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Research Article

Efficient Micropropagation protocol for *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum* Grown Naturally in Turkey

Mehmet TÜTÜNCÜ[1], Başar SEVİNDİK[2*], Tolga İZGÜ[3], Özer YILMAZ[4], Gönül KAYNAK[4], Pembe ÇÜRÜK[5], Yeşim YALÇIN MENDİ[6]

ABSTRACT

Lamiaceae family includes annual and perennial 250 genus which are commonly used for their medicinal aromatic properties due to its high biologically active compounds. *Lamium garganicum L.* subsp. *striatum* (Sm.) Hayek var. *striatum* (Telbalıcak) is a member of *Lamium* genus spread over in Turkey. *Lamium* species are used for cure since ancient times. Tissue culture provides important advantages for the conservation and sustainability of genetic resources such as clonal and mass production in a limited area. Micropropogation is one of the most significant alternatives for rapid clonal propagation in a short time. In this study, nodes include one or two leaves were placed on MS medium including different levels of BA and GA_3 (0, 0.5, 1, 1.5, 2, 2.5 mg/L) for propagation. The explants regenerated to the shoots with the response of 82.5% and 80% in MS medium including 2 mg/L BA +2 mg/L GA_3, 2 mg/L BA +1.5 mg/L GA_3 respectively. Rooting experiment was carried on MS medium supplied with 0, 0.5, 1, 1.5, 2 mg/L Indole-3- Butyric Acid (IBA) for root induction. Maximum root formation was observed as 45% on MS medium including 0.5 mg/L IBA. Plantlets were acclimatized and 80% of the plants are survived after acclimatization.

Keywords: *Lamiaceae, Lamium* sp., micropropagation, rooting, genetic resources

Türkiye'de Doğal Olarak Yayılış gösteren *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum* Türü İçin Etkili Mikroçoğaltım Protokolü

ÖZ

Lamiaceae familyası, yüksek bioaktif bileşenleri sayesinde tıbbi ve aromatik özelliklerinden dolayı halk tıbbında kullanılan tek yıllık ve çok yıllık 250 cins içermektedir. *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum* Türkiye'de doğal olarak yayılış gösteren Lamium cinsinin bir üyesidir. Lamium türleri eski çağlardan beri tedavi amaçlı kullanılmaktadır. Doku kültürü, genetik kaynakların korunması ve sürdürülebilirliği açısından klonal çoğaltım ve sınırlı alanda kitlesel üretim gibi önemli avantajlar sağlar. Mikroçoğaltım kısa sürede klonal çoğaltım için çok önemli bir alternatiftir. Bu çalışmada bir ya da iki yaprak içeren nod eksplantları çoğaltım amacıyla farklı seviyelerde BA ve GA_3 (0, 0.5, 1, 1.5, 2, 2.5 mg/L) içeren MS besi yerine yerleştirilmiştir. Eksplantlar %82.5 ve %80 oranında sırasıyla 2 mg/L BA +2 mg/L GA_3, 2 mg/L BA +1.5 mg/L GA_3 içeren MS besi yerinde sürgüne rejenere olmuşlardır. Köklenme denemeleri 0, 0.5, 1, 1.5, 2 mg/L Indol-3- Bütirik Asit (IBA) içeren MS besi yerinde gerçekleştirilmiştir. Maksimum kök oluşumu 0.5 mg/L IBA içeren MS besi yerinde %45 olarak gözlemlenmiştir. Bitkiler dışa aktarılmış ve bitkilerin aklimatizasyondan sonra %80 oranında hayatta kaldığı belirlenmiştir.

Anahtar Kelimeler: Lamiacaeae, Lamium sp., mikroçoğaltım, köklenme**,** genetik kaynak

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[1]University of Ondokuz Mayıs, Faculty of Agriculture, Department of Horticulture, 55270, Atakum, Samsun, Turkey
[2]University of İzmir Demokrasi, Vocational School of Higher Education, 35140, Karabağlar, İzmir, Turkey
[3]National Research Council of Italy (CNR), IBE/Institute of BioEconomy, 50019, Florence, Italy
[4]University of Uludağ, Faculty of Art and Science, Department of Biology, 16059, Nilüfer, Bursa, Turkey
[5]University of Korkut Ata, Kadirli School of Applied Sciences, Kadirli, Osmaniye, 80760, Turkey
[6]University of Çukurova, Faculty of Agriculture, Department of Horticulture, 01130, Sarıçam, Adana, Turkey
*E-mail: basar.sevindik@idu.edu.tr

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ORCID ID (Yazar sırasına göre)
0000-0003-4354-6620, 0000-0002-1448-6617, 0000-0003-3754-7694, 0000-0003-1498-5827, 0000-0002-1294-7854, 0000-0003-4764-7242, 0000-0002-4587-5156

