration system for *Haworthia* from callus derived from inflorescence explants. The best shoot proliferation rates were on media with 1.0 mg/L BA and 0.0 - 0.4 mg/L NAA (65.57 - 81.01%) under a light intensity of 45 μmol $m^{-2}s^{-1}$. In another study, the effect of BA on adventitious shoot initiation of *Haworthia* 'Natalie', 'Musin', and 'Tiffany x Fertenon B Com' from inflorescence-derived callus were conducted. The highest number of shoot multiplications (20.8 ± 0.29) was observed for 'Tiffany x Fertenon B Com' on a medium containing 1.4 mg/L BA (Reshma *et al.*, 2020). The results obtained in our study showed a significant improvement in the percentage of shoots and the number of shoots formed compared with some previous studies.

In addition, many somatic embryos formed and developed into shoots when callus clusters were further sub-cultured on solid MS medium supplemented with 2.0 mg/L BA. Numerous globular somatic embryos were visible around the surface of the callus after 2 weeks of culture (Fig. 2c). After that, these somatic embryos continuously developed into shoots (Fig. 2f). This suggests that the shoot formation of *H. retusa* can be followed by two pathways; i) shoots formed through the callus; ii) shoots formed through the somatic embryo. This result can be explained during the liquid-shake culture period in the medium supplemented with auxin stimulated the formation of pre-embryonic cells, and then the explants transferred to the medium without auxin, or adding cytokinin enhanced somatic embryogenesis. This is also one of the novelties of this study; propagation via somatic embryos can produce this plant on a large scale. However, this issue needs to be further investigated to confirm the appropriate auxin and cytokinin concentrations and the corresponding embryonic stages in this species. Previous reports have shown that auxin is a plant hormone that is indispensable for the initiation of somatic embryogenesis, and cytokinin is often combined with auxin in a culture medium to enhance somatic embryo formation in most plants, including *Harworthia* species (Kim *et al.,* 2019; Reshma *et al.*, 2020). According to Kim *et al.* (2019), the somatic embryogenesis of plants depends on various types of cytokinins and auxins as well as their optimal concentration. This finding shows that adventitious shoot regeneration by somatic embryogenesis is effective in *H. retusa* micropropagation. Because *Harworthia* shoot regeneration is a time-consuming procedure, using this method can reduce time and improve the efficiency of *H. retusa* micropropagation.

Besides BA, KIN plays a vital role in the shoot morphogenesis of *Harwothia* species. For example, the shoot and callus induction percentages were affected by kinetin (KIN) and BA in *H. splendens* and 'White Wolf' (Reshma *et al.*, 2020). However, in the present study, the percentage of shoot induction, the number of shoots per explant, and the shoot height obtained in the treatments using KIN were lower compared with BA (Table 1). The positive effect of BA on shoot formation may be attributed to the ability of plant tissues to metabolize BA more readily than other plant growth regulators or to the ability of BA to induce the production of natural hormones, such as zeatin, within the tissue (Malik *et al.*, 2005).

Root induction and plantlets formation

Effect of culture medium on the root induction of *H. retusa*

Vitamins and nutrients in the culture medium play a significant role in the growth of *in vitro* plants. After 4 weeks of culture, *H. retusa* shoots induced roots on full-strength MS, half-strength MS, full-strength SH, and half-strength SH medium without plant growth regulator. On a half-strength MS medium, the highest rate of rooting explant (89.3%), number of roots/ explant (4.7), and root length (7.4 mm) were recorded (Table 2). Plants form roots to acquire water and minerals. Nutrient deficit acts as a powerful stimulant for root induction. The half-strength MS is to induce some level of nutrient stress, this resulted in the best root induction of *H. retusa* shoots. Whereas MS and SH are nutrient-rich media that are not suitable for the rooting of *H. retusa;* therefore, root induction on these media was lower than the half-strength MS medium. In addition, using a half-strength MS medium

