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DEVELOPMENT OF A MICROPROPAGATION PROTOCOL FOR ENDANGERED *HYPERICUM BILGEHAN-BILGILII* BAŞKOSE & SAVRAN (HYPERICACEAE) SPECIES, LOCAL ENDEMIC TO TURKEY

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Abstract

Hypericum species, are medicinally important and well studied for their pharmaceutical values. These species are gradually losing ground due to habitat loss, serious anthropogenic reasons as well as excessive collection from nature. *Hypericum bilgehan-bilgilii* is a local endemic species in Turkey. The population size is small and facing a threat. Keeping this in view there was a need to develop an *In vitro* ex-situ conservation protocol. Therefore an attempt was made to develop a protocol for callus induction, shoot growth, rooting and acclimatization for this endemic species. The nodal parts of the plant were preferred as the source of explant. These were cultured on a MS medium containing 2,4-Dichlorophenoxy acetic acid (2,4-D) and Kinetin (Kin) in different concentrations and combinations. The best callus induction was recorded in MS medium including 1.0 $mg.L^{-1}$ Kin and 0.5 $mg.L^{-1}$ 2,4-D with 6.2 shoots per callus. MS media with different Kin concentrations were used for shoot growth. The best shoot growth was observed on the MS medium with 2.0 $mg.L^{-1}$ Kin. Shoots grown *In vitro* were subcultured to MS medium containing 0.5 and 1.0 $mg.L^{-1}$ indole-3-butyric acid (IBA) for rooting. The best-rooting frequency (90 %) and rooting were determined in MS medium containing 1.0 $mg.L^{-1}$ IBA. There were 10 root formations per shoot. The micropropagated plantlets were acclimatized in pots filled with peat and by this way, an effective *In vitro* tissue culture protocol for the species studied was developed and its sustainable conservation way was confirmed.

Key words: *Hypericum bilgehan-bilgilii*, Endemic, Ex-situ conservation, Callus culture, Micropropagation.

Introduction

Medicinal plants are globally a valuable resource for drug production (Hamilton, 2004; Balunas & Kinghorn, 2005). With the rise in herbal medicine demand, natural health products and active ingredients of medicinal plants, the usage of these valuable herbs is increasing at a fast pace. The International Union for Conservation of Nature (IUCN) and the World Wildlife Fund (WWF) state that approximately 5.10^4 to 8.10^4 angiosperms around the world are used for medicinal purposes. It was stated that ~15.000 species among them are face to face with an extinction risk due to over-harvesting and habitat destruction (Bentley, 2010). Despite the awareness of this threat over the past two decades, the number of economically and medicinally important plants is steadily decreasing. These plants are often collected in excessive amounts from natural populations and the demand for harvesting from natural habitats is going up annually by 8-15% in the world (Ross, 2005).

The protection and sustainable use of such plants are of great importance in terms of economical input (Uprety *et al*., 2012). Many taxa are identified as endemic or endangered. Several sustainable and feasible programs are followed globally to protect the existing biodiversity. In the conservation strategies, special attention needs to be paid to the medicinally endemic plants because of their restricted distribution (Corral *et al*., 2011).

Various practices such as in-situ (within natural habitats) and ex-situ (outside of natural habitats) conservation methods are applied for the valuable plants in general. The disappearance of large natural areas due to threats from natural disasters, pests, pathogens, changing government policies and uncontrolled urban development make in-situ conservation nearly impossible. In the case of ex-situ conservation, the rule applied is to transfer endangered plant species to a place under controlled environmental conditions or a different natural environment. Creating continuous callus and shoot cultures with micropropagation in botanical gardens, seed banks, gene banks and plant tissue culture laboratories are the important ex-situ conservation methods.

Hypericum species have been used for hundreds of years in traditional medicine as natural medicinal plants for the prevention of diseases and their treatment. The species belonging to this genus are highly important because of the secondary compounds they contain and the therapeutic effects of the relevant compounds such as wound healing (Nowacki *et al*., 2015; Elisabetta *et al*., 2017), antibacterial (Li *et al*., 2018), anti-inflammatory (Raziq *et al*., 2015), diuretic/sedative (Khodayari *et al*., 2015), anticarcinogenic (Singh *et al*., 2019), antimicrobial (Li *et al*., 2018; Avci & Gergeroglu, 2019), antidepressant (Mejía-Agudelo *et al*., 2019), effects on neurodegenerative diseases (Zirak *et al*., 2019) and use as antioxidants (Eruygur *et al*., 2019). The species belonging to this genus are also regarded as a very valuable plant group from an economical perspective (Yaylacı *et al*., 2013).