Introduction

Turkey has a rich floral diversity both medicinal and aromatic plants and also ornamental plant due to its geographic properties (Aka Kaçar et al. 2020; Güner 2012). *Lamiaceae* family with seven subfamilies is one of the widest family in the world that contains 250 genus and 7852 species (The Plant List 2019; Atalay, 2016; Koyuncu et al. 2010; Kahraman et al. 2009). *Lamium* genus known as deadnettle, belongs to the *Lameioidae* subfamily, distributed across from North Africa to Europe, Australia, South West Asia and South America with 40 annual and perennial species which are mainly utilized for their medicinal and aromatic properties (Sajjadi and Ghannadi 2012; Dimitrova et al. 2011; Alipieva et al. 2003). The genus *Lamium* includes perennial and annual species as shrubs or herbs. Some of these species used in folk medicine, traditional health therapy, and medicine industry such as *Lamium album* L., *Lamium maculatum*, *Lamium purpureum*, due to their therapeutic, anti-inflamutar, astringent, antiseptic, antibiotic spasmolytic and anti-proliferative activity. Especially *Lamium purpureum* used for the treatment of injury, fractures, decomposition, palsy, leucorrhoea, hypertension and women afflictions (Akkol et al. 2008; Paduch et al., 2007; Matkowsi and Piotrowska, 2006; Trouillas et al., 2003; Shuya et al., 2003; Bremness, 1995; Weiss, 1988). Moreover, *Lamium album* has an important effect on the cure of the bronchitis and pharyngitis due to its antispasmodic and mucolytic properties (Paduch et al., 2006; Mazza 2000). Additionally, some of the *Lamium* species such as *Lamium album* used as alternative food in China, Japan and some European countries during time of scarcity (Sturtevants, 1919; Turner et al., 2011). Some of these medicinal and aromatic plants such as *Lamium album*, *Lamium maculatum*, and *Lamium purpureum* has been used for tonic and cure to reduce pain, rheumatism and arthritic ailments (Baytop, 1999; Özaydin et al., 2006). Extracts obtained from shoot, leaves, flowers were used for different cure in folk medicine. Turkey has a rich flora in terms of vascular plants and medicinal and aromatic plants diversity (Güner 2012; Davies 1982) In Turkey, Lamiacae familycontains O45 genus and 574 species (Kahraman et al. 2009). The main characteristics of this genus are possessing perennial plants that reach 0.5-1 m length, green four angled stems, long, triangular leaves, serrated margins and soft hairs. White flowers contained two lips, located on the upper part of the stem. Most of the Lamiaceae family members possess flowers that are rich in nectars (Atalay 2016; Harley et al. 2004). The genus *Lamium* contains self-pollinated species which is pollinated by entomophilous pollinators and so they attract bumblebees, honey bees. *L. garganicum* is native and important genetic resource because of its remarkable bioactive ingredients such as sesamoids, shanzhiside methly ester, barlerin and lamalbide. Uludağ mountain located in north west of Turkey has a big floral diversity and *L. garganicum* is one of the important species among these species.

Preservation and sustainability of the plant genetic resource is an important subject for the plant biologist. Different biotechnological methods have been applied for the ex situ conservation of the genetic resources for a long time. Tissue culture is significant method to preserve genetic resources via the multiplication and clonal production of valuable plants (Aka Kaçar et al. 2019). Developing efficient conservation protocols for grown naturally species is in demand to prevent loss of genetic resources. Additionally, tissue culture provides clonal, virus free plant, rapid mass propagation in limited areas. Various studies were carried out for micropropagation of *Lamium* sp. Effect of different plant hormone, their combinations and different medium have been investigated for micropropagation of *Lamium* sp. For instance; Dimitrova et al. (2011) investigated the impact of different plant hormone on 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) on

micropropagation, secondary metabolite accumulation and their medicinal aromatic properties. Researchers developed an efficient micropropagation protocol for *Lamium album* derived from Lozan mountain. In last decades, micropropagation studies are mainly focused on *Lamium album.* In another study, Stefanova et al. (2011) reported that the effect of different growth regulators (BAP and IBA) on *Lamium album*. Although there have been some reports on different *Lamium* sp. such as *L. album*, *L. amplexicaule* (Lord et al. 1982), to our knowledge, there is no any published report about *in vitro* propagation of *L. garganicum.* Aim of this study was to micropropagate the *L. garganicum* species which is important medicinal aromatic plants for Turkey. In this study, effect of various dosage of giberellic acid (GA_3) and BA on shoot regeneration, shoot multiplication, shoot length were investigated. In addition, the effect of various concentrations of IBA were examined by means of rooting and root length. This species was microprogated, rooted and acclimatized by using *in vitro* micropropagation method.

