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Clonal propagation of *Dorystoechas hastata* via axillary shoot proliferation

Bengi BABA ERDAĞ*, Yelda ÇALMAZ EMEK, Serap KURT AYDOĞAN

Department of Biology, Faculty of Arts and Science, Adnan Menderes University, 09010 Aydın - TURKEY

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Abstract: A protocol for the in vitro seed germination, seedling development, and axillary shoot propagation of *Dorystoechas hastata* Boiss. & Heldr. ex Bentham is described. By employing sterilised seeds that were transferred onto in vitro media, the effects of various in vitro media, photoperiods, and temperatures on germination were investigated. At the end of these experiments, a final protocol was determined for the in vitro germination of *D. hastata* seeds. With this protocol, the highest germination rate was obtained with distilled water supplemented with vitamins and 1 mg L^{-1} of gibberellic acid (GA_3), with a 16:8 photoperiod at 24 ± 2 °C. Germinated seeds were transferred onto various in vitro media for further seedling development, and the appropiate medium for seedling development was found to be half-strength Murashige and Skoog medium (MS). In axillary shoot propagation experiments, the highest shoot number per explant was obtained with half-strength MS medium supplemented with 0.5 mg L^{-1} of N^6-Benzyladenine (BA). Solitary shoots, removed from stock cultures, were transferred onto half-strength MS media supplemented with various concentrations of indole-3-butyric acid (IBA) or naphthalene acetic acid (NAA) for rooting. The maximum rooting rate was obtained with half-strength MS medium supplemented with 1 mg L^{-1} of IBA. Rooted plantlets were gradually acclimatised to the external environment.

Key words: *Dorystoechas hastata*, endemic species, in vitro, germination, propagation

Dorystoechas hastata'nın aksillar sürgün proliferasyonu yoluyla klonal çoğaltımı

Özet: Bu çalışmada, *Dorystoechas hastata* Boiss. & Heldr. ex Bentham'ın in vitro tohum çimlenmesi, fide gelişimi ve aksiller sürgün çoğaltımı için bir protokol tanımlanmıştır. In vitro ortamlara aktarılan sterilize edilmiş tohumlar kullanılarak farklı in vitro ortamların, ışığın-karanlığın (fotoperiyodun) ve sıcaklığın çimlenme üzerine etkileri araştırılmıştır. Denemelerin sonunda *D. hastata* tohumlarının in vitro çimlenmesi için kesin protokol belirlenmiştir. Bu protokole göre en yüksek çimlenme yüzdesi 1 mg L^{-1} gibberellik asit (GA_3) içeren vitamin destekli distile suda, 16:8 fotoperiyot koşulları altında 24 ± 2 °C'de elde edilmiştir. Çimlenmiş tohumlar ileri fide gelişimi için farklı in vitro ortamlara aktarılmış ve fide gelişimi için en uygun ortamın yarı-güçlü Murashige-Skoog (MS) ortamı olduğu gözlenmiştir. Aksiller sürgün çoğaltımı denemelerinde, eksplant başına en yüksek sürgün sayısı 0.5 mg L^{-1} N^6-Benziladenin (BA) içeren yarı-güçlü MS ortamında elde edilmiştir. Stok kültürlerden ayrılan tek sürgünler köklenmeleri için indol-3-butirik asit (IBA) veya naftalenasetik asit (NAA)'in farklı konsantrasyonlarını içeren yarı-güçlü MS ortamlarına aktarılmıştır. En yüksek köklenme yüzdesi 1 mg L^{-1} IBA içeren yarı-güçlü MS ortamında elde edilmiştir. Köklenen bitkicikler aşamalı bir şekilde dış ortama aktarılmıştır.

Anahtar sözcükler: *Dorystoechas hastata*, endemik tür, in vitro, çimlenme, çoğaltım

* E-mail: berdag@adu.edu.tr

Introduction

Wasteful or destructive uses of natural habitats menace many plant species at different levels, ranging from threatened to extinction. The International Union for Conservation of Nature (IUCN) and the World Wildlife Fund (WWF) estimate that up to 60,000 higher plant species could become extinct or nearly extinct by the year 2050 if the current trends of utilisation continue (Etkin, 1998).

Natural plant species are one of the most important sources of healing and medicinal substances. Nearly 80% of people in developing countries use traditional medicine for their basic health requirements, and 85% of these traditional medicinals contain plant extracts (Vieira & Skorupa, 1993). In the last decades, increasing attention to natural plant material and unreasonable harvesting activities have led to an increase in the existing pressure and danger on natural plant species.

