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Research Article

In vitro Micropropagation and Flowering of the Endemic Plant *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis**

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Abstract

Linaria known as “nevruz otu” has a wide natural distribution area in Anatolia. The *Linaria* plant, which has been widely used in folk medicine in Anatolia for many years, has the potential to be used as an ornamental plant. Biotechnological methods are valuable tools in the mass propagation of the plants grown in the nature. Tissue culture is widely used to propagation of medicinal plants for active compounds. Also, *in vitro* techniques serves protect to genetic resources especially endemic species. The aim of this study was to make rapid propagation of endemic plant of *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis, native to Anatolia. Plant growth regulators TDZ and BAP were added into ½ MS medium alone or in combination with GA_3 to improve shoot propagation. The effect of plant growth regulators on shoot induction, rate of shoot, shoot number per explant and shoot length were determined. Considering both the shoot length and the number of shoots of *in vitro* propagation of *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis plants, it was concluded that the best results (32.6 shoots per explant) for obtaining plantlets could be achieved by adding 0.2 mg l^{-1} TDZ into the ½ MS medium. The highest shoot length was 41.4 mm on the media containing 0.5 mg l^{-1} BA which was closely followed by control treatment (34.6 mm). Healthy root development with shoot growth was observed in all media tested. Plantlets were grown on a mixture of peat:perlite in a 3:1 ratio. Rooted plants were successfully transferred to a greenhouse. 80% of the plants survived and acclimatized in ex vitro conditions.

Key words: *Linaria genistifolia*, *in vitro* propagation, endemic plant.

Endemik *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis Bitkisinin *In vitro* Mikroçoğaltımı ve Çiçeklenmesi

Öz

Anadolu'da geniş bir yayılım alanına sahip olan *Linaria* türleri "Nevruz otu" adıyla bilinmektedir. Anadolu'da halk tıbbında uzun yıllardır yaygın olarak kullanılan *Linaria* bitkisi süs bitkisi olarak kullanılma potansiyeline sahiptir. Biyoteknolojik yöntemler doğada var olan bitkilerin kitlesel üretimine olanak sağlayan önemli bir araçtır. Doku kültürü, aktif komponentlerinden dolayı tıbbi bitkilerin çoğaltılmasında yaygın olarak kullanılmaktadır. Ayrıca *in vitro* teknikler endemik türler başta olmak üzere genetik kaynakların korunmasına hizmet etmektedir. Bu çalışmanın amacı, *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis endemik bitkisinin hızlı bir şekilde çoğaltılmasına olanak sağlamaktır. Sürgün çoğaltmayı teşvik için bitki büyüme düzenleyicisi TDZ ve BAP tek başına veya GA_3 kombinasyonu ile MS besi ortamı içerisine ilave edilmiştir. Bitki büyüme düzenleyicilerinin sürgün indüksiyonu, sürgün oranı, eksplant başına sürgün sayısı ve sürgün uzunluğu üzerine etkisi belirlenmiştir. *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis'in *in vitro* çoğaltılmasının hem sürgün uzunluğu hem de sürgün sayısı dikkate alındığında bitkicik elde etmek için en iyi sonuçların, ½ MS

ortamına 0.2 mg l^{-1} TDZ eklenmesiyle (eksplant başına 32.6 sürgün) elde edildiği sonucuna varılmıştır. En yüksek sürgün uzunluğu 41.4 mm ile 0.5 mg $^{-1}$ BA içeren besi ortamından elde edilmiştir ve bunu kontrol uygulaması (34.6 mm) yakından takip etmiştir. Test edilen tüm besi ortamlarında sürgün büyümesi ile birlikte sağlıklı kök gelişimi gözlenmiştir. Bitkicikler, 3:1 oranında torf:perlit karışımı üzerinde büyütülmüştür. Köklenen bitkiler başarıyla seraya aktarılmıştır. Bitkilerin %80'i hayatta kalmıştır ve ex vitro koşullarda aklimatize olmuştur.

Anahtar kelimeler: *Linaria genistifolia*, in vitro çoğaltım, endemik bitki.

Introduction

Members of the genus Linaria which are included in the Plantaginaceae family, are perennial herbaceous plants. The genus Linaria, which has 150 species in the world, is represented by 34 taxa, 12 of which are endemic in Turkey. *Linaria* known as "nevruz otu" has a wide natural distribution area in Anatolia. One of the endemic species, *Linaria genistifolia* (L.) Miller ssp. praealta (Boiss.) P.H. Davis is grown in the Eastern Mediterranean Region of our country (Davis, 1978).