Material and Method

Plant Material

Lamium garganicum L. subsp. *striatum* (Sm.) Hayek var. was gathered from Uludağ mountain (40°05'27.55" N (long.), 29°10'26.26" E (lat.), 1.6 km altitude at August 2012. Totally 20 individual shrubs were collected from the nature and cultured in the Çukurova University, Adana, Turkey. Each plant was placed into pots including mixture of peat, sand, perlite (1:1:1, v/v/v) at greenhouses, irrigated by 300 ml tap water/pot three times a week each morning. Donor plants were cultured 30°C temperature, 70% humidity and 2-11 MJ m^{-2} day^{-1} light condition before the *in vitro* culture period.

Method

Explant Surface sterilization

Fresh nodes (2 cm length) obtaining from one year old plants containing two leaves were used as explant. Nodes including two leaves were steep under tap water for 20 min. Nodes were transferred to the sterile cabinet (Thermo Fischer Scientific Laminar Flow- USA) and treated with 70% EtOH (Interlab - 96% Ethanol) 1-2 minutes. After rinsed one time with autoclaved distilled water, node explants were immersed in 20% NaOCl (4.6% active chlorine) for 20 min. Node explants derived from the donor plant were washed three times with sterilized deionized water in the laminar flow cabinet. All equipment (lancet, forcepts filter paper, distilled water, medium) used in the experiments were sterilized at the autoclave (Hirayama HV- 50 L) (121°C, 15 min. 1.05 atm pressure).

Culture conditions, medium ingredients and incubation

Node explants were cultured into the test tubes (15 cm) (Sigma Aldrich) including 10 ml MS medium (Murashige and Skoog 1962) (Duchefa RV) supplied with 30 g/L sucrose, 7 g/L agar (Duchefa, P1001), 10 ml/L Fe-EDHA. MS medium combined with various dosages and combinations of the BAP (0, 0.5, 1, 1.5, 2, 2.5 mg/L) and GA_3 (Merck, Darmstadt, Germany) (0, 0.5, 1, 1.5, 2, 2.5 mg/L) for micropropagation. pH was stabilized to 5.6- 5.8 with 1 N KOH and 1 N HCl before autoclave. Nodes including 2 leaves were cultured in the growing room at 25 ± 2°C, 60% humidity and 16 h photoperiod under (40 µmol m^{-2} s^{-1} light density) F36W/54-765 daylight 2350 Lm. (Philips Lighting Holding B.V.) during 4 weeks. Micropropagated plants derived from node explants were cultured and multiplied on micropropagation medium after each subculture. After two times subculture and multiplication, plantlets were transferred to MS medium supplied with 30 g/L sucrose, 7 g/L agar and various concentration (0, 0.5, 1, 1.5, 2, 2.5, 3 mg/L) IBA for root initiation. Plants were cultured into the test tubes including 15 ml rooting medium at the same conditions (25 ± 2°C, 60% humidity and 16 h photoperiod (40 µmol m^{-2} s^{-1} light density) during 6 weeks. Subculture were not applied to the rooting plants.

Acclimatization

The caps of the tubes containing 4 months old and 10-25 cm length rooted plants were removed gradually from the test tubes. Roots of the plantlets were washed under tap water to

remove the medium from the roots and roots immersed to the fungicide (1g/1L; Captan). Plants were cultured into 13 cm diameter x 20 cm tall plastic pots including disinfected sand, peat and perlite (1:1; v/v). The potted explants were covered with a transparent plastic in the growing room (± 25°C and 16 /8 day/night photoperiod 2-11 MJ m^{-2} day^{-1}). Distilled water was sprayed to the plants under the transparent plastic three times in a day to provide optimum humidity and 300 ml water were sprayed every spray. Plants were individually cultured on the pots after 2 weeks later.

Statistical analyses

Node explants in the experiment were established as a completely randomized design, each plantlet located in tubes. In this study, experiments were set up as 10 replicates × 25 combinations of plant growth regulators (PGRs). JMP® program ver. 5.00 (SAS Institute, Cary, NC) were used for statistical analyses. Means were separated by LSD test to determine significant of difference at the 0.001 level of probability. Arcsine transformation was applied to the percentage values before analyses.