In recent years, there has been an increased interest in in vitro techniques, which offer powerful tools for germplasm conservation and the mass multiplication of many threatened plant species (Murch et al., 2000). In vitro propagation presents a very important alternative because it has higher rates of multiplying clean (pest- and disease-free) plant material and requires a smaller area for a larger number of plants (Khan et al., 2002). Moreover, cultivation of medicinal plants for the purpose of extraction of active constituents may face certain limitations, such as climate, season, water availability, diseases and pests, and scarcity of natural dense populations (Pierik, 1987). Such limitations have led to the use of tissue culture techniques for the production of active constituents (Arıkat et al., 2004).

The family *Lamiaceae* includes approximately 200 genera of cosmopolitan distribution, which are important because of their etheric oil production capacity. Most genera of the family are thus rich sources of terpenoids and/or iridoid glycosides and flavonoids (Valant-Vetschera et al., 2003). *Dorystoechas hastata* Boiss. & Heldr. ex Bentham is a tertiary relict monotypic plant from the family *Lamiaceae*, endemic to Antalya, Turkey. The species is under protection only in Termessos National Park, but lives in other localities under extreme conditions and germination difficulty. It is known as chalba tea (çalba çayı) around the province of Antalya, and fresh or dried leaves are used by local people for treating colds or flu. Many of the *Lamiaceae* members have been called "mountain tea" (Dağ çayı), but *D. hastata* was considered to be the true chalba by Baytop (1999). As with many of the *Lamiaceae* members, the etheric oils of *D. hastata* have been investigated, and 1,8-cineol has been found to be the main compound of its etheric oils (Başer & Öztürk, 1992). In another study, diterpenes and norditerpenes from its roots were described (Ulubelen et al., 2004). Additionally, Karagözler et al. (2008) studied the proline and antioxidant contents of the leaves and showed that the species may serve as a natural source of proline and antioxidants.

In spite of numerous studies of the genera of the family *Lamiaceae*, there is no published study on the cultivation and tissue culture of the taxon.

The present investigation was carried out with the following objectives: a) to determine the seed vigour and in vitro seed germination process of *D. hastata*, and b) to develop an effective in vitro propagation protocol via axillary shoot propagation.

Materials and methods

Plant material

Mature seeds were collected from Antalya, Turkey (Antalya, Beydağları, ascent to Feslikan Yayla above Geyik Bayırı, open mixed (*Pinus nigra* var. *caramanica, Cedrus libani, Juniperus excelsa*) limestone, 1420 m, in June and July 2008). The seeds were stored in paper bags at room temperature (20-25 °C in the dark).

Seed vigour test

Seed vigour was tested with the tetrazolium test (Agrawal et al., 1973). Halved seeds were treated in tetrazolium solution (TTC, 0.1%) for 2 h at room temperature and red-staining embryos were evaluated as alive.

Seed sterilisation

For seed sterilisation, intact seeds were washed under running tap water for 30 min. Subsequently, seeds were surface sterilised in 70% (w/v) ethanol

(EtOH) for 10 min followed by a 4.5% (w/v) sodium hypochloride solution (NaOCl) for 15 min, and then rinsed 3 times in sterile distilled water in a laminar flow hood.

In vitro germination

In vitro germination experiments were used to investigate the effects of different in vitro media, photoperiods, and temperatures on the germination of *D. hastata* seeds. The sterilised seeds were cultured in 210 mL glass baby food jars containing 50 mL of culture medium. Paper bridges were used as physical support for the seeds.

Three different in vitro media were used to test the effect of medium compositions on the germination of *D. hastata* seeds: Murashige and Skoog (MS) (Murashige & Skoog, 1962), White's medium (W) (White, 1963), and distilled water (DW) as the control. DW was supplemented with 0.1 mg L^{-1} of thiamine-HCl, 0.5 mg L^{-1} of pyridoxine-HCl, 0.5 mg L^{-1} of nicotinic acid, 2.0 mg L^{-1} of glycine, 100.0 mg L^{-1} of myo-inositol, and 30 g L^{-1} of sucrose. Different concentrations of gibberellic acid (GA_3: 1.0, 2.0, and 3.0 mg L^{-1}) were added to all media, and a GA_3-free medium was prepared to use as a control. The pH of the media was adjusted to 5.8 for MS and DW and 5.5 for W with 0.1 M of NaOH and 0.1 M of HCl before autoclaving at 105 kPa and 121 °C for 15 min. Cultures were maintained at 24 ± 2 °C with a 16:8 photoperiod, with illumination provided by cool white fluorescent lamps at 40 μE m^{-2} s^{-1}.

