

CHAPTER 6
IN VITRO MICROPROPAGATION OF
ALYSSUM NEZAKETIAE AYTAÇ & H. DUMAN

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INTRODUCTION

Anatolia has a very important potential in terms of biodiversity due to its different geographical characteristics and diversity. In fact, while 2,750 of a total of 12,000 plant species are endemic in Europe, Anatolia hosts approximately 11,000 plant taxa, 1/3 of which is endemic (Ekim et al., 2000). Serious studies are carried out to protect endemic species in the world and to ensure the continuation of their generation. Studies on this subject in Turkey began to gain value between the years 1992-1997. Endemic species, which continue their lives in a very limited area, are faced with some threats in order to survive. In particular, factors such as urbanization, industrialization, tourism, overgrazing, unconscious expansion of agricultural areas, fires, and the use of herbicides pose a great danger to plants.

The soil structure of the region where the provincial center of Çankırı has located consists of the gypsum (gypsum) series (Figure 1). According to the records of the first 10 volumes of the Flora of Turkey, a total of 357 species, 119 of which are endemic, live in this region, where many endemic plants are found (Davis, 1965-1988). It is estimated that the number of species is over 1000 with various flora studies carried out later.

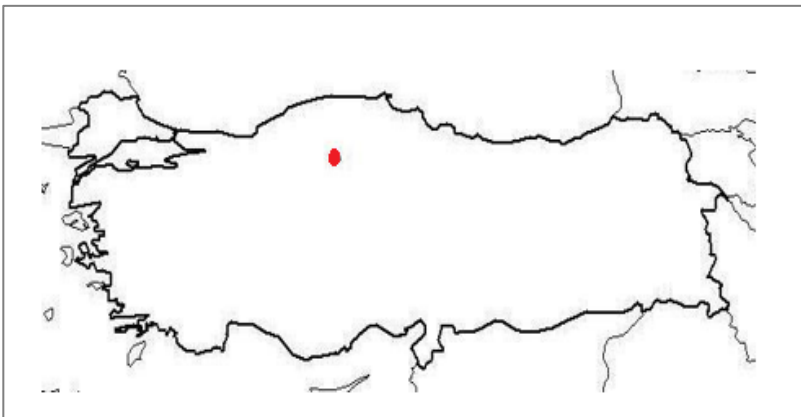


Figure 1. Distribution area of *A. nezaketiae*, Çankırı/TÜRKİYE (Bakis et al. 2011)

It has a local and endemic distribution in the arid and semi-arid climate of Çankırı and is not yet in the danger category according to the International Union for Conservation of Nature (IUCN) data. However, *Alyssum nezaketiae* AYTAÇ & H. DUMAN (Aytaç and Duman, 2000), which is known to be a candidate for the CR (Critical Endangered to Endangered Extinction) category, is very important due to its habitat size of around 100 km² and the minimal exact boundaries of its habitat (Figure 2).

These individuals, which belong to the Brassicaceae and are usually annual or perennial in small and herbaceous forms, spread over a wide area worldwide, excluding the Antarctic continent (Koch and Kiefer, 2006). Today, Brassicaceae has approximately 320 genera and 3,700 species belonging to these genera (Anonymous, 2016).

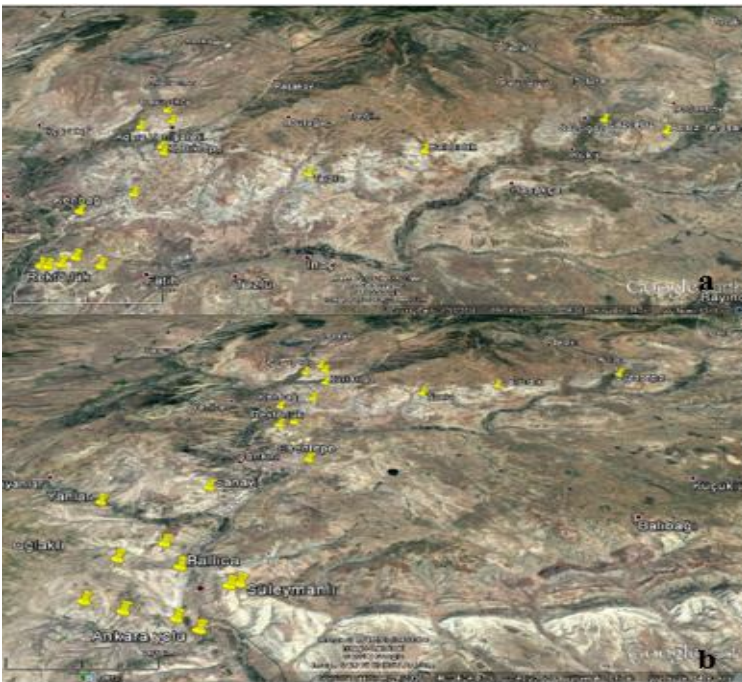


Figure 2. Satellite image of the points where the species was detected (Anonymous, 2016)

While classified as vascular plants as a group, the Brassicaceae taxonomy classification, which is also a member of the flowering plants family, is as follows (Mabberley, 1987);

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Brassicales

Family: Brassicaceae

While there are about 210 valid species belonging to the genus *Alyssum* in this family (Figure 3), 110 of them are located in Turkey. About 50 of these plants are endemic and are generally seen in mountainous and hilly areas. Although it is mostly seen in the Mediterranean environment throughout the world, it is known that a few species are also found in North Africa, Central Asia, Siberia and North America (Dudley, 1964; Dudley, 1965).

It is very important both to protect the habitats of endemic species with limited natural habitats and to ensure species continuity. For this purpose, it is very important to carry out studies on culturing these plants.

Alyssum nezaketiae, which is locally endemic in the province of Çankırı, is unique to gypsum areas (Aytaç and Duman, 2000). Individuals of this species have been found even in very extreme conditions where erosion is severe, organic matter in the soil is low, and the bedrock dissolves on the surface.



Figure 3. *Alyssum nezaketiae* a) flowers b) habitat

Factors such as overgrazing, field clearing, road construction works, and urbanization due to the rapidly increasing population, which spread around a residential area and therefore are likely to be exposed to human influence, endanger the extinction of *A. nezaketiae*. In addition to all these, although it spreads in extreme areas threatened by erosion, it is possible to say that such rapid erosion limits the areas where the plant can live day by day. Considering all these, it is very important to propagate this species vegetatively in a short time using in vitro techniques.

In this study, shoot tips and nodes of the *A. nezaketiae* plant were used as explant sources, and they were propagated rapidly and clonally by tissue culture micropropagation technique. *A. nezaketiae* explants were cultured in vitro, and shoot regeneration was performed by rooting with 36 different combinations of gelrite as a solidifier, 2 different basal media, and plant growth regulators (PGR). The plants showing root and shoot development were taken to the acclimatization stage, but the plants could not adapt to the external conditions. This study was the first to study the reproduction of *A. nezaketiae* in vitro conditions.