The *Hypericum* L. taxon belongs to the Hypericaceae family. It includes around 500 species distributed on all continents except for the poles, Antarctica, low altitude tropical areas and arid areas (Robson, 2012). The family includes 9 genera. The genus *Hypericum* represents approximately 80% of the species diversity in the family (Crockett & Robson, 2011). In Turkey there are 107 taxa and 49 of these are endemics (Başköse & Savran, 2018).

The *Hypericum bilgehan*-bilgilii Başköse & Savran, is an endemic distributed on calcareous rocks in the Central Anatolian region of Turkey mainly Beyşehir / Konya, at an altitude of 1600-2000 m asl (Guner *et al*., 2012; Başköse & Savran, 2018). As per the IUCN levels of protection status this local endemic needs to be protected as it is a Critically Endangered (CR) species (Başköse & Savran,

2018). Therefore, traditional conservation measures together with universal conservation strategies are needed to protect this taxon.

Recently, *In vitro* tissue culture techniques have been preferred as a method in conservation studies of endemic plants (Isıkalan *et al*., 2020). There is sufficient information in the literature about *In vitro* micropropagation of different *Hypericum* species (Cardoso & De Oliveira, 1996; Ayan & Cirak, 2006; Montes-Villegas & Pedroza Manrique, 2008; Banerjee *et al*., 2012; Coste *et al*., 2012; Swain *et al*., 2016; Abdollahpoor *et al*., 2017; Cirak *et al*., 2020; Yamaner & Erdağ, 2020). However, no *In vitro* study has been reported on the local endemic *H. bilgehan-bilgilii*. In view of this, we investigated the effective *In vitro* tissue culture protocol for this critically endangered endemic species.

Material and Methods

Plant material and explant source: *H. bilgehan-bilgilii* was collected from the calcareous rocks from Beyşehir district of Konya (1600 – 2200 m, N 37º 29’ 21.66’’–E 31º 19’ 35.81’’). The plant parts were taken for tissue culture in required numbers without damaging the plants. The determination and identification of the plants were carried out by Dr. Ahmet SAVRAN, one of the authors of *H. bilgehan-bilgilii*, as per Davis, (1965). The media containing various PGRs were prepared before going to the fieldwork. MS and the seeds obtained from the fieldwork were used for callus induction studies. The seeds of *H. bilgehan-bilgilii* have very strong dormancy like other *Hypericum* species (Cirak *et al*., 2004; Pérez-García *et al*., 2006; Cirak *et al*., 2011; Carta *et al*., 2016). Because of this, the relevant plant was first kept for 24 hours in a 1.5 $mg.L^{-1}$ gibberellic acid medium and it was grown for six months in a peat-containing pot in the climate chamber. Explants (nodes) taken from the plant were grown in the growth chamber and used for callus induction studies. The explants were kept in 70% ethanol for 30 seconds and in 40% commercial bleach (5% NaOCl) for 20 minutes and after each process, washed five times with sterile distilled water.

Callus induction: Node explants for callus induction of *H. bilgehan-bilgilii*, were transferred onto the Petri dishes containing MS media (Murashige & Skoog, 1962) with different Kinetin concentrations (0.5; 0.8; 1.0; 2.0 $mg.L^{-1}$) and 2,4-D (0.5; 0.8; 1.0 $mg.L^{-1}$), which are known in the literature as the most commonly used PGRs for callus induction of *Hypericum* species. In this study, 4.405 $g.L^{-1}$ MS with vitamins (duchefa) and 30 $g.L^{-1}$ saccharose were used. The pH of all media was adjusted to 5.8 with the help of sodium hydroxide (NaOH) and hydrochloric acid (HCl), then sterilized in an autoclave at 121°C for 20 minutes. Callus induction of the explants was carried out in the plant growth chamber under a temperature of 25±2°C, 16/8 photoperiod, 27 μmol $m^{-2}s^{-1}$ light intensities and constant humidity of 50±5%. After 2 weeks, the cultures were subcultured on the medium with the same concentrations. Callus formation frequency, callus morphology and the shoot numbers per callus were recorded.