The same experiments were conducted in dark conditions and the effects of the dark on germination in different in vitro media were investigated at 24 ± 2 °C.

In order to test the effect of temperature on germination success, the seeds were incubated with DW medium, containing various vitamins and supplemented with 1.0 mg L^{-1} of GA_3, at 10, 15, 20, 25, and 30 °C in the light.

Two replicates were conducted, consisting of 10 explants per jar, and the experiments were repeated twice. The best result was used as the standard in the next experiment. The seeds were considered germinated when the emerging radicles were visible. The germination rate was calculated after 6 weeks of culturing.

Determination of the most suitable in vitro medium for sterile seedling development

Germinated seeds were transferred onto MS, half-strength MS, B_5 (Gamborg et al., 1968), and White's medium under aseptic conditions to determine the most suitable in vitro medium for seedling development. These media were solidified with 0.8% agar-agar (Merck). After 4 weeks, the development of the seedlings was evaluated considering morphological criteria such as seedling length, root length, root number, and leaf number. Cultures were incubated at 24 ± 2 °C with a 16:8 photoperiod provided by cool white fluorescent lamps.

Axillary shoot propagation

Approximately 4-week-old sterile seedlings were separated from primary roots and transferred onto half-strength MS medium containing different concentrations of BA or kinetin (KIN) (0.2, 0.5, 1.0, and 2.0 mg L^{-1}) for axillary shoot propagation. A cytokinin-free treatment was also included as a control. Cultures were incubated at 24 ± 2 °C with a 16:8 photoperiod provided by cool white fluorescent lamps. At the end of the 4-week period, the number of shoots per explant was evaluated for each cytokinin type and concentration. Some of the seedlings were subcultured at 2-week intervals to determine the effect of subculture period on the rate of necrotic seedlings.

Shoot rooting and acclimatisation of plantlets

After 4 weeks (subculturing at 2-week intervals), axillary shoots were excised from the stock culture and transferred onto half-strength MS media with or without different concentrations (0.1, 0.5, 1.0, and 2.0 mg L^{-1}) of auxins (IBA or NAA) for rooting. The results of the rooting experiments were expressed as percentages.

After 4 weeks, the plantlets were removed from the medium, and then the agar was carefully washed off the rooted plantlets to minimise pathogen attack. The plantlets were planted into plastic plant pots, 10 cm in diameter containing garden soil, and were kept in a growth chamber at 24 ± 2 °C with a 16:8 photoperiod. After 4 weeks, the plantlets were maintained at normal laboratory conditions.

Statistical analysis

The experiments involving seedling development were repeated twice with each experiment containing 5 replicates of 4 explants each, while shoot propagation and rooting experiments, conducted with 10 replicates of 1 explant per jar, were repeated twice. All data were subjected to ANOVA, and means were compared using Duncan's Multiple Range Test at the 0.05 level of probability.

Results and discussion

Seed vigour

According to the results of the tetrazolium test, 40% of the seeds collected in June and 94% of the seeds collected in July were alive. This obvious difference between survival percentages may have resulted from the time of seed collection. Seeds go through several developmental phases, including histo-differentiation (initial morphogenesis), maturation (seed expansion), and maturation drying (desiccation) (Muntz, 1982; Kermode, 1990, 1995). The reaching of the drying phase signals the discontinuation of the zygotic embryo program and the beginning of the germination process (Kermode, 1990). In our experiments, the low level of response to the tetrazolium test given by seeds collected in June may be an indication that collecting seeds in this month caused an interruption in the zygotic embryo development program, leading to death. The seeds collected in July, on the other hand, completed their developmental phases and therefore responded positively to the tetrazolium test at a rate of 94%. This reveals that the best time to collect *D. hastata* seeds is July. However, the fact that the life cycle of the plant changes according to environmental factors should be taken into consideration.

The germination rate of up to 40% for the seeds collected in June was in concordance with the tetrazolium test results (40% seed viability). This implies that the test is suitable for determining the viability of *D. hastata* seeds and led us to use the seeds collected in July as the starting material for the in vitro germination experiments.