In vitro Propagation Studies of Different Species in Brassicaceae

Ceylan (2015), examined the effects of NAA and BAP at different doses in order to perform micropropagation in *Crambe orientalis* L. in his study. *C. orientalis* seeds were cultured in PGR-free Murashige and Skoog (MS) medium. Node and shoot were used as explant sources in seedlings that developed after 14 days. The explants were cultured in an MS medium containing 12 different combinations of BAP and NAA. Thus, the percentage of callus and shoot formation rates, the number of explants/mean shoots, and their effects on shoot length were determined. Callus formation was observed at all BDM concentrations by culturing shoot tips. The maximum average shoot length was reached in MS-BAP (2 mg/L). Rooted the regenerated shoots in MS basal medium containing different concentrations of NAA, but it was observed that the rooting of the regenerated shoots was difficult in general.

Özdemir and Yıldırım (2016), in their study, aimed to micropropagation *Crambe maritima* L., which they used as an explant source from hypocotyl parts of plants grown in vitro. Explants were cultured in MS basic nutrient medium formed with 9 different combinations of BAP (0.50-1-2 mg/L) and NAA (0-0.25-0.5 mg/L). The average percentage of callus formation was in MS medium containing the most BAP (0.5 mg/L) + NAA (0.5 mg/L), and the average percentage of regenerated shoots was MS-BAP (2 mg/L) + NAA (0.25 mg/L) average shoot number produced by each explant in maximum MS-BAP (1 mg/L) + NAA (0.5 mg/L) basal medium, average shoot length maximum MS-BAP (2 mg/L) in the basal medium they have recorded. Shoots with good growth were rooted in a medium containing MS-NAA (1 mg/L).

Memon and Memon (2021), in their study, created an effective plant regeneration protocol for *Brassica nigra*, which is known to be a Cu accumulator. Nodes taken from 10-day-old mature plants were used

as explants. The highest percentage of callus formation (100%) was observed in MS media containing 0.1 mg/L BAP + 0.5 mg/L NAA, 0.6 mg/L + BAP 0.2 mg/L NAA. The highest shoot formation percentage (100%) was obtained in 0.6 mg/L BAP + 0.05 mg/L IBA, 0.2 mg/L IBA + 0.2 mg/L NAA media. The highest number of shoots per explant (3.25) was obtained in MS medium supplemented with 0.6 mg/L BAP + 0.05 mg/L IBA. Plants growing in MS were transferred to soil and grown in the plant growth chamber for one month.

Yıldızhan (2007), aimed to in vitro micropropagation of the *Brassica nigra*. In the study, a low-cost and efficient regeneration protocol was developed with intensive production of *B. nigra* for phytoremediation purposes. Sterilized *B. nigra* seeds were cultured in a PGR-free MS medium for 30 days; transferred the plants in this period to MS media containing different doses of BAP and NAA. High callus formation was detected in hypocotyl, apex, and stem explants, while it detected both direct and indirect shoot regenerations in apex explants. While it provided direct shoot formation in all combinations of NAA and BAP, indirect shoot formation from the apex was detected in media where NAA and BAP growth regulators were in appropriate proportions. Indirect and direct shoots were transferred to 5 different MS media for root regeneration and root development was observed within 7-20 days. The plants that completed the regeneration were transferred to the soil.

Demir (2020), aimed to reveal an effective in vitro clonal production protocol by providing regeneration of endemic *R. carnosula* by somatic embryogenesis or organogenesis. Hypocotyl and cotyledons of plantlets obtained from embryos were used as explant sources. Explants were planted in 73 different regeneration media with various concentrations of auxin group IAA, NAA, and 2,4-D, cytokinin group BAP, TDZ, and Zeatin. Thus, the interaction of explant types and regeneration media alone and with each other; investigated the effects of explant response, amount of callus, type of callus (embryogenic or

organogenic), anthocyanin formations, direct or indirect somatic embryogenesis and organogenesis including shoot-root. At the end of the study, somatic embryo, shoot, and root structures were observed in control environments.

Various Studies on *Alyssum* sp.

Oturgan (2007), investigated 11 species of plants belonging to 5 genera in terms of their biological activities. In his study on plants, it was stated that two of them were potentially cytotoxic, one was DPPH, and all of them had reducing properties and were antioxidants according to their water purification status, and the microbiological activities of eight plants were significant. Hamed et al., (2015) and Erdogan et al., (2015) stated in their studies that some *Alyssum* species are used as natural stabilizers in the food industry, as well as have some important biological activities in some species. Bayramoglu et al., (2012) found that the chemically modified biomass of *Alyssum discolor* T.R. Dudley & Hub.-Mor. can be used as a biological solvent for the separation of reactive yellow textile dye that can be found in aqueous media. Akdemir et al., (2019) investigated the mass reduction coefficients and molar extinction coefficients in the root, aerial and flower parts of *A. pateri* subsp. *prostratum* plant belonging to the genus *Alyssum*. They determined that the above-ground and flower structures had a higher radiation absorption ability than the root structure. Babaoğlu (2008), emphasized that *A. corsicum* has a very important role in the bioremediation and recovery of heavy metals, especially nickel, as a candidate plant for use in herbal treatment studies. It was observed that the plant accumulated more nickel metal in its stem than in its roots, and with the increase in nickel metal concentration, changes occurred in leaf hair density and structure. In addition, Adıgüzel and Reeves (2012), reported that *Alyssum pterocarpum* T.R. Dudley, *Alyssum cypricum* Nyár, *Alyssum discolor* T.R. Dudley & Hub-Mor. *Alyssum huber-morathii* T.R. Dudley, *Alyssum masmenaeum* Boiss.,

Alyssum caricum T.R. Dudley, *Alyssum davisianum* T.R. Dudley, *Alyssum pinifolium* (Nyár.) T.R. Dudley, *Alyssum peltarioides* Boiss. sub sp. *peltarioides*, *Alyssum callichroum* Boiss. & Balansa, *Alyssum crenulatum* Boiss., *Alyssum samariferum* Boiss. & Hausskn species are the prominent Ni-accumulator *Alyssum* species. In the study of Tozyilmaz (2019), the antibacterial and antifungal activities of the extracts obtained from *Alyssum discolor* Dudley & Hub-Mor., which grows endemic in Turkey, against eighteen bacterial and two fungal strains by disc diffusion method. *Alyssum discolor* Dudley & Hub-Mor. while it showed the lowest effect in the antimicrobial study, it could not show any effect against fungi. It also showed the lowest activity in the anti-biofilm study. Tajbakhsh et al., (2012) investigated the phenolic content, antioxidant properties and activities in gram positive and negative bacteria in methanol extracts of *Alyssum*. Kürşat et al., (2008) reported that *Alyssum* species, especially the perennial ones, can be used as a pioneer plant in erosion studies because they are drought resistant and content with soil requirements. Ertuğ (2014), reports that the leaves of the *Alyssum constellatum* Boiss. species called Goramaz are consumed as food in Turkey. Amiri et al., (2012; 2013) and Parsaei et al., (2016), *Alyssum minus* Rothm. seeds as a relaxant and laxative; *Alyssum campestre* L. leaves are used as a relaxant and cough suppressant; *Alyssum alyssoides* L. stated that it is used as an antipyretic, relaxing, anti-pharyngitis, cough suppressant and voice suppression.