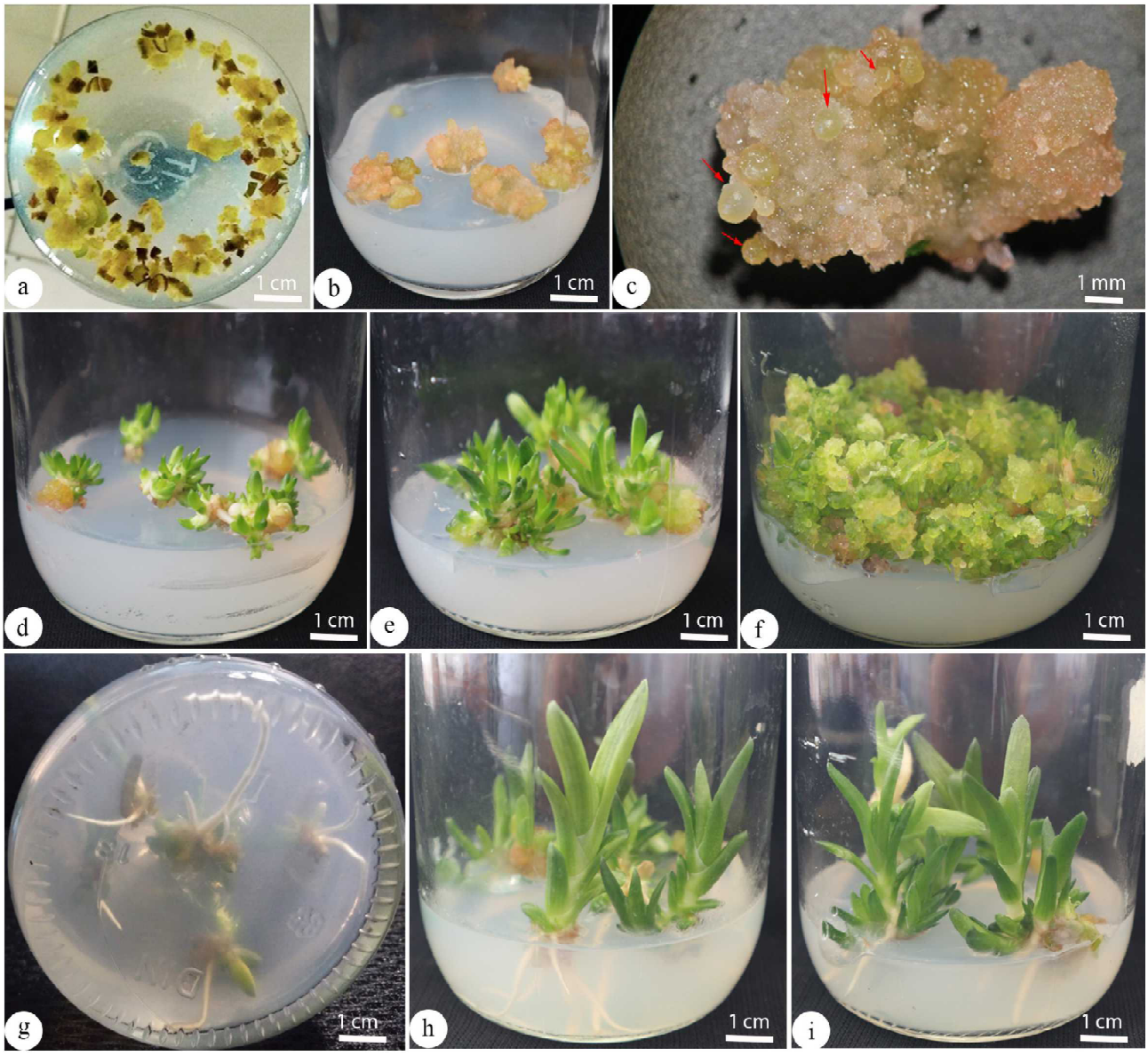

Fig. 2 : The *in vitro* plantlet regeneration protocol of *H. retusa* from leaves. a: leaf fragments induced to form a callus and adventitious roots in liquid shaking medium (MS + 2.0 mg/L IBA); b: callus proliferation on a solid medium (MS + 2.0 mg/L IBA); c: Somatic embryogenesis on the surface of callus. Arrows showed globular stage somatic embryo; d, e: shoots formation on MS medium supplemented 2.0 mg/L KIN (d) and 1.0 mg/L BA (e); f: somatic embryos developed into shoots; g, h, i: root induction and plantlet.

Table 2 : Effect of culture medium on the root induction of *H. retusa* after 4 weeks of cultured

Mineral medium	Rooting of explants (%)	Number of roots/explant	Root length (mm)
MS	82.0 ± 2.6^{b*}	3.3 ± 0.3^{b}	6.3 ± 0.1^{b}
½MS	89.3 ± 1.6^{a}	4.7 ± 0.4^{a}	7.4 ± 0.2^{a}
SH	70.3 ± 2.5^{c}	3.1 ± 0.3^{b}	6.4 ± 0.2^{b}
½SH	65.7 ± 4.0^{c}	1.8 ± 0.2^{c}	5.8 ± 0.3^{c}
P	*	*	*

*Means in the same column that is followed by different letters are significantly different ($p \leq 0.05$) using LSD's multiple range test

for the rooting stage also improves the acclimatization and survival rate of plantlets when they are transferred to the greenhouse because it reduces the difference in nutrient concentration between *in vitro* and soil conditions. Similarly, as in our experiments, the half-strength MS medium is effective in root induction in various species including *Cereus jamaica* (Monostori *et al.*, 2012), *Mentha spicata* (Fadel *et al.*, 2010), *Portulaca pilosa* (Chen *et al.*, 2020); *Rhus coriaria* (Amiri and Mohammadi, 2021).