Linaria species have been researched due to the various components they contain such as monoterpenes, diterpenes, iridoids, flavonoids and alcholoids. In addition, Linaria plants are widely used in traditional medicine in Anatolia, Japan and India. *Linaria* species have been reported to be used in the treatment of anti-diabetic, diuretic and eczema diseases (Baytop, 1984; Singh and Prakash, 1987; Kitagawa et al., 1973). There is also research on the content of polyphenols and flavonoids in some species such as *Linaria tingitana*, *Linaria corifolia, Linaria scariosa* (Hanfer et al., 2016; Gul et al., 2017). Some *Linaria* species are valued as ornamentals. There are revision, anatomical, morphological, ecological, palynological studies on the *Linaria* plant (Erdemoğlu, 1998; Juan et al., 1999; Tatlıdil et al., 2004; Temel, 2006). Studies on the productive and vegetative reproduction of *Linaria* species are scarce (Nadeau and King, 1991; Vujnovic and Wein, 1997; Necajeva and Ievinsh, 2008). Today, with the increase of consumer awareness and the adverse effects of synthetic substances, the demand for natural products of plant origin has increased rapidly. According to the report of the World Health Organisation (WHO), 80% of the increasing populations in the world primarily use medicinal plants for health. For this purpose, it is stated that 20,000 herbs and 4 thousand herbal drugs are used and 400 of them are actively traded. There are 140 medicinal plants registered in the Turkish codex (Kitagawa et al., 1978; Ceylan, 1995; Güler et al., 2011).

Figure 1. *Linaria genistifolia* (L.) Miller subsp. *praealta* plants in nature.

The plant material used in the present study, named as *Linaria genistifolia* (L.) Miller subsp. *praealta* (Boiss.) Davis and is endemic to Turkey (Figure 2) and it has potential for ornamental usage. This species grown in South and

South East Anatolia, and flowering period ranges from May to August (Glasby, 1991).

For the sustainability of our genetic diversity, it is very important to determine the reproduction methods of endemic plants. The introduction of this herbaceous plant with perennial, yellow flowers and the taxa of its genus to ornamental plants should also be evaluated economically. Biotechnological methods are valuable tools in the mass propagation of the plants grown in the nature. *In vitro* micropropogation studies have been carried out on some endemic species (*Origanum sipyleum, Muscari azureum, Erodium somanum, Silene bolanthoides, Iris sari*) with limited distribution in valuable biodiversity of our country (Sevindik et al., 2018; Urnabey, 2010; Çetin et al., 2016; Çördük et al., 2018; Doğan and Çağlar, 2020).