Shoot formation and propagation: Calluses obtained from node explants for shoot formation were transferred onto the MS including vitamins and varied concentrations of Kinetin (0.5; 1.0; and 2.0 $mg.L^{-1}$). After adjusting the pH to 5.8, shoot formation studies were carried out in the same environment as optimized for callus induction. The shoot formation data was noted 6 weeks after the transfer.

Rooting and acclimatization: Micro shoots (1.5-2.0 cm) with complete shoot growth and propagation were subcultured in MS medium (pH 7.8) supplemented with 0.5 $mg.L^{-1}$ and 1.0 $mg.L^{-1}$ IBA. MS medium without hormone served as the control. The cultures were kept in the same environment for six weeks in a climatic chamber under the conditions mentioned above. Rooting rate, the root numbers per shoot and root length data were recorded. Healthy seedlings which completed their root development were taken from Petri dishes and gently cleared with running tap water to remove residual MS medium. The seedlings were transferred to pots (five centimeters in diameter) containing peat to adapt to the soil (16/8 photoperiod). The small plants in the pot were covered with a transparent plastic nylon wrap, which prevented moisture loss and provided high humidity (Fig. 4). During six weeks, plastic nylon wraps were gradually opened and adaptation to the soil was followed.

Statistical analysis

The experiments were repeated three times for each medium. The number of shoots per callus, shoot length and nodal segment numbers recorded in the shoot data were statistically evaluated as per $p \leq 0.05$ in Tukey's (1954) test. Standard error and standard deviation values were determined in the same program.

Results

Callus Induction: *H. bilgehan-bilgilii* nodal explants were cultured in MS added with different cytokinin (CK) and auxin (Aux) concentrations to induce callus induction (Table 1).

Table 1. Impact of Aux and CK concentrations on callus induction in *H. bilgehan-bilgilii*.

Treatment groups	PGR concentrations ($mg.L^{-1}$)	Callus formation frequency (%)	Callus morphology	Number of shoots per callus
TC_1	MS hormone free (Control)	0	Not observed	0^{x}
TC_2	MS + 0.5 Kin + 0.5 2.4-D	50	Light green, transparent structure	2.2 ± 0.75^{y}
TC_3	MS + 1.0 Kin + 0.5 2.4-D	100	Dark green color, compact callus structure and amorphous	6.2 ± 0.87^{z}
TC_4	MS + 0.5 Kin + 1.0 2.4-D	30	Whitish color and transparent structure	2.0 ± 0.82^{xy}
TC_5	MS + 0.8 Kin + 0.4 2.4-D	100	Light greenish color, compact callus structure	4.1 ± 0.83^{t}
TC_6	MS + 2.0 Kin + 0.5 2.4-D	20	Whitish color, compact callus with transparent structure	1.5 ± 0.50^{xy}

Different letters in the columns point out a significant difference ($p<0.05$)

The callus induction was observed in all groups subjected to varying applications, except "TC_1", on MS media including 2,4-D as Aux and various concentrations of Kin as CK. The best callus induction in the species studied here was observed in the application group TC_3 with 5-7 shoots per callus in 7-14 days (Fig. 1). It was observed that the callus formed was dark green and compact. The application groups with the highest callus formation frequency were determined as TC_3, TC_5 and TC_2, respectively.

Shoot development from callus: Callus structures obtained from the plant used here were subcultured on the MS supplemented with different concentrations of kinetin to develop shoots (Table 2).

The shoot development was examined on the MS media including various Kin concentrations as CK. The best shoot development was recorded on MS + 2.0 $mg.L^{-1}$ Kin media with the formation of 12-14 nodal segments in the whole plant in 10-15 days (Fig. 2). After the first shoot development, the lateral shoot structures developing from the same shoot were recorded as one and four in the MS + 0.5 $mg.L^{-1}$ Kin and MS + 2.0 $mg.L^{-1}$ Kin application groups, respectively.

Rooting and acclimatization: Auxin plays an effective role in the root development of *Hypericum* species used in this study. Micro-seedlings which completed shoot development were subcultured in MS medium containing IBA at different concentrations. The root development data is presented in Table 3.

In the study, while rooting did not occur in IBA-free (control) MS medium, root formation occurred in all media containing IBA. The best root development was found in the application group supplemented with MS + 1.0 $mg.L^{-1}$ IBA (10 roots per shoot) (Fig. 3), the best root formation was 90 percent in MS including 1.0 $mg.L^{-1}$ IBA. It was observed that root percentage, root length and the number of roots per shoot increased in parallel with the increase in IBA concentration added to MS medium.