In vitro Propagation Studies in *Alyssum* sp.

Winterhalter et al., (2008) studied with *Alyssum murale* and revealed that the ideal medium for shoot formation is MS medium containing 0.46 μ M Kinetin. Root formation was observed on the explants after approximately one month. Roots were isolated and propagated in PGR-free medium. While they observed rapid growth in some explants, they observed spontaneous shoot formation.

In the study of Bayat (2017), micropropagation of *Alyssum pinifolium* (Nyar.) TR. Dudley species, which is distributed in Çanakkale, was carried out. As an explant source, healthy nodes, internodes, and leaves of in vitro-grown plants were used and cultured in an MS medium. BAP and IAA were added to the culture medium in different concentrations and combinations. The best results in callus formation were achieved in the medium of MS-BAP (2 mg/L) and IAA (0.1 mg/L). Indirect organogenesis was obtained as a result of subcultures.

Material Supply of *Alyssum nezaketiae* in vitro Propagation

Alyssum nezaketiae was first collected by Prof.Dr.Hayri Duman in 1995 from Kenbağ Mevkii on the Çankırı-Kastamonu road. In later studies, the plant was also found on the Çankırı-Ankara road, in Balıca (Vural and Şahin, 2013). In Turkey, only around the provincial center of Çankırı; It is a small and pleasant-looking plant, about 10 cm tall, with yellow flowers, which is known to be a candidate for the Critically Endangered (CR) category, growing on gypsum soils on sloping hills near Ankara road, Kastamonu road, and Yapraklı road.

A.nezaketiae, which started to appear in the field in spring (April-May) with the beginning of the vegetation period, usually begins to bloom in mid-May. Plants collected from the area with coordinates 40°28'54"N-33°38'48"E around Çankırı Karatekin University Campus were brought to the laboratory in a way that their natural habitat and reproduction would not be adversely affected (Figure 4).



Figure 4. a) Full flowering view of *A.nezaketiae* b) *A.nezaketiae* collected from the field and brought to the laboratory c) flowers, leaves and shoots of *A.nezaketiae*

Sterilization of Plants After the Field Study

Alyssum nezaketiae, collected from the growing area, was brought to the laboratory, and the required surface sterilization protocol of the plant was applied as follows before planting in vitro conditions (Sezgin and Dumanoğlu, 2014) (Figure 5):

- First, the plant was washed under tap water for 20 min.
- It was treated in ethanol (70%) for 3 min.
- Tween 20 (8-10 mg/L) was added to a 20% solution of commercial sodium hypochlorite (NaOCl containing 15% chlorine) to increase the effectiveness and treated for 10 min.
- In order to remove NaOCl from the tissues, it was rinsed with sterile distilled water for 5 min under aseptic conditions and this process was repeated 3 times.

Basal Media and Plant Growth Regulators

The shoot tip and the nodes on the shoot of the *A.nezaketiae* plant, on which surface sterilization was performed, were used as explant sources. For this purpose, different basic nutrient media and combinations of auxin and cytokinin group plant growth regulators (PGR) with different doses were used in the initial stage (Şan et al., 2007; Yıldızhan, 2007; Sezgin and Dumanoglu, 2014). At this stage, the initial effects of combinations of media and PGR were tested (Table 1).

- 1) Initial stage media
 - a. MS (Murashige and Skoog, 1962)
 - b. Woody Plant Medium (WPM) (Lloyd and McCown, 1981)
- 2) Plant growth regulators and doses
 - a. 6-benzylaminopurine (BAP)-(0 mg/L, 0.5 mg/L, 1 mg/L)
 - b. Kinetin (KIN)-(0 mg/L, 0.5 mg/L, 1 mg/L)
 - c. Indole-3-acetic acid (IBA)-(0 mg/L, 0.25 mg/L, 0.5 mg/L)
 - d. α -naphthaleneacetic acid (NAA)-(0 mg/L, 0.25 mg/L, 0.5 mg/L)

Sucrose (30 g/L) as a carbon source and gelrite (2.1 g/L) as a solidifier were added to the basal media prepared in 36 different combinations with 2 basal media, 6 cytokinin, and 6 auxin doses for *A.nezaketiae*. The pH of the media was adjusted to 5.7 (Babaoğlu et al., 2001).

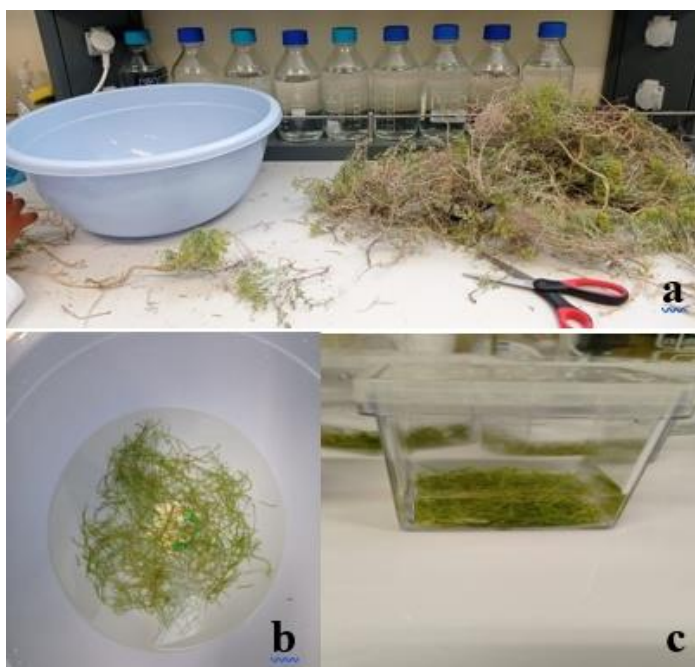


Figure 5. Sterilization of *A. nezaketiae* a) separation of shoots of the plant b) EtOH application c) explants ready for planting

All combinations were prepared in ten repetitions. In order to prevent possible contamination after planting, Plant Preservative Mixture (PPM-Duchefa[®]) (1 ml/L) was added to the media. The media containing appropriate amounts of PGR and solidifier were divided into the erlenmeyers into equal amounts (120 ml) and autoclaved in order to ensure sterilization. In the autoclave, the media was sterilized at 121°C and 1.2 atm pressure for 20 min (Sezgin and Dumanoglu, 2014). After sterilization, the media was divided into sterile tubes of 130x10 mm size, approximately 10 mL each, before the gelrite solidified in a laminar airflow cabinet.