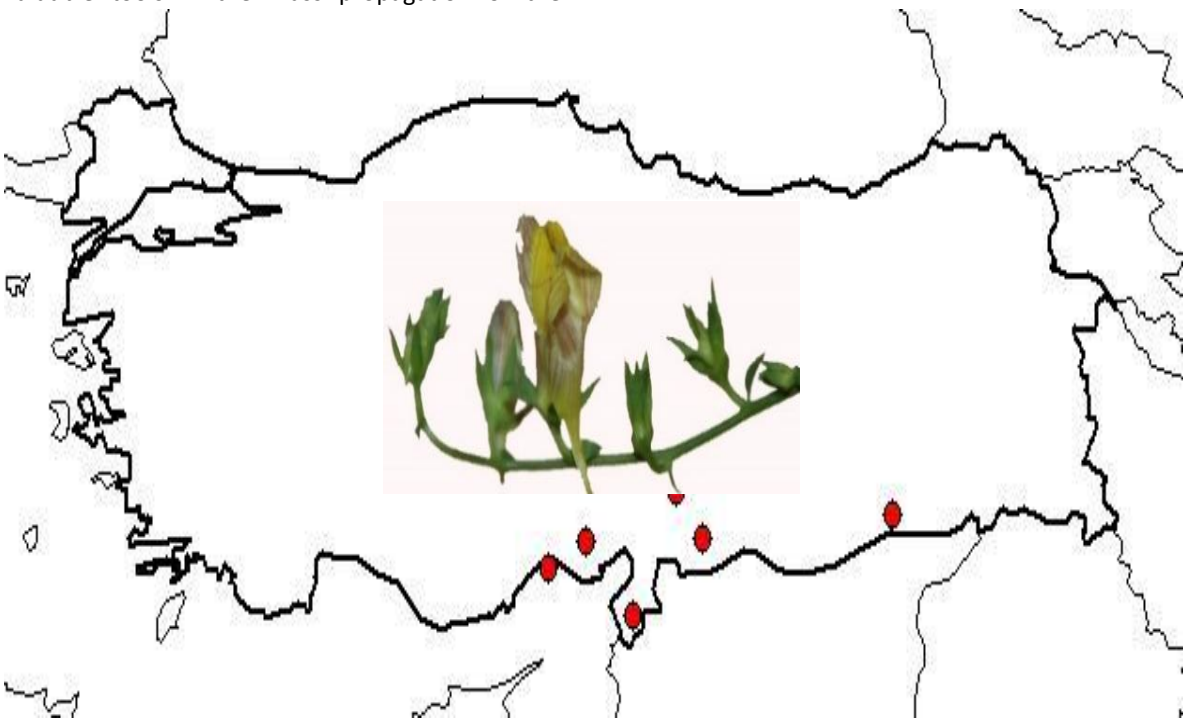

Figure 2. Distribution of *Linaria genistifolia* (L.) MILLER subsp. *praealta* in Turkey (Anonymous, 2021).

Among them *in vitro* tissue culture is the most popular tool for this aim. Tissue culture is based on concept of totipotency; the ability of plant cells and tissues to develop into whole new plant (Fowler et al., 1993). *In vitro* culture techniques offer an alternative route for multiplication or other treatments such as embryo culture. *In vitro* micropropagation of medicinal plants are provide of higher rate of multiplication and produce available all year round. Also tissue culture technique can provide production of secondary metabolite and conservation of threatened plant species. For the production of secondary metabolites in the *Linaria* species, which is very rich in secondary metabolites, it is necessary to study plant cell and tissue culture methods, which are advantageous especially compared to classical methods. Guiding technological methods are being developed to adapt these wild plants to different growing conditions. With this study, it is aimed to rapidly reproduce endemic species, which are a part of natural formation, in *in vitro* culture against many factors threatening the environment for the continuity of plant genetic diversity

For this purpose, the effects of different plant growth regulators on *in vitro* propagation of *Linaria genistifolia* (L.) Miller subsp. *praealta* was investigated in order to culture and develop it for ornamental usage.

Materials and Methods

Surface sterilization of plant material

The study was conducted at the biotechnology laboratory of the horticulture department, Kahramanmaras Sutcu Imam University. Fresh stem parts of *Linaria genistifolia* (L.) Miller subsp. *praealta* plants were collected from naturally grown in Başkonuş National Park (Kahramanmaras-Turkey) during the month of April-May and grown in greenhouse. Nodal parts were used as initially explants. In the present study, stem parts (have at least 5 nodes and except shoot tips) were rinsed three times with distilled

water and surface sterilized for 1 minute in 70% ethanol and for 15 minute in 5% sodium hypochlorite solution containing few drops of Tween-20 with continuous stirring. Then rinsed with sterilized distilled water 4-5 times in a sterile cabinet. Finally, stem parts were dried on tissue paper.

Culture of explants and shoot multiplication

Explants containing 1 node were divided into parts (approximately 1.5 cm in length) under sterile conditions. Explants were placed onto ½MS (Murashige and Skoog, 1962) medium (salt and organic compounds strength) containing sucrose (3% w/v) as the carbon source and solidified with 7% (w/v) agar, consisting of different concentration of 6- benzylaminopurine (BAP; 0.0, 0.5, 1.0, 2.0 mg l^{-1}) and Thidiazuron (TDZ; 0.0, 0.05, 0.1, 2.0 mg l^{-1}) alone or combined with 0.1 mg l^{-1} GA_3. A total of 12 different medium combinations were tested. MS medium without any growth hormones were used as control. These media were supplemented with growth regulators and adjusted to pH 5.7-5.8 by 1 N KOH and 1 N HCl and autoclave under the condition of 1.2 atm. pressure and 121°C temperature for 15 min. Nodal explants were placed in magentas GA-7 (Sigma) containing culture medium of 40 ml. Explants were placed on the growth media under sterile conditions and all cultures were incubated at 25±1°C, under cool white fluorescent light with 16-h (day)/8-h (night) photoperiods. The cultures were kept at 3500 lux of light intensity. Cultures were subcultures in fresh nutrient media at the and 6 weeks. The number of shoots per explant and shoot length was recorded after 2 subculture. Plantlets with well-developed roots were washed sterile water to clean media, transplanted to viols containing sterile perlite-peat (3:1).