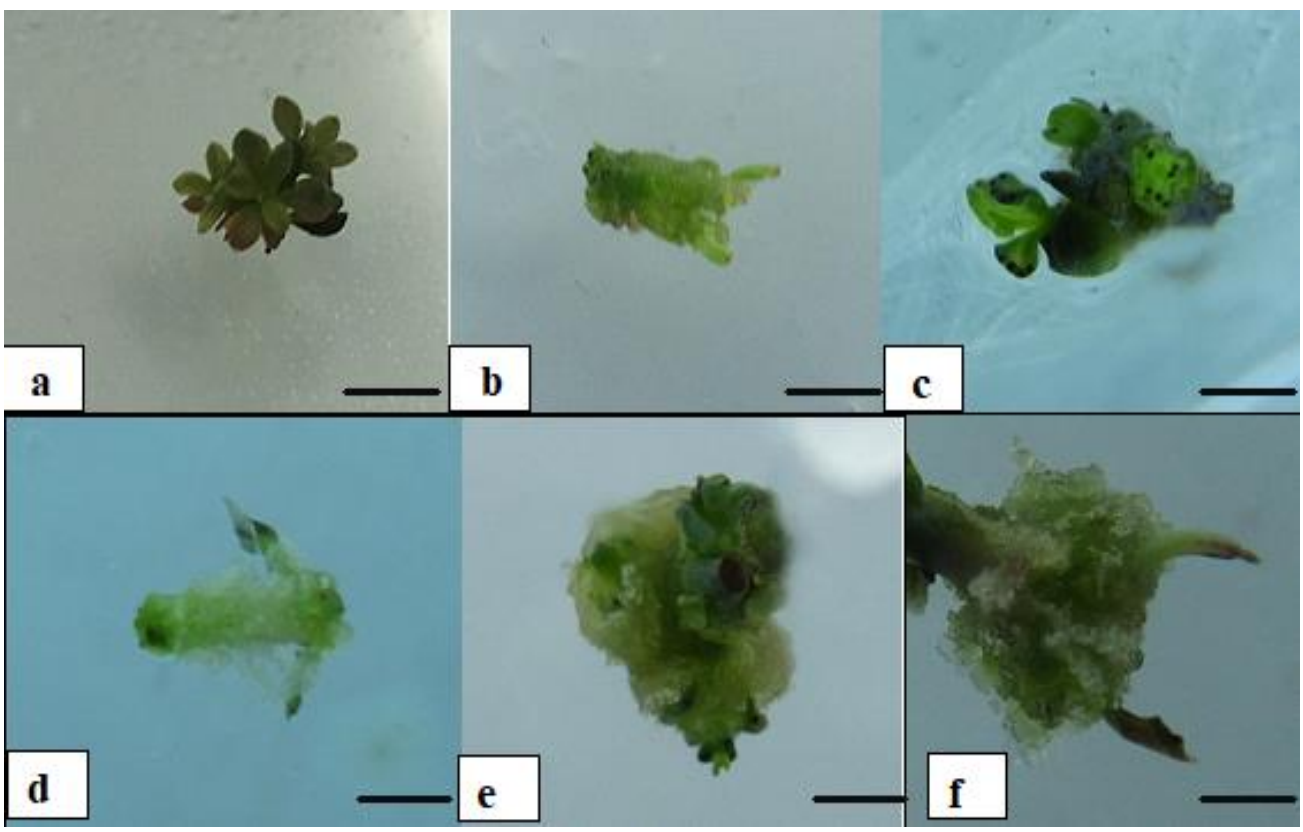

Fig. 1. (a) MS hormone-free (Control), Callus induction on the medium containing (b) MS with 0.5 $mg.L^{-1}$ Kin + 0.5 $mg.L^{-1}$ 2,4-D, (c) MS with 1.0 $mg.L^{-1}$ Kin + 0.5 $mg.L^{-1}$ 2,4-D (d) MS with 0.5 $mg.L^{-1}$ Kin + 1.0 $mg.L^{-1}$ 2,4-D (e) MS with 0.8 $mg.L^{-1}$ Kin + 0.4 $mg.L^{-1}$ 2,4-D (f) MS with 2.0 $mg.L^{-1}$ Kin + 0.5 $mg.L^{-1}$ 2,4-D. (The dark line/bar below the photos is 1cm)

Table 2. The impact of cytokinin concentrations on shoot development in *H. bilgehan-bilgilii*.