Table1. PGR combinations

1	BAP (0mg/L) + IBA (0mg/L)	19	KIN (0mg/L) + IBA (0mg/L)
2	BAP (0mg/L) + IBA (0.25mg/L)	20	KIN (0mg/L) + IBA (0.25mg/L)
3	BAP (0mg/L) + IBA (0.5mg/L)	21	KIN (0mg/L) + IBA (0.5mg/L)
4	BAP (0.5mg/L) + IBA (0mg/L)	22	KIN (0.5mg/L) + IBA (0mg/L)
5	BAP (0.5mg/L) + IBA (0.25mg/L)	23	KIN (0.5mg/L) + IBA (0.25mg/L)
6	BAP (0.5mg/L) + IBA (0.5mg/L)	24	KIN (0.5mg/L) + IBA (0.5mg/L)
7	BAP (1mg/L) + IBA (0mg/L)	25	KIN (1mg/L) + IBA (0mg/L)
8	BAP (1mg/L) + IBA (0.25mg/L)	26	KIN (1mg/L) + IBA (0.25mg/L)
9	BAP (1mg/L) + IBA (0.5mg/L)	27	KIN (1mg/L) + IBA (0.5mg/L)
10	BAP (0mg/L) + NAA (0mg/L)	28	KIN (0mg/L) + NAA (0mg/L)
11	BAP (0mg/L) + NAA (0.25mg/L)	29	KIN (0mg/L) + NAA (0.25mg/L)
12	BAP (0mg/L) + NAA (0.5mg/L)	30	KIN (0mg/L) + NAA (0.5mg/L)
13	BAP (0.5mg/L) + NAA (0mg/L)	31	KIN (0.5mg/L) + NAA (0mg/L)
14	BAP (0.5mg/L) + NAA (0.25mg/L)	32	KIN (0.5mg/L) + NAA (0.25mg/L)
15	BAP (0.5mg/L) + NAA (0.5mg/L)	33	KIN (0.5mg/L) + NAA (0.5mg/L)
16	BAP (1mg/L) + NAA (0mg/L)	34	KIN (1mg/L) + NAA (0mg/L)
17	BAP (1mg/L) + NAA (0.25mg/L)	35	KIN (1mg/L) + NAA (0.25mg/L)
18	BAP (1mg/L) + NAA (0.5mg/L)	36	KIN (1mg/L) + NAA (0.5mg/L)

Planting and Incubation of the Explants

After the sterilization stage, the node and shoot tips of *A.nezaketiae* plant were used as explant sources and these explants were planted in tubes. One explant was planted in each tube. From this stage onwards, all cultures were taken into the climate cabinet at 25±1°C, 16/8 hours of light/dark (35 µmol·m⁻²·s⁻¹) conditions, and their incubation was ensured by being checked regularly for one month (Figure 6).

Subculture

The explants of *A.nezaketiae*, which were cleaned from the leaves, sterilized under appropriate conditions, and then planted, were incubated in the climate room and controlled every other day. Explants, media, and PGR combinations were subcultured, adhering to the initial combinations, and their growth and development were followed.

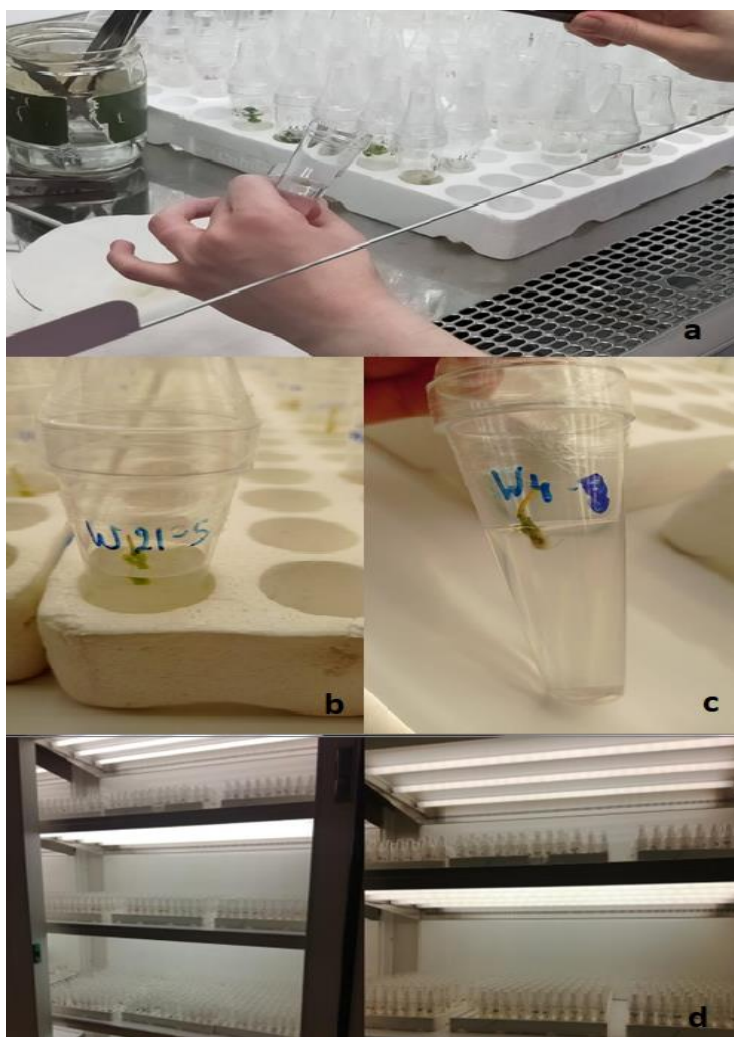


Figure 6. Planting of *A. nezaketiae* a) planting explants into tubes in a laminar air flow cabinet b-c) explants after planting d) incubated explants (Growth chamber)

The explants were subcultured 4 times at 28-day intervals in order to enable them to develop shoots and roots after callus formation. The subculture studies of the explants were continued in the ongoing process, with the medium MS and solidifier. Some explants showed shoot and root development. At this stage, data on root number and length and shoot number and length were recorded. Explants showing

direct shoot growth were transferred to rooting media for root development (Figure 7).