Statistical methods

In this study, twelve different combinations of plant growth regulators such as BAP, GA_3, TDZ were tested. The data were analyzed statistically using factorial completely randomized design consisting of each combination was three replicates consisted of six explants. Data was statistically analyzed using the JMP 8.0. Means were separated according to the least significant difference (LSD) test at the 0.05 level of probability. Variance analysis was done according to the literature (Steel and Torrie, 1980; Yurtsever, 1984). The data (shoot number, length of shoots, percent of multiplication) were statistically analyzed using one-way analysis of variance Duncan's multiple range test (p=0.05).

Figure 3. Shoot tips and nodal segments.

Figure 4. *In vitro* cultured *Linaria* explants.

Result and Discussion

Multiple shoot induction

In the present study, nodal explants of *Linaria genistifolia* (L.) Miller subsp. *praealta* were multiplicated on all of the culture media. Multiplication potential of nodal segments was explored on ½MS medium supplemented with various plant growth regulators and results are summarized in Table 1. All nodal explants cultured on MS medium supplemented with different concentrations of TDZ and BAP individually or combination with GA_3 have developed healthy shoots. In terms of shoot multiplication, TDZ was found to be superior over BAP. Nodal explants cultured on ½MS medium added with BAP+GA_3 induced multiple shoots at a lesser frequency compared to the media supplemented with combined treatments of TDZ + GA_3. Among all the treatments examined, TDZ at 0.2 mg l^{-1} showed 53.2% response with maximum number of multiple shoots per explant (32.6). Among all the combinations tested, the best number of shoots (31.2) were obtained on medium containing 0.2 mg l^{-1} TDZ with 0.1 mg l^{-1} GA_3. According to the statistical analysis results, these two combinations were significant and different from the others. In addition, the number of shoots in the medium supplemented with TDZ varied between 6.8 and 30.5, while it varied between 20.4 to 30.8 in the nutrient medium supplemented with TDZ+GA_3. However, the number of shoots in the nutrient medium supplemented with BAP was only between 6.5 and 9.8, and similarly, it was observed that it varied between 7.3 and 14.5 in the nutrient medium supplemented with BAP+GA_3. When the effect of growth regulators added to the length of the shoots was examined, shoot lengths ranging from 8.8 to 9.1 mm in TDZ containing media developed between 7.0 and 11.5 mm in nutrient media containing TDZ+GA_3 combination. In the nutrient medium supplemented with BAP, shoot lengths were observed between 16.1-20.6 mm, while in the nutrient medium containing the combination of BAP+GA_3, the shoot lengths improved between 18.6-34.17 mm. In the *in vitro* propagation study of *Linaria genistifolia* (L.) Miller ssp. *praealta* (Boiss.) Davis considering both shoot number and shoot length, the best growing content was added to ½MS medium 2.0 mg l^{-1} BAP+0.1 mg l^{-1} GA_3 observed that it can be obtained by adding.

Considering both the shoot length and the number of shoots of in vitro propagation plants, it was concluded that the best results for obtaining plantlets could be achieved by adding into the. There was very less shoot multiplication observed in ½MS media without any growth hormone(control). The lowest shoot numbers were obtained as 2.3 and 5.1 per explant in ½ MS media (control) and medium supplemented with 0.5 mg l^{-1} BA, respectively (Table 1, Figure 6). Similar observation were made by Golle et al. (2017) in *Eugenia involucrate,* in which there was an increase in the number of shoots produced by nodal explants due to the addition of growth medium with TDZ. Similarly, the positive effect of TDZ, promoted the highest multiplication rate for *Cassia angustifolia* nodal segments, either alone or in combination with NAA-IAA (Siddique and Anis, 2007). Also, for many plant species, TDZ has been

used as an efficient alternative for the combination of cytokinins and auxins. Some researches have stated that, TDZ is less necessary than other plant growth regulators (i.e., BAP, NAA, KIN) to achieve the result (Ahmad and Anis, 2007; Faisal et al., 2005). In an early study Isah (2020), explored that in maximum formation of the Ginkgo biloba shoot buds was observed on TDZ supplemented solid medium at 3.0 mg l^{-1}, with 11.45 ± 0.31 shoots/explant using herbaceous nodal segment explant. Similarly, combination with TDZ treatment had promoted high frequency plant multiplication in, *Cicer arieinum*, (Kumari et al. 2018), and its promoting effect on shoot morphogenesis competence have been reported in woody plants such as *Eucalyptus grandis* X *E. urophylla* (Barrueto Cid et al., 1999), many plant species (e.g. Malik and Saxena, 1992; Bakshi et al., 2012; Graner et al., 2013).