PGR Con. ($mg.L^{-1}$)	Shoot number occurring during shoot development	Shoot/s length (cm)	Number of nodal segments per shoot
MS hormone free (Control)	0	0^{x}	0^{x}
MS + 0.5 Kin	1	1.67 ± 0.62^{xy}	1.67 ± 0.94^{xy}
MS + 1.0 Kin	0	1.67 ± 0.94^{xy}	2.67 ± 0.47^{xy}
MS + 2.0 Kin	4	2.33 ± 0.24^{y}	4.67 ± 1.70^{y}

Different letters in the columns point out a significant difference (p<0.05)

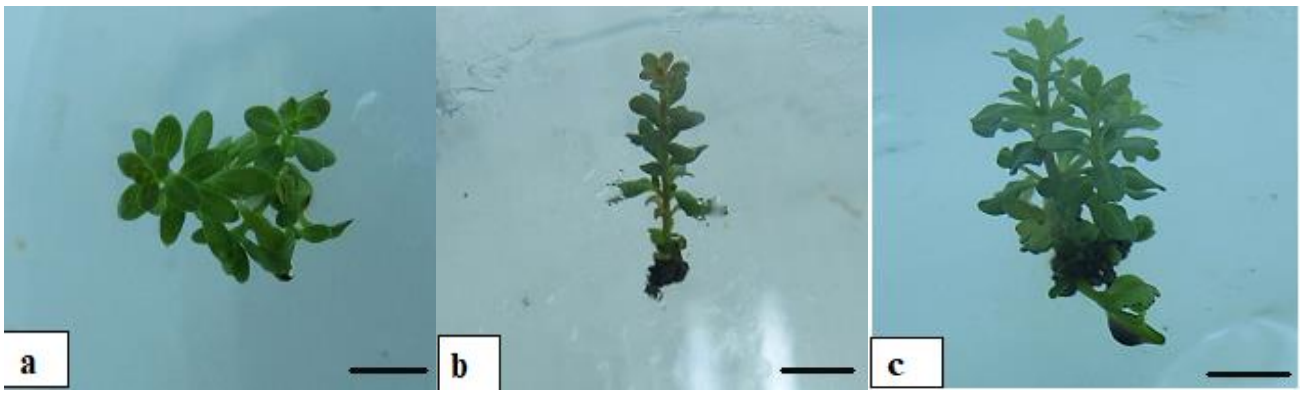

Fig. 2. Shoot development on the (a) MS + 0.5 mg.L^{-1} Kin (b) MS + 1.0 mg.L^{-1} Kin (c) MS + 2.0 mg.L^{-1} Kin mediums (the dark line/bar below the photos is 1cm).

Table 3. The impact of different Aux concentrations on root growth in *H. bilgehan-bilgilii.*

PGR Con. (mg.L^{-1})	Root percentage (%)	Root/s length (cm)	Number of roots per shoot
MS hormone free (Control)	0	0 ^{x}	0 ^{x}
MS + 0.5 IBA	70	3 ^{y}	6 ^{y}
MS + 1.0 IBA	90	5 ^{z}	10^{z}

Different letters in the columns point out the significant difference (p<0.05)

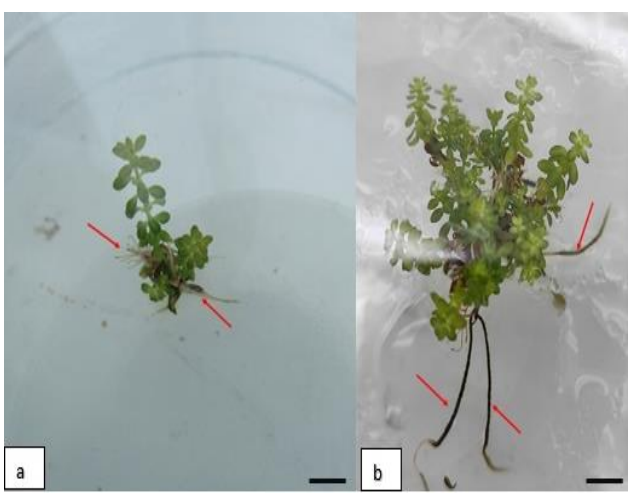

Fig. 3. Root formation in (a) MS + 0.5 mg.L^{-1} IBA (b) MS + 1.0 mg.L^{-1} IBA (The dark line/bar below the photos is 1cm, The red arrow shows the root structures formed on the shoots).