In vitro germination

In tissue culture studies, seeds are preferred as starting materials in establishing cultures so that genetic diversity can be displayed at a maximum (Fay, 1994). In addition, in vitro germinated seeds supply several aseptic seedlings, which can then be used in tissue culture.

According to the results of our experiments, the highest germination rate (70%) was found with vitamin-supplemented distilled water containing 1 mg L^{-1} of GA_3. White's medium and MS medium, both with 1 mg L^{-1} of GA_3 added, showed lower germination rates, 45% and 35%, respectively (Figure 1). Our study revealed a germination rate that was inversely proportionate to the salt content of the media. The reason for little or no germination with a high salt content in the medium is that an increased percentage of salt leads to negative osmotic potential (Kauffman, 1969).

Germination is the first step in the beginning of the life of most flowering plants. This step starts with the uptake of water (imbibition) by the dry seed, which is in the resting phase, and generally ends with the emergence of an embryonic axis like a radicle (Zielinski et al., 2006). In experiments conducted under in vitro conditions, seeds that are not dormant can generally germinate in in vitro media with low salt content (Padilla & Encina, 2003). The salt contents of the in vitro media used in our experiments were different from each other. MS medium, which has a high salt content, especially potassium and nitrate salts, is the most widely used medium in tissue culture studies (Maliro & Kwapata, 2000). White's medium, on the other hand, has a low salt formulation. Vitamin-supplemented distilled water is different from the other media in that it is salt-free.

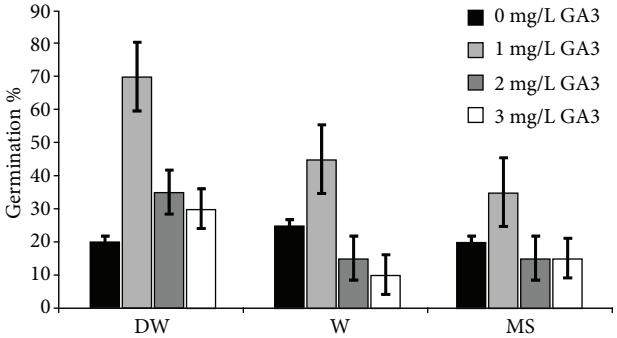

Figure 1. Germination percentage of *D. hastata* seeds on different in vitro media at 24 ± 2 °C, 16:8 photoperiod.

In in vitro propagation studies, successful seed germination can be obtained by using a low-salt medium, thus an advantage for the subsequent steps of the experiments. However, in cases where, as in our experiment, water uptake in the beginning of germination occurs at a maximum rate and the germination rate is almost doubled, use of vitamin-supplemented distilled water medium is important so that sufficient sterile seedlings can be obtained for use as starting material in in vitro experiments. In addition, this situation clearly shows that minerals are not necessarily needed in order for *D. hastata* seeds to germinate.

The lower germination rate on GA_3-free media may be related to dormancy. We may suggest that gibberellic acid added to the media breaks dormancy and hastens germination of *D. hastata* seeds, as Khan (1977) also reported that gibberellins have an impact that breaks dormancy in some seed species and accelerates germination in non-dormant seeds.

The same experiments were established in dark conditions and the effects of the dark on germination on different in vitro media were investigated at 24 ± 2 °C. The results of this experiment indicated that 50% of *D. hastata* seeds germinated in the dark (Figure 2), whereas the rate was 70% for the seeds in the light (Figure 1), revealing a clear distinction between light and dark applications. Investigating the germination of 5 *Origanum* species endemic to Antalya, a study by Ünal et al. (2004), in which germination under light-dark conditions is higher than germination in dark conditions, confirms our results.

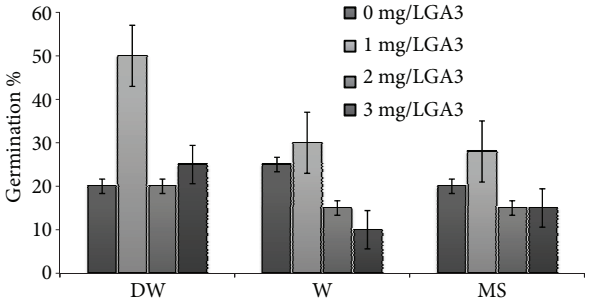

Figure 2. Germination percentage of *D. hastata* seeds on different in vitro media at 24 ± 2 °C, darkness.