Table 1. The effects of different plant growth regulators on the number, length and percentage of shoots as well as rooting and flowering status of *Linaria* plantlets grown *in vitro*.

Treatments (mg l^{-1})	Shoot number*	Shoot length (mm)**	Percentage of shoots < 5 mm	Root occurrence	Flower occurrence
½ MS	2.3 **e**	34.6 **ab**	19.0	+	+
½ MS+ BA 0.5	5.1 **e**	41.4 **a**	22.8	+	-
½ MS+BA 1.0	7.5 **de**	16.2 **cde**	29.6	+	+
½ MS+BA 2.0	11.7 **cd**	15.5 **cde**	18.5	+	-
½ MS+BA 0.5 + GA_3 0.1	6.7 **de**	20.8 **cd**	20.3	+	+
½ MS+BA 1.0 + GA_3 0.1	8.2 **de**	25.5 **bc**	18.9	+	+
½ MS+BA 2.0 + GA_3 0.1	15.5 **bc**	21.5 **cd**	5.7	+	-
½ MS+TDZ 0.05	7.2 **de**	10.8 **de**	44.7	+	-
½ MS+TDZ 0.1	17.4 **bc**	10.9 **de**	40.3	+	-
½ MS+TDZ 0.2	32.6 **a**	7.5 **e**	53.2	+	-
½ MS+TDZ 0.05 + GA_3 0.1	20.8 **b**	14.9 **cde**	17.7	+	-
½ MS+TDZ 0.1 + GA_3 0.1	14.3 **c**	14.9 **cde**	15.5	+	-
½ MS+TDZ 0.2 + GA_3 0.1	31.2 **a**	7.8 **d**	33.3	+	-

*: LSD (1%): 8.4; **: LSD (1%): 5.7

In this study, inversely correlation was observed between number of shoots and length of shoot developed. So, a gradual decrease was observed in mean shoot length with increased concentrations of all plant growth regulators combinations. The shoot lengths were positively improved by adding GA_3 plus cytokine into the media. There was a significant difference in shoot length when cultured control. The highest shoot length was 41.4 mm on the media containing 0.5 mg l^{-1} BA which was closely followed by control treatment (34.6 mm). However, the lowest number of shoot was also in the same MS concentration (0.5 mg l^{-1} BA). The result of this experiment was similarly with the findings of Ridzuan et al. (2020). The shoot length of *Moringa oleifera* L. was the best MS media containing 0.5 mg l^{-1} BAP, 1.0 mg l^{-1} BAP and control.

½ MS+TDZ 0.2 + GA_3 0.1 mg l^{-1}

½ MS+TDZ 0.2 mg l^{-1}

½ MS+BA 0.5 mg l^{-1}

½ MS

Figure 5. Shoot development on different growing media.

The shortest shoots were obtained as 7.5 and 7.8 mm on the media containing 0.2 mg l^{-1} TDZ and 0.2 mg l^{-1} TDZ+ 0.1 mg l^{-1} GA_3, respectively (Table 1). Fifty percent of the shoots were shorter than 5 mm on the media containing only TDZ. The shorter shoots on the other media ranged from 5.7 to 33.3%. The best result regarding to rate of shorter shoots (<5 mm) to total number of shoots (5.7%) was obtained on ½MS media containing BA 2.0 + GA_3 0.1 mg l^{-1} (Table 1).

Healthy root development along with shoot growth was observed in all media tested. Flowering was present both in the control and BA added media, but none in TDZ added media (Figure 7). Vitrification was observed (up to 30%) in the highest concentration of TDZ (Table 1).

Considering both the number and length of shoots of *in vitro* propagated *Linaria genistifolia* (L.) Miller subsp. In *Praealta* plants, we concluded that the best result was obtained on ½ MS media supplemented with 2.0 mg l^{-1} BA+0.1 mg l^{-1} GA_3.