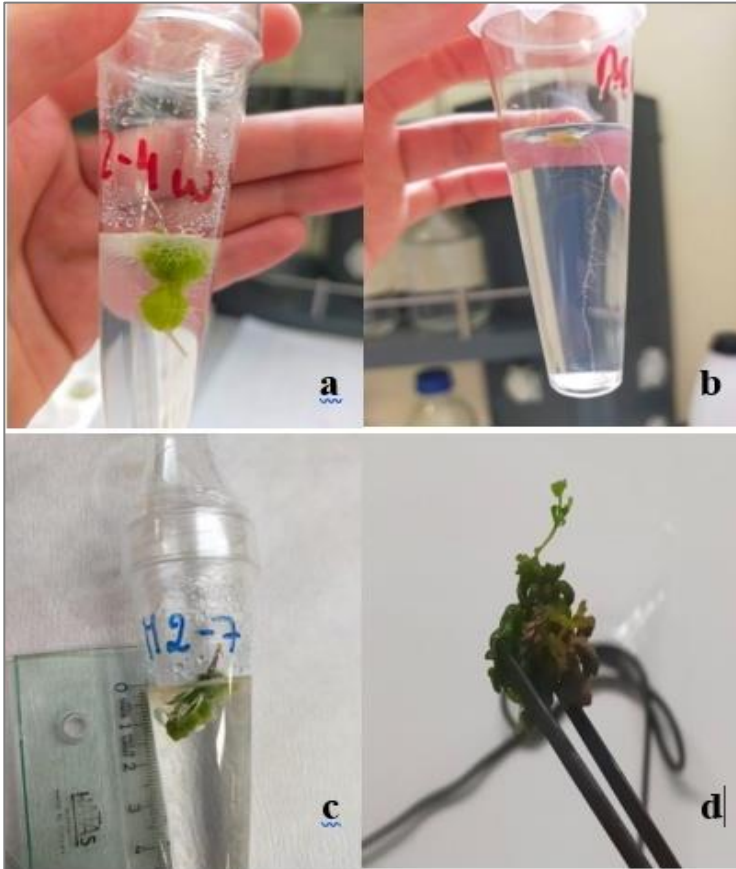


Figure 7. Growth of explants a) root growth in WPM-BAP (0 mg/L) + IBA (0.25 mg/L) b) root formation in MS-BAP (0 mg/L) + NAA (0.5 mg/L) c) callus formation in MS-BAP (0 mg/L) + IBA (0.25 mg/L) d) shoot formation in WPM-KIN (0.5 mg/L) + IBA (0 mg/L)

Rooting

Explants, which also showed shoot development during the rooting stage, were added to the MS and WPM media with different doses of KIN and IBA as a solidifier and planted. At this stage, the explants were planted in 125x65x80 mm sterile tubes containing 120 mL of basal medium (Figure 7). Rooting stage was carried out in

twenty-eight days periods. At this stage, the plants planted for the rooting stage were incubated at in the climate cabinet (Mandal et al., 2013). In explants showing root formation, the number of roots and the lengths of the roots (cm) and the number of shoots and shoot lengths were recorded as data (Figure 8).

Acclimatization

The shoots and roots of *A.nezaketiae* explants that started to develop were taken to the acclimatization stage. The plants, which were carefully taken from their media, were thoroughly washed with distilled water in order to clean the media residue on them. Growing plants were planted in a mixture of flower soil, sand and perlite (3:1:1 v/v). This mixture was first sterilized in an autoclave at 121°C and 1.2 atm pressures for 90 minutes to prevent possible contamination from soil. After the plants were planted in the small plastic pots, they were watered with approximately 50 mL of distilled water every other day.



Figure 8. Regenerated *A. nezaketiae* a) Root and shoot formation in MS-KIN (1 mg/L) + IBA (1 mg/L) medium b) Acclimatization of regenerated plants

Statistical Analysis

Analysis of variance was performed using SPSS program and Game-Howell non-parametric Post-Hoc test as the mean of repetitions according to the data of the study ($P < 0.05$, 0.01, 0.001). In order to determine the statistically significant differences, the Duncan Multiple Comparison Test was applied according to the 5% error limit and the differences were expressed with the help of letters.

The shoot tips and nodes of *A. nezaketiae* were used as explants, and the regeneration of these explants was observed depending on the media they were in and the PGR combinations they contained. For this purpose, numerical data were recorded by observing the callus, shoot, and root structures that developed after each subculture.

Shoot Formation in Subcultures

In vitro propagation of *A.nezaketaiae*, the number of shoots formed by the regeneration of callus in media was observed and the data were evaluated statistically. In this sense, the interaction between 'basal medium x PGR combinations' is statistically significant ($P \leq 0.05$). In the explants, the values of the shoot formation formed from the callus after the culture start stages are given in Table 2. In the interaction between callus shoot formation "basal medium x PGR combinations" in *A.nezaketaiae*, MS-KIN (1 mg/L) + IBA (0.25 mg/L) medium together with WPM-KIN (0.5 mg/L), the best values for shoot formation were obtained in L) + NAA (0.5 mg/L) medium. In addition, regeneration of shoots in MS-KIN (1 mg/L) + IBA (0.5 mg/L) medium and WPM-BAP (0.5 mg/L) + IBA (0.25 mg/L) medium was found to be statistically significant.

Table 2 Interaction of "basal medium x PGR combination" of shoot regeneration from callus in *A.nezaketaiae* ($P \leq 0.05$)

Basal Medium			Basal Medium		
PGR	MS	WPM	PGR	MS	WPM
1	0±0c	0.3±0.6cd	19	0.8±1.8 cd	0±0d
2	1.4±3.4 cd	1±2.3cde	20	1.3±2.2 cd	2.7±3.7bcde
3	2.8±4.3 abcd	5.3±8.4abcd	21	2.7±3.6 abcd	5.1±13.1abcde
4	0±0c	0±0d	22	0.7±1.6 cd	2.6±4.5bcde
5	2.9±3.6 abcd	6.3±8.1ab	23	1.3±1.6 cd	4.6±6.6abcde
6	1.9±3.10abcd	2±3.6bcde	24	3.4±4.9 abcd	5.5±7.6abc
7	1.6±4.3bcd	0±0d	25	0.1±0.3 c	0.1±0.3cd
8	1.7±3.6 abcd	1.3±3.7bcde	26	4.9±3.7 a*	1.3±2.7bcde
9	1.3±3.7cd	1.5±1.7bcde	27	4.7±4.7 ab	2±5bcde
10	1.1±2.5 cd	0.8±2.2cde	28	1±2.5 cd	0±0d
11	2.6±4.2 abcd	2.4±7.5bcde	29	1.2±3.7 cd	0.1±0.3cd
12	0.5±1.2 c	0.3±0.9cd	30	1.6±2.2 bcd	2.9±6.2bcde
13	1.3±2.0 cd	1.8±5.6bcde	31	0.2±0.4 c	0±0d
14	3.9±5.2 abc	1.1±2.6cde	32	1.8±3.5 abcd	1.5±4.4bcde
15	2.1±2.4 abcd	1.5±1.9bcde	33	2.7±4.1 abcd	8±8.5a*
16	2.1±3.9 abcd	0.1±0.3cd	34	1.9±2.0 abcd	1.6±2.9bcde
17	0.2±0.4 c	0.2±0.4cd	35	0.8±1.2 cd	2.7±4bcde
18	0.1±0.3 c	0.3±0.6cd	36	0±0 c	1.8±1.9bcde

* Different letters indicate the difference between applications.