As a result, vitamin-supplemented distilled water with 1 mg L^{-1} of GA_3 added was found to be the most suitable medium for in vitro germination of *D. hastata* seeds in light-dark conditions, and subsequently it was used for examining the effects of temperature on in vitro germination.

In the third experiment, the effect of temperature on in vitro germination of *D. hastata* seeds was examined. Seeds incubated at 20 °C and 25 °C showed the highest germination percentages (70%), whereas the germination rate below and above this temperature showed a decreasing tendency (Figure 3). Thanos and Doussi (1995) studied the seed germination of *Origanum dictamnus* L., *Sideritis syriaca* L. subsp. syriaca, *Salvia pomifera* L. subsp. pomifera, and *Salvia fruticosa* Miller, and found that 15, 20, and 25 °C were the most ideal temperatures. Our findings seem to agree with these results.

Based on the combined results of these experiments, the final protocol revealed for the maximum germination percentage of *D. hastata* seeds in in vitro conditions can be summarised as follows:

After the seeds are washed under running tap water for 30 min, they are exposed to 70% ethanol for 10 min. They are then treated with 4.5% sodium hypochlorite for 15 min for surface sterilisation, followed by rinsing with sterilised distilled water 3 times. Sterilised seeds are then transferred onto vitamin-supplemented distilled water medium containing 1 mg L^{-1} of GA_3, and kept at 24 ± 2 °C with a 16:8 h photoperiod. This protocol successfully allows *D. hastata* seeds to germinate at a rate of 70%.

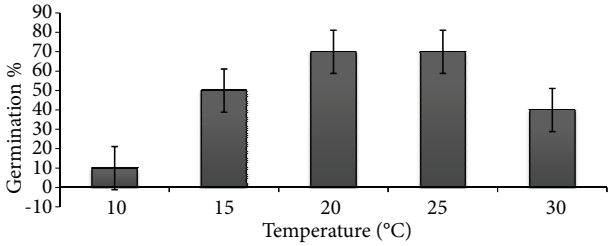

Figure 3. Germination percentage of *D. hastata* seeds on vitamin-supplemented distilled water containing 1 mg L^{-1} GA_3 at different temperatures, 16:8 photoperiod.

Determination of the most suitable in vitro medium for the development of sterile seedlings

In in vitro micropropagation studies, healthy sterile seedlings must be obtained to use as starting material. To determine the most suitable medium for seedling development, sterile seedlings were transferred onto different in vitro media.

In trials with White's medium, seedlings lost their vigour after a while, likely resulting from the low mineral content of this medium. Seedling growth on B_5 medium exhibited less satisfying results in terms of parameters such as plant height and number of leaves when compared to MS and half-strength MS media. On the other hand, no evident morphological difference was determined between seedlings grown on MS and half-strength MS media. However, in our experiments, during the subculturing period of 4 weeks, which is a general approach, chlorosis began to appear in leaves, followed by necrotic shoots. When necrotic shoot rates from the MS and half-strength MS media are compared, it is seen that half-strength MS medium gave rise to the lowest rate of necrotic shoots. Considering some parameters such as seedling development and low necrotic shoot rate, the half-strength MS was found to be the best medium for seedling development (Table 1).

Problematically, in vitro shoot necrosis is related to some culture conditions, including salt formulation of the medium, plant growth regulators, materials added to the medium, and culture period. In our experiments, we overcame shoot necrosis to some degree by decreasing the salt concentration of the media. However, further research is needed concerning this issue.

Axillary shoot propagation

From the experiments in which 4-week-old sterile seedlings were used as explants, the optimum type and concentration of cytokinin was determined for axillary shoot propagation.

All media, with or without plant growth regulator, allowed axillary shoot propagation in the seedlings, which were separated from their primary roots and transferred onto shoot propagation media (Table 2). The highest axillary shoot number per explant (4.60 ± 1.26) was observed in the shoots transferred onto half-strength MS medium containing 0.5 mg L^{-1} of BA. According to the results, cytokinin BA can be said

Table 2. Effects of different concentrations of BA or KIN on axillary shoot propagation of *D. hastata* (shoots were counted after 4 weeks of incubation).