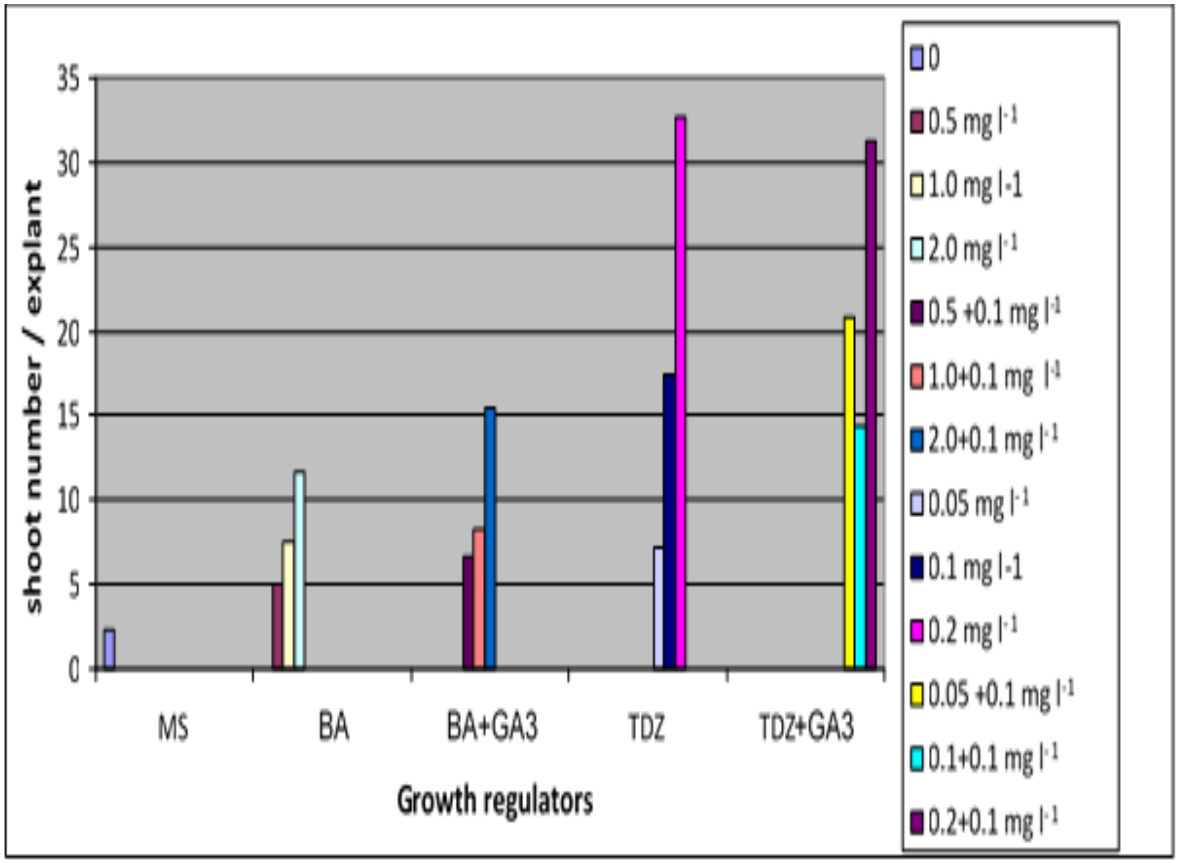

Figure 6. The number of shoots per explant obtained with different growth regulators added to ½ MS media.

Conclusion

In addition to its rich plant biodiversity, Turkey is also in a very important position in terms of endemic plants. Otherwise, some endemic species are in danger of extinction and conservation strategies need to be developed. Unfortunately, comprehensive studies on appropriate propagation methods of certain endemic plants are either insufficient or still not available. Therefore, further studies are needed to develop species-specific *in vitro* propagation protocols. Plant growth regulators, plant genotype, plant age, plant harversting time, habitat and explant type are among the most important factors that directly affect *in vitro* plant regeneration (Nasircilar et al., 2009; Özcan, 2002; Doğan and Çağlar, 2020). In this study, the addition of GA_3 or in combination with BAP or TDZ in the growing media improved the multiplication success of the *Linaria genistifolia* subsp (L.) Miller ssp. *praealta* (Boiss.) explants. The findings of the present study might also be used in the further studies for obtaining a better multiplication rate in the other *Linaria* species as well as in other genetic resources plants.