Effect of IBA and NAA on the root induction of *H. retusa*

The results showed that IBA was more efficient in root induction than NAA after six weeks of culture (Table 3). When NAA (0.5 - 2.0 mg/L) was used to induce roots, a mass of callus was produced on the shoot and callus formation increased with increasing concentration of NAA. Callus production resulted in decreased rooting frequency, therefore, the percentage of rooting explants in treatments using NAA was lower than the control. The number of roots/explant and root length were not significantly different between NAA treatments. In contrast, callus formation was not observed in the IBA-supplemented treatments, and shoots induced roots with a high frequency. In *in vitro* culture, the addition of auxin at appropriate concentrations to the culture medium stimulated root formation, but higher concentrations stimulated callus formation. The auxin activity of NAA was higher than IBA, which was the reason for the shoots of *H. retusa* induced roots on the medium containing IBA and induced callus when NAA was used in this experiment.

In the treatment with 0.5 mg/L IBA, the percentage of rooting explant reached 100%, and it gradually reduced with higher IBA concentrations (1.0 - 2.0 mg/L). On a half-strength MS medium containing 0.5 mg/L IBA, the highest proportion of rooted explants, number of roots per explant, and root length were found among all treatments evaluated (Table 2). Previous studies showed that shoots of *Harwothia* species such as *H. attenuata* (Richwine *et al.*, 1995) and *H. turgida* (Liu *et al.*, 2017) formed roots on a medium supplemented with auxin. IBA has also been reported to be suitable for rooting in succulent species such as *Aloe adigratana* Reynolds (Niguse *et al.*, 2020); *Haworthia* 'Sansenjyu' (*H. obtuse* x *H. comptoniana*) (Chen *et al.*, 2019), *Haworthia* 'Natalie', 'Musin', 'Tiffany x Fertenon B Com' (Reshma *et al.*, 2020), *Stenocereus thurberi, Carnegiea gigantea* and *Pachycereus pringlei* (Pérez-Molphe-Balch *et al.*, 2002). In addition, it was observed that shoots produced several secondary shoots along with rooting when they were cultured on media for root formation (Fig. 2h, i).

Table 3 : Effect of IBA and NAA on the root induction of *H. retusa* after 6 weeks of culture

IBA (mg/L)	NAA (mg/L)	Rooting of explant (%)	Number of roots/explant	Root length (mm)
0	-	91.3 ± 3.2^{c*}	4.8 ± 0.2^{a}	10.3 ± 0.4^{a}
0.5	-	100.0 ± 0.0^{a}	5.0 ± 0.2^{a}	10.6 ± 0.8^{a}
1.0	-	98.0 ± 2.0^{ab}	4.7 ± 0.3^{a}	10.4 ± 0.3^{a}
1.5	-	95.3 ± 2.5^{b}	4.3 ± 0.2^{b}	10.2 ± 0.3^{a}
2.0	-	91.7 ± 1.2^{c}	4.1 ± 0.1^{b}	10.5 ± 0.3^{a}
-	0.5	33.7 ± 2.1^{d}	1.7 ± 0.2^{c}	3.7 ± 0.2^{b}
-	1.0	27.0 ± 1.0^{e}	1.7 ± 0.1^{c}	3.7 ± 0.3^{b}
-	1.5	21.3 ± 1.5^{f}	1.6 ± 0.2^{c}	3.6 ± 0.2^{b}
-	2.0	17.0 ± 2.6^{g}	1.7 ± 0.3^{c}	3.5 ± 0.4^{b}
P		*	*	*

Means in the same column that is followed by different letters are significantly different ($p \leq 0.05$) using LSD's multiple range test.

CONCLUSION

This study established an efficient plantlet regeneration system by indirect somatic embryogenesis through callus derived from the leaf tissues in a time span of 16-18 weeks. It included 5-6 weeks for callus induction and proliferation, six weeks for shoot regeneration, and 5-6 weeks for rooting and plants.

The time for this procedure can be shortened by 5-6 weeks for subsequent regeneration procedures because of the available source of callus and shoots. The process can be commercially exploited for the large-scale production of *H. retusa* plants.

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