Plant growth regulators (mg L^{-1})		Average number of shoots/explant
BA	KIN	
-	-	1.20 ± 0.42 e
0.1	-	2.70 ± 1.25 bc
0.5	-	4.60 ± 1.26 a
1	-	3.10 ± 0.99 b
2	-	1.80 ± 0.42 cde
-	0.1	1.40± 0.84 de
-	0.5	2.30 ± 1.42 bcd
-	1	1.60 ± 0.97 de
-	2	1.30 ± 0.67 e

The mean ± SD of 2 replicates. Values followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

Table 1. Seedling development of *D. hastata* on different in vitro media.

Medium	Shoot length (cm)	Leaf number	Root number	Root length (cm)	*Necrotic shoot %
MS	5.80 ± 1.39 a	7.20 ± 0.91 a	2.70 ± 0.95 a	3.55 ± 0.95 a	60 ± 0.52 ab
½ MS	6.00 ± 0.70 a	7.50 ± 1.91 a	2.40 ± 1.43 a	3.62 ± 1.61 a	30 ± 0.48 b
B5	3.10 ± 0.84 b	4.30 ± 0.95 b	2.45 ± 0.83 a	3.60 ± 1.00 a	60 ± 0.52 a
W	-	-	-	-	100 ± 0.00 a

* The mean % ± SD (standard deviation) of 2 replicates.
The mean ± SD of 2 replicates. In each column, the means with different letter(s) are significantly different at the 0.05 probability level using Duncan's multiple range test.

to be more effective than cytokinin KIN for axillary shoot propagation of *D. hastata*. Similar results were also reported from the studies of axillary shoot propagation in many medicinal plants from the family *Lamiaceae*, including *Orthosiphon spiralis* (Lour.) Murr. (Elangomathavan et al., 2003), *Orthosiphon stamineus* Benth. (Lai-Keng & Leng, 2004), *Mentha piperita* L. (Sunandakumari et al., 2004), and *Melissa officinalis* L. (Tavares et al., 1996).

Necrotic shoots were also observed during the axillary shoot propagation studies. In order to determine the effect of subculture period on necrotic shoot rates, cultures were transferred onto half-strength MS medium containing 0.5 mg L^{-1} of BA, which is the optimum concentration, and an obvious decrease in necrotic shoot rate was observed by subculturing at 2-week intervals (15% necrotic shoots). This procedure indirectly enabled the average number of axillary shoots per explant to increase. The necrotic symptoms observed in plantlets in our experiments may have resulted from the release of toxic phenolic compounds into the medium and/or a high level of ethylene accumulation, which affects growth and development (Biddington, 1992). However, this should be investigated in detail in further studies.

Rooting of axillary shoots

Axillary shoots obtained in the experiments were transferred onto half-strength MS media containing 0.1, 0.5, 1.0, and 2.0 mg L^{-1} of IBA or NAA for rooting. No rooting was observed with half-strength MS medium that was free of plant growth regulator; however, all concentrations of IBA or NAA induced rooting (Table 3). According to our findings, auxins are needed for root formation in *D. hastata*. Furthermore, we found that IBA is more effective for root formation than NAA, since half-strength MS medium containing 1 mg L^{-1} of IBA provided the highest rooting rate (70 ± 0.44). Similar findings were also obtained for *Cunila galioides* Benth., a *Lamiaceae* member (Fracaro & Echeverrigaray, 2001). Another study by Pattnaik and Chand (1996), employing different *Ocimum* spp., found in rooting experiments that IBA was an effective auxin for some species, whereas NAA was effective for the others.

Table 3. Effects of IBA or NAA on rooting of *D. hastata* seedlings obtained from axillary shoots propagation.

Plant growth regulators (mg L^{-1})		% Rooting
IBA	NAA	Mean ± SD
-	-	0 ± 0.00 b
0.1	-	50 ± 0.50 ab
0.5	-	55 ± 0.44 ab
1.0	-	70 ± 0.44 a
2.0	-	53 ± 0.46 ab
-	0.1	35 ± 0.41 ab
-	0.5	45 ± 0.37 ab
-	1.0	38 ± 0.41 ab
-	2.0	33 ± 0.42 ab

The mean ± SD of 2 replicates. Values followed by different letters are significantly different at the 0.05 probability level using Duncan's multiple range test.

Rooted plantlets were gradually acclimatised to the external environment for approximately 4 months, and it was seen that they preserved their vigour at the end of this period.

The defined protocol here explains the in vitro propagation process of monotypic endemic *Dorystoechas hastata*. The results of this study will form the basis for further studies on micropropagation of that plant, with enormous potential as a medicinal plant.

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