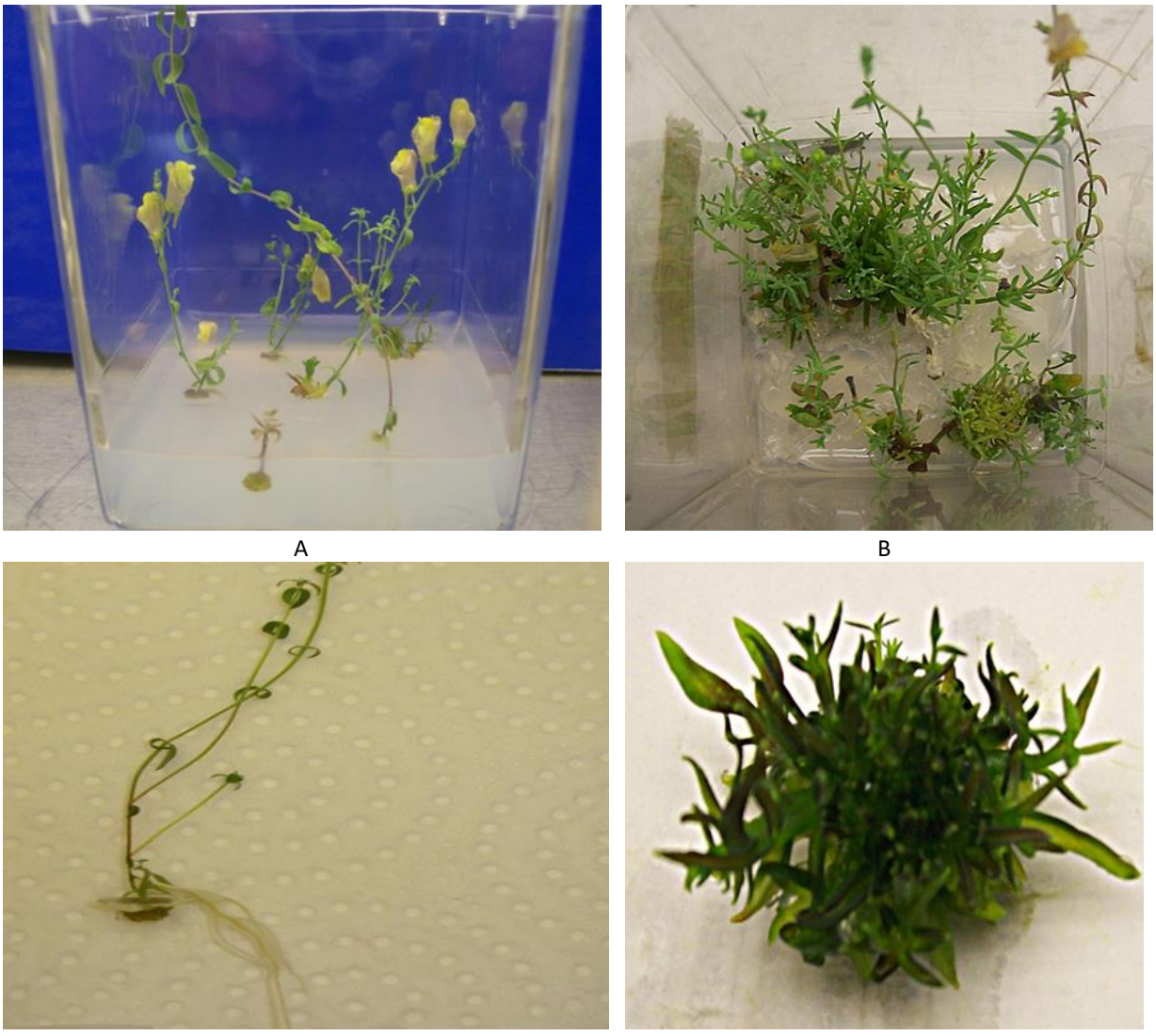

Figure 7. A- Flowering, B- Best result media (2.0 mg l^{-1} BA+0.1 mg l^{-1} GA_3), C- Rooting, D- Hyperhydricity.

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Conflict of Interest Statement: The authors of the article declare that there is no conflict of interest between them..

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