Number of Shoots in Subcultures

In vitro propagation of *A.nezaketaiae*, the data on the number of shoots formed in the media of the explants were taken and statistically evaluated. In this sense, the interaction of 'basal medium x PGR combinations' turned out to be statistically significant ($P\leq0.05$). The statistical averages of the data on the number of shoots occurring in the explants are given below (Table 3). The interaction of “basal medium x PGR combinations” was found to be significant in the number of shoots in explants. In addition to MS-KIN (1 mg/L) + IBA (0.25 mg/L) medium, WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium, in terms of shoot number, the best data has been obtained. In addition, shoot numbers in MS-KIN (1 mg/L) + IBA (0.25 mg/L) medium and WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) medium, were statistically significant.

Table 3. Interaction of “basal medium x PGR combinations” of shoot numbers in *A.nezaketaiae* explants ($P\leq0.05$)

Basal Medium			Basal Medium		
PGR	MS	WPM	PGR	MS	WPM
1	0±0c	0±0c	19	0±0c	0±0c
2	0.2±0.6c	2.2±4.6abc	20	0.4±1.2c	4.4±7.6abc
3	0.7±1.4bc	2.6±3.6abc	21	1.2±1.9abc	2.7±5.1abc
4	0.1±0.3c	0±0c	22	0.8±2.5bc	0.7±1.6c
5	0.5±1.2c	1.1±2bc	23	0.2±0.6c	0.2±0.6c
6	0.7±1.4bc	3.3±5.9abc	24	1.2±2.5abc	5.8±5.8a*
7	0.3±0.9c	0±0c	25	0±0c	0.1±0.3c
8	0.4±1.2c	1.4±3.7abc	26	2.4±3.5ab	1.8±5.6abc
9	0.3±0.9c	4.2±6.9abc	27	2.6±3.8a*	2.5±5abc
10	0±0c	1.2±3.1bc	28	0±0c	0±0c
11	0.7±1.6bc	3.8±8.6abc	29	0.4±1.2c	0±0c
12	0±0c	1.4±4.4abc	30	1.1±2.3abc	2.1±4.3abc
13	0.8±2.5bc	1.5±3.8abc	31	0±0c	0±0c
14	0.4±0.6c	5.6±7.3ab	32	1.4±2.6abc	1.3±4.1abc
15	1.6±2.7abc	3.5±5.2abc	33	0.7±2.2bc	1.8±2.1abc
16	0.9±1.9abc	2.8±5.1abc	34	1.4±1.7abc	3±5.5abc
17	0±0c	0±0c	35	0±0c	0.6±1c
18	0±0c	0±0c	36	0±0c	0.1±0.3c

* Different letters indicate the difference between applications.

Length of Shoot in Subcultures

The data on the shoot lengths occurred in the explants during the subculture stage of the in vitro propagation of *A.nezaketiae* were taken and after statistical evaluation, the interaction of "basal medium x PGR combinations" emerged as statistically significant ($P \leq 0.05$) (Table 4).

Table 4. Interaction of "basal medium x PGR combinations" of shoot length in *A.nezaketiae* explants ($P \leq 0.05$)

Basal Medium			Basal Medium		
PGR	MS	WPM	PGR	MS	WPM
1	0±0b	0±0e	19	0±0b	0±0e
2	0.14±0.4b	0.29±0.6abcde	20	0.2±0.6ab	0.53±0.8abcde
3	0.16±0.3b	0.6±0.5abcde	21	0.25±0.5ab	0.43±0.7abcde
4	0.04±0.1b	0±0e	22	0.08±0.2b	0.13±0.2de
5	0.1±0.2b	0.24±0.4bcde	23	0.05±0.1b	0.04±0.1e
6	0.47±1ab	0.41±0.6abcde	24	0.27±0.4ab	0.86±0.7a*
7	0.03±0b	0±0e	25	0±0b	0.1±0.3e
8	0.45±1.4ab	0.22±0.4bcde	26	0.24±0.3ab	0.15±0.4cde
9	0.06±0.1b	0.6±0.9abcde	27	0.34±0.4	0.46±0.8abcde
10	0±0b	0.16±0.3cde	28	0±0b	0±0e
11	0.26±0.6ab	0.32±0.6abcde	29	0.06±0.1b	0±0e
12	0±0b	0.22±0.6bcde	30	0.16±0.3b	0.28±0.4abcde
13	0.16±0.5b	0.19±0.4bcde	31	0±0b	0±0e
14	0.22±0.3ab	0.81±1ab	32	0.15±0.2b	0.12±0.3e
15	0.37±0.7ab	0.77±0.9abc	33	0.1±0.3b	0.64±0.76abcde
16	0.2±0.4ab	0.36±0.6abcde	34	0.65±0.7a*	0.75±1.2abcd
17	0±0b	0±0e	35	0±0b	0.17±0.3cde
18	0±0b	0±0e	36	0±0b	0.04±0.1e

* Different letters indicate the difference between applications.

Shoot length data in explants "basal medium x PGR combinations" interaction, best shoot on MS-KIN (1 mg/L) versus WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium length is reached. In addition, shoot length in media with a combination of MS-BAP (0.5

mg/L) + NAA (0.25 mg/L) and BAP (0.5 mg/L) + NAA (0.5 mg/L) BDM was statistically significant.

Root Number in Subcultures

In vitro propagation of *A.nezaketiae*, the numbers of roots formed by explants in subcultures were taken as data and in the statistical evaluation, the interaction of "basal medium x PGR combinations" did not reveal a difference that could be considered statistically significant ($P\leq0.05$) (Table 5).

Table 5.Interaction of “basal medium x PGR combinations” of root number in *A.nezaketiae* explants ($P\leq0.05$)

Basal Medium			Basal Medium		
PGR	MS	WPM	PGR	MS	WPM
1	0±0a	0.5±1.5a	19	0.1±0.3a	0±0a
2	1.4±3.5a	6.5±13.6a	20	0.2±0.6a	7.9±15.3a
3	2.7±5a	4±10.2a	21	0.8±1.1a	4.4±7.5a
4	0.1±0.3a	0±0a	22	0.7±2.2a	0.7±1.4a
5	0±0a	0±0a	23	0.1±0.3a	0.1±0.3a
6	1.3±2.6a	4.9±12.3	24	2.2±6.2a	3.3±5.7a
7	0.3±0.9a	0±0a	25	0.1±0.3a	0.5±1.5a
8	2±6.3a	4±9.2a	26	1.1±2.8a	6.6±20.8a
9	2±6.3	4.4±8.4a	27	0.8±1.6a	5±15.12a
10	0±0a	1.2±3.7a	28	0±0a	0±0a
11	3.2±7.5a	7.4±15.9a	29	0±0a	0±0a
12	0.2±0.6a	0.6±1.8a	30	0.1±+0.3a	5.9±13.2a
13	0.9±2.8a	2.9±9.1a	31	0.1±0.3a	0±0a
14	0.9±2.2a	6.5±10.4a	32	0.4±1.2a	0.4±1.2a
15	2.2±4.5a	6.8±14.6a	33	0.4±0.8a	0.7±1.4a
16	0±0a	2.5±7.9a	34	2.1±3.5a	3.3±8.5a
17	0.2±0.6a	0±0a	35	0±0a	0.4±0.6a
18	0±0a	0±0a	36	0±0a	0.6±1.8a

* Different letters indicate the difference between applications.