The seedlings which completed their root development were acclimatized under non-sterile conditions (Fig. 4). During the acclimatization period, it was determined that shoot lengths increased and the leaf blades expanded. The acclimatization rate of the seedlings for which soil adaptation studies were performed was recorded as 80 percent for ten seedlings. No morphological problems were observed in the growth of seedlings that got adapted to the soil.

Discussion

Callus induction and shoot development: Callus induction and proliferation for the protection of endangered or dormant plants are known as a highly effective means for micropropagation of plants concerned. In this study, an effective callus induction and shoot development protocol was determined for the endangered *H. bilgehan-bilgilii* species. Different proportions of Aux and CK combinations were used for callus induction and different CK concentrations were used for shoot development. Basal MS medium is not sufficient to initiate callus induction, and medium/media containing various PGRs are needed. Relevant MS media should contain different concentrations of auxin and CK. Kocak, (2015) has evaluated sterile seedling nodes as an explant source for *H.perforatum* L. and reported that optimum callus induction was observed on the MS with 0.5 BAP and 0.5 mg.L^{-1} 2,4-D. In the study where internodes and leaf parts were used as the explant source for the *H.bupleuroides* Griseb. (Ayan & Kevseroglu, 2007), it has been reported that the optimum callus formation was on the MS media containing 1.0 mg.L^{-1} BA + 0.1 mg.L^{-1} 2,4-D and 1.0 mg.L^{-1} BA + 1.0 mg.L^{-1} 2,4-D. According to these workers, the internode explants were more effective than leaves in callus induction. The varied auxin and cytokine concentrations added to the MS media have stimulated callus induction in the species *H. perforatum* and *H. bupleuroides* (Cirak, 2006), *H. triquetrifolium* Turra, (Akcam Oluk *et al*., 2010), *H.adenotrichum* Spach, (Yamaner & Erdağ, 2020), *H. spectabile* Jaub. & Spach, (Isıkalan *et al*., 2011), *H. brasiliense* Choisy, (Cardoso & De Oliveira, 1996), *H.heterophyllum* Vent., (Ayan & Cirak, 2006) and *H. perforatum* (Pretto & Santarém, 2000; Ayan *et al*., 2005; Banerjee *et al*., 2012;). Cell suspension cultures used in secondary metabolite enhancement studies are usually developed from callus tissues. The callus tissues are used as an intermediate stage for plant micropropagation through somatic embryogenesis or organogenesis (Nunez-Palenius *et al*., 2005).

Varghese *et al*., (2016) have determined that the best shoot development in *H. hookerianum* Wight & Arn.; a critically endangered species spread in the Western Ghats region of India; was on the MS medium including 2.325 µM Kin with 3.6 shoots per explant. Yamaner, (2011) subcultured the calli obtained from *H. adenotrichum* on the MS with 0.5 mg.L^{-1} Kin and observed that shoot growth was in the relevant application. Similar results were reported in the study

carried out by Namli *et al*., (2010). A study has also been carried out on *H. retusum* Aucher, Jaub. & Spach, and shoot development determined on the MS + 1.5 mg.L^{-1} Kin. Baruah *et al*., (2001) have obtained similar results for *H. patulum* Thunb., an endemic species in the Himalayan Mountains. Cytokinins mediate in the induction and propagation of shoots obtained from meristematic tissues via *In vitro* tissue culture studies (Swain *et al*., 2016). In the shoot development studies of *H. scabroides* N.Robson & Poulter, (Surmus, 2013); *H. polyanthemum* Klotzsch ex H. Reich., an endemic species of Southern Brazil (Bernardi *et al*., 2007); *H. mysorense* B. Heyne, found in the Western Ghats of India as an important medicinal plant for the region (Shilpashree & Rai, 2009); *H. umbellatum* A.Kern. and *H. richeri* ssp. *transsilvanicum*, endemic and endangered species in Romania (Coste *et al*., 2012); *H. scabroides*, an endemic species in Turkey (Asan *et al*., 2015); *H. gaitii*, a medicinal plant in the Himalayas including, Afghanistan, Bhutan, Nepal and Pakistan (Swain *et al*., 2016); *H. aviculariifolium* Jaub. & Spach, and *H. pruinatum* Boiss. & Balansa ex Boiss., endangered species in the flora of Turkey (Cirak *et al*., 2020); and *H. adenotrichum* (Yamaner & Erdağ, 2020) under *In vitro* conditions, different types of cytokines and their different combinations have been used, and they report that the cytokine hormone is quite effective for shoot development.