There was no statistically significant difference in the interaction of root numbers in explants between “basal medium x PGR combinations”. The obtained data revealed that all combinations and media had the same effect on root numbers.

Root Length in Subcultures

In vitro propagation of *A.nezaketaiae*, the length data of the roots formed in the subcultures were taken and statistically evaluated, and the interaction between the "basal medium x PGR combination" was statistically significant ($P \leq 0.05$) (Table 6).

Table 6. Interaction of "basal medium x PGR combinations" of root length in *A.nezaketaiae* explants ($P \leq 0.05$)

Basal Medium			Basal Medium		
PGR	MS	WPM	PGR	MS	WPM
1	0±0a	0.1±0.3bcd	19	0.5±1.5a	0±0d
2	0.3±0.6a	0.65±1.1abcd	20	0.15±0.47a	0.8±1.1abcd
3	0.7±1.1a	0.37±0.6abcd	21	0.49±0.9a	0.58±0.9abcd
4	0.05±0.1a	0±0d	22	0.17±0.5a	0.3±0.6abcd
5	0±0a	0±0d	23	0.002±0a	0.08±0.2bcd
6	0.27±0.4a	0.28±0.7abcd	24	0.19±0.4a	0.96±1.3ab
7	0.15±0.4a	0±0d	25	0.03±0a	0.1±0.3bcd
8	0.2±0.6a	0.62±1.3abcd	26	0.31±0.6a	0.3±0.9abcd
9	0.35±1.1a	0.62±1.1abcd	27	0.2±0.4a	0.45±1.1abcd
10	0±0a	0.04±0.1cd	28	0±0a	0±0d
11	0.39±0.8a	0.47±0.9abcd	29	0±0a	0±0d
12	0.5±1.5a	0.15±0.4bcd	30	0.02±0a	0.4±0.8abcd
13	0.15±0.4	0.24±0.7abcd	31	0.03±0	0±0d
14	0.2±0.4a	1.14±1.2a*	32	0.1±0.3a	0.28±0.8abcd
15	0.385±0.6a	0.45±0.9abcd	33	0.06±0.1a	0.5±1.2abcd
16	0±0a	0.23±0.5bcd	34	0.57±0.8a	0.95±2abc
17	0.2±0.6a	0±0d	35	0±0a	0.29±0.5abcd
18	0±0a	0±0d	36	0±0a	0.04±0.1cd

* Different letters indicate the difference between applications.

In the "basal medium x PGR combinations" interaction of root length data in explants, the best data were obtained in terms of root length in WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) medium. In addition, WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium is also statistically significant.

Rooting Stage

In vitro propagation of *A.nezaketiae*, the explants were taken into rooting media following the subculture step. At this stage, the effects of auxin and cytokinin group PGR and their different combinations, which gave the best results in the subculture stage, were examined and the effects of media on plant growth were examined (Table 7 and 8). The data on the number and length of shoots, root numbers and lengths formed by the explants were recorded and statistically evaluated.

Table 7. Application table of PGR doses used in rooting stage

PGR DOSES	
1	KIN (0 mg/L) + IBA (0 mg/L)
2	KIN (0.25 mg/L) + IBA (0.25 mg/L)
3	KIN (0.5mg/L) + IBA (0.5mg/L)
4	KIN (1mg/L) + IBA (1 mg/L)

Table 8. The effect of “PGR doses” on root number and length and shoot number and length during rooting stage in *A.nezaketiae* explants ($P\leq0.05$)

PGR DOSES		Shoot number	Shoot length	Root Number	Root length
1	KIN (0 mg/L) + IBA (0 mg/L)	0.2±0.5c	0.05±0.15b	0.05±1.3b	0.05±0.15c
2	KIN (0.25 mg/L) + IBA (0.25 mg/L)	0.9±1.2ab	0.82±1.16a*	13.75±25.17a b	0.48±0.71ab
3	KIN (0.5mg/L) + IBA (0.5mg/L)	1.4±1.18a*	0.87±0.89a*	21.75±35.62a *	0.65±0.87a*
4	KIN (1mg/L) + IBA (1 mg/L)	0.70±0.9bc	0.66±0.92a*	4.25±13.69b	0.15±0.47bc

When the effect of PGR doses on root numbers-lengths and shoot numbers-lengths in rooting stage of *A.nezaketiae* explants was examined, the best root number (21.75) and the maximum root length (0.65) and the best shoot number (1.4) were examined. It was reached in the presence of KIN (0.5 mg/L) + IBA (0.5 mg/L) BDM dose (Figure 9). There was no statistically significant difference between the

PGR doses regarding the lengths of the shoots. The effect of media on shoot numbers, lengths, root numbers and lengths during the rooting phase is given in Table 9.

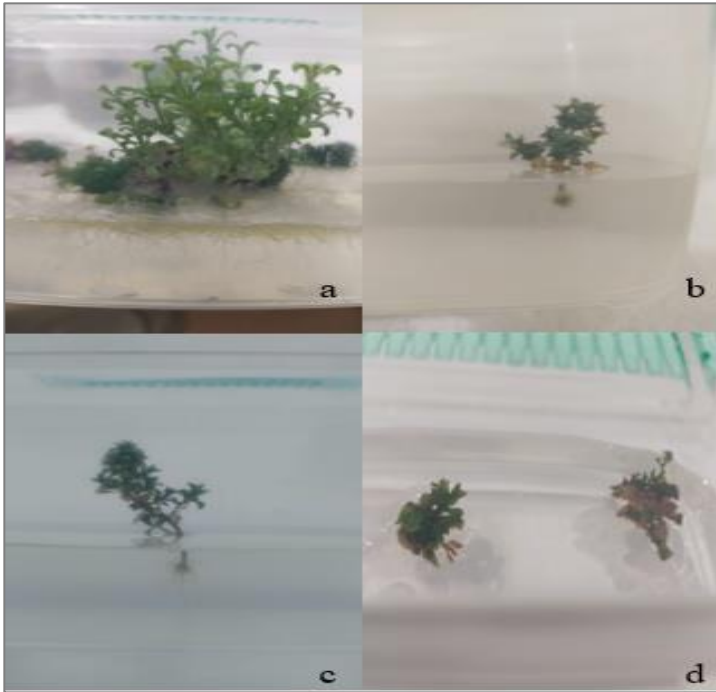


Figure 9. Root and shoot growth in rooting medium a) MS-KIN (0.5 mg/L) + IBA (0.5 mg/L) b) MS-KIN (0.25 mg/L) + IBA (0.25 mg/L) c) MS-KIN (1 mg/L) + IBA (1 mg/L) d) WPM-KIN (0.5 mg/L) + Shoot and root development in IBA (0.5 mg/L)

Table 9. The effect of “media” on root number and length, shoot number and length in rooting stage of *A. nezaketiae* explants ($P \leq 0.05$)

	Media	N	Mean
Shoot number	MS	40	1.125±1.20
	WPM	40	0.475±0.81
Shoot length	MS	40	0.62±0.88
	WPM	40	0.585±0.95
Root number	MS	40	16.52±31.38
	WPM	40	3.62±9.47
Root length	MS	40	0.43±0.69
	WPM	40	0.2425±0.6

When the effects of “media” on root number and length and shoot number and length were examined in the rooting stage of *A. nezaketiae* explants, all 4 dependent variables (root number 16.52; root length 0.43; shoot number 1.125; shoot length 0.62) for MS medium was found to be statistically significant.

Acclimatization Stage

Eight specimens of *A. nezaketiae* showing the best root and shoot development were selected. It was observed that the plants incubated in the climate chamber could not develop under external conditions.

Result and Discussion

The basal media containing PGR in different concentrations, in which shoot tips and nodes are used as explant sources in the *A. nezaketiae* plant belonging to the genus *Alyssum*, which is known to be a candidate for the critically endangered (CR) category and is endemic to Çankırı, micropropagation is the aim of the study.

As in many plant species, shoot tip, node, hypocotyl, and cotyledons have been used as explant sources in many studies for in vitro propagation of plants belonging to the Brassicaceae family under aseptic conditions (Yıldızhan, 2007; Ceylan, 2015; Özdemir and Yıldırım, 2016; Demir, 2020; Memon and Memon, 2021).

A. nezaketiae, which is locally endemic in Çankırı province, started to be seen in the field at the beginning of the vegetation period of 2021. It was found in full flowering from the middle of May. The flowering plants, which have completed their physiological development, were taken from the field towards the end of May for in vitro propagation and brought to the laboratory. The simple sterilization protocol applied was effective, and infection was rare during the study. After the sterilization step, MS and WPM were added to the basal media. It was ensured that the media were solidified with gelrite.

In the study, plant regeneration was achieved from the shoot tip and node explants of *A. nezaketiae*, but it was observed that the

obtained shoots and roots developed very slowly. As a result of each subculture applied after the initial stage, it was observed that some explants carried out root and shoot development, but the development continued in a limited way. In addition, it is thought that infections that are thought to develop during the planting of explants make the process difficult.

'Basal medium x PGR combinations' of shoot regeneration consisting of callus obtained at the beginning of reproduction in *A. nezaketiae* explants, in interaction (Table 2), MS-KIN (1 mg/L) + IBA (0.25 mg/L) and WPM-KIN (0.5 mg/L) + NAA (0.5 mg/L) shoot formation data were the best results. In addition, shoot regeneration was statistically significant in MS-KIN (1 mg/L) + IBA (0.5 mg/L) and WPM-BAP (0.5 mg/L) + IBA (0.25 mg/L) was found to be significant. The most successful results were achieved at different BDM doses in WPM. Bayat (2017), added BAP and IAA in different amounts and combinations to the MS medium in his study. It performed shoot formation from healthy callus by subcultures, and determined that the best shoot formation was in MS medium supplemented with BAP (2.0 mg/L) and IAA (0.1 mg/L) from callus obtained from node explants. In our study, WPM was used together with MS.

At the beginning of propagation in explants, the data on the number of shoots in the "basal medium x PGR combinations" interaction (Table 3), MS-KIN (1 mg/L) + IBA (0.25 mg/L) in medium (shoot number 2.6) and WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium (shoot number 5.8), the best values for shoot number were reached. At the same time, MS-KIN (1 mg/L) + IBA (0.25 mg/L) (shoot number 2.4) and WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) (shoot number 5.6) data obtained from shoot numbers were evaluated as statistically significant. Ceylan (2015), examined the effects of only BAP and NAA concentrations in his study with *Crambe orientalis* L., unlike our study. PGR-free MS was used as a source of cotyledon node and hypocotyl explant of plants obtained from seeds cultured in basal

medium. When hypocotyl explants were cultured in MS, the maximum number of explants/mean shoots was 27.34 ± 0.69 in MS-BAP (1.0 mg/L) + NAA (0.25 mg/L). When cotyledon explants were cultured in MS, the maximum number of explants/mean shoots was determined in MS-BAP (2 mg/L) + NAA (0.25 mg/L) (18.14%).

There was no statistically significant difference in the interaction of root numbers between “basal medium x PGR combinations” in *A. nezaketiae* explants. The obtained data revealed that all combinations and media had the same effect on root numbers. Although the data obtained after subculture was meticulously collected, the consistency between the data was not found statistically significant. The best data were obtained in terms of root length in WPM-BAP (0.5 mg/L) + NAA (0.25 mg/L) medium, in the interaction of the lengths of the roots at the beginning of reproduction, “basal medium x PGR combinations”. In addition, root length was found to be statistically significant in WPM-KIN (0.5 mg/L) + IBA (0.5 mg/L) medium. Ceylan (2015), *Crambe orientalis* L. var. *orientalis* rooted the regenerated shoots in MS medium containing different concentrations of NAA, but observed that the rooting of the regenerated shoots was difficult in general.

In our study, data on root number and length, shoot number and length that occurred during the rooting stage after subculture applications were obtained and statistically evaluated. At this stage, the effects of KIN and IBA used in different doses and the medium were examined. The effects of media and PGR doses during the rooting stage were found to be statistically significant. In all trials, data on the best root number (root number 21.75) and maximum root length (root length 0.65), and the best shoot number (shoot number 1.4) were obtained in a medium with 1 mg/L dose of KIN and IBA while obtaining; the data obtained did not reveal a significant difference in the effects of BDM doses on shoot length. However, the effect of media on the rooting stage was found to be statistically significant. MS medium was found

to be successful in all trials (root number 16.52; root length 0.43; shoot number 1.125; shoot length 0.62).

For the acclimatization stage, the most successful plants in terms of shoot and root development were selected and transferred to external conditions. Root and shoot development occurred in plants obtained by in vitro propagation of *A. nezaketiae*, but the developments remained within certain limits. Therefore, the plants transferred to the external conditions kept at the optimum level could not develop within the framework of these conditions, so abiotic stress factors could not be applied to the plants.

The goal of "Developing technologies for the characterization of gene resources, conservation and protection of biological diversity" prepared by TUBITAK (The Scientific and Technological Research Council of Türkiye) between 2003 and 2023 and in line with the sustainability of development goals, is also an important issue in the National Science and Technology Policies Strategy Document (Anonymous, 2011). In this study, *Alyssum nezaketiae*, which is known to be a candidate for the CR (Critical Endangered to Endangered) category in terms of distribution criteria, due to its habitat size of around 100 km² and the very small habitat boundaries, although it is not yet in the endangered category according to IUCN data, in addition to its aromatic properties in terms of in vitro reproduction (Aytaç and Duman, 2000). This thesis topic, which is a comprehensive optimization study, can be used as a source for in vitro propagation of *Alyssum nezaketiae* and other endemic plants.

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