Fig. 4. Covering *H. bilgehan-bilgilii* with plastic nylon wrap and providing soil adaptation.

Rooting and acclimatization: An increase in habitat destruction is posing a great threat to plant diversity. The use of biotechnological methods for producing *İn vitro* cultures makes an important contribution to the conservation of endangered plant taxa and the creation of germplasm resources (Coste *et al*., 2012). Plant growth regulators are used to modify growth and development in plant tissue culture studies. Two of the most preferred and used growth regulators are the auxins and cytokines (Khlifa *et al*., 2016). Indole acetic acid (IAA), IBA, 2,4-D and Naphthalene acetic acid (NAA) are the most commonly used auxins in *İn vitro* propagation studies. These are generally used to stimulate root formation and can also be preferred in callus induction studies.

Sarropoulou *et al*., (2018) have reported that the optimum root development in *H. empetrifolium* Willd. subsp. *empetrifolium In vitro* ex-situ conservation study has been in MS with 0.1 mg.L^{-1} IBA. In the micropropagation study of *H. perforatum*, the maximum root formation number has been found in the medium containing ½ MS + IBA 4.9 µM (Fascella *et al*., 2017). Abdollahpoor *et al*., (2017) have found that the optimum root formation in *H. perforatum* plant in MS media including 0.5 mg.L^{-1} IBA has been with five roots per shoot. The best root growth in *H. gaitii*; another endangered medicinal plant; is in a medium including 0.5 mg.L^{-1} and 1.0 mg.L^{-1} IBA. (Swain *et al*., 2016). Similar results have been recorded in micropropagation studies of endemic and endangered species *H. richeri* ssp. *transsilvanicum* as well as *H. umbellatum* in Romania. Coste *et al*., (2012) report that the best root formation for both species has been in the medium containing 2.45 µM IBA. In *H. heterophyllum* (Ayan & Cirak, 2006) and *H. spectabile* (Karakus, 2011); endemic taxa in Turkey; as well as *H. aviculariifolium* and *H. pruinatum* (Cirak *et al*., 2020); endangered species in Turkey; *H. retusum* (Namli *et al*., 2010), *H. maculatum* (Băcilă *et al*., 2010), *H. triquetrifolium* (Akcam Oluk *et al*., 2010) and *H. perforatum* (Cristea *et al*., 2015), various auxin concentrations have been used and all are reported to stimulate root formation.

Acclimatization is one of the last stages in micropropagation studies. It is one of the most difficult steps, as the plants transferred to the soil are very sensitive to factors such as moisture loss, biotic and abiotic stresses. In this study, the adaptation rate of *H. bilgehan-bilgilii* to the acclimatization process was found to be 80 percent. Swain *et al*., (2016) developed a micropropagation protocol for *H. gaitii* and determined the adaptation rate of the plant to the acclimatization process as 50 percent. According to Ayan & Cirak, (2006), the rate of adaptation of *H. heterophyllum* to the soil is 40 percent. A study conducted by Onlu, (2019) on *H. pruinatum* and an endemic *H. heterophyllum* has

revealed that the acclimatization process of the seedlings obtained *In vitro* in the *H. pruinatum* species was successfully carried out, the adaptation of the seedlings of the *H. heterophyllum* species has not been successful.

Conclusions

Ex-situ *İn vitro* conservation of local endemic and endangered species under *In vitro* conditions using plant tissue culture techniques is attracting much attention lately by biotechnologists. In this study, effective *İn vitro* propagation protocols were developed for the local endemic *H. bilgehan-bilgilii* for the first time. In our opinion, the development of an effective and reliable *İn vitro* tissue culture protocol is very significant for the cultivation or conservation of endemic and endangered plant taxa. We believe that the data of this study will shed light on different *İn vitro* tissue culture and ex-situ conservation studies to be conducted on endemic and endangered taxa in the future for drug development.

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