Result and Discussion

Efficient shoot induction were obtained from the nodes placed into MS medium including 30 g/L sucrose, 7 g/L agar, 2 mg/L BA +2 mg/L GA_3 and 2 mg/L BA +1.5 mg/L GA_3 as 82.5% and 80% respectively at the end of the two subculture. In the first subculture efficient shoot multiplication determined as 4.7±0.84 on MS medium including 2 mg/L BA +2 mg/L GA_3. After the second multiplication, highest plant number determined as 5.7±0.33 on the MS medium including 2 mg/L BA +1.5 mg/L GA_3 (Table 1). Regeneration were obtained after 6 weeks of cultivation at the light condition 25 ± 2°C, 60% humidity and 16h photoperiod (40 $\mu molm^{-2}s^{-1}$) (Table 1; Figure 1). Totally 994 plants were obtained at the end of the multiplications and micropropagation. In the control medium, absence of the plant growth regulators, no shoot regeneration was detected. After shoots reached 10 cm tall they were transferred to the rooting medium. Maximum root induction was detected on MS medium including 30 g/L sucrose, 7 g/L agar, 0.5 mg/L IBA as 45% (Table 1; Figure 1). Also rooting was obtained on MS medium supplemented with 1 mg/L or 1.5 mg/L IBA in response to 35% at both medium. In the control medium, micropropagated shoots were not rooted. At the beginning of the rooting, roots were white and bright but after 6 weeks later of the cultivation some of the roots turned brownish. Plantlets that was 10-25 cm tall, were acclimatized to the *ex vitro* conditions after 4 months of the cultivation. Rooted plants derived from different micropropagation medium were acclimatized and cultured in the pots including sand, peat and perlite (1:1; v/v) in the greenhouse (±25°C and 16/8; day/night photoperiod 30 µmol m^{-2} s^{-1}). Survival rate of the acclimatized plants were observed as 80% after one month of the cultivation.

Table 1. Micropropagation and Multiplication *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum*

Plant Growth Regulator	Shoot Formation (%)	No of shoots after 1th multiplication	No of shoots after 2nd multiplication
Control	0	0	0
0 mg/L BA +0.5 mg/L GA_3	0 l (0)	0	0
0 mg/L BA +0.5 mg/L GA_3	0 l (0)	0	0
0 mg/L BA +1.5 mg/L GA_3	0 l (0)	0	0
0 mg/L BA +2 mg/L GA_3	0 l (0)	0	0
0 mg/L BA +2.5 mg/L GA_3	5kl (6)	0.5±0.34	0.3±0.21

1 mg/L BA +0.5 mg/L GA_3	12.5hıjkl (13.5)	1.3±0.55	0.7±0.33
1 mg/L BA +1 mg/L GA_3	10ıjkl (12)	0.9±0.37	1.1±0.54
1 mg/L BA +1.5 mg/L GA_3	32.5def (34.5)	2.2±0.24	2.1±0.34
1 mg/L BA +2 mg/L GA_3	20ghıjk (19.5)	1.2 ±0.41	1.1±0.45
1 mg/L BA +2.5 mg/L GA_3	7.5jkl (9)	0.4±0.22	0.4±0.22
1.5 mg/L BA +0.5 mg/L GA_3	5kl (6)	0.4±0.26	0.3±0.21
1.5 mg/L BA +1 mg/L GA_3	32.5defg (31.5)	2.2 ±0.44	1.5±0.37
1.5 mg/L BA +1.5 mg/L GA_3	45cde 40.5	2.9±0.45	2.6±0.45
1.5 mg/L BA +2 mg/L GA_3	10ıjkl (12)	0.5±0.22	0.3±0.15
1.5 mg/L BA +2.5 mg/L GA_3	17.5ghıjk (19.5)	0.8±0.24	0.6±0.26
2 mg/L BA +0.5 mg/L GA_3	17.5ghıjk (19.5)	0.6±0.26	0.6±0.22
2 mg/L BA +1 mg/L GA_3	42.5cde (40.5)	2.1±0.43	2.6±0.68
2 mg/L BA +1.5 mg/L GA_3	80a (69)	4.4±0.63	5.7±0.33
2 mg/L BA +2 mg/L GA_3	82.5a (70)	4.7±0.84	5.3±0.53
2 mg/L BA +2.5 mg/L GA_3	20fghıj (22.5)	1.1±0.31	1.3±0.39
2.5 mg/L BA +0.5 mg/L GA_3	25efgh (27)	1.2±0.29	1.2±0.29
2.5 mg/L BA +1 mg/L GA_3	60bc (51)	2.5±0.30	2.3±0.33
2.5 mg/L BA +1.5 mg/L GA_3	75ab (64.5	4.2±0.94	3.8±0.57
2.5 mg/L BA +2 mg/L GA_3	45cd (42)	2.1±0.69	2.5±0.87
2.5 mg/L BA +2.5 mg/L GA_3	25fghı (24)	1.1±0.34	0.9±0.4

Different letters with in a column are significantly different
Values in parenthesis indicate arcsin transformed % values
LSD_{shoot}:14.61 $p<0.001$,

Table 2. Rooting of *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum*

Rooting Medium (IBA)	Rooting (%)
Control	0
0.5 mg/L	45.00a (42.05)
1 mg/L	35.00a (36.22)
1.5 mg/L	35.00a (36.22)
2 mg/L	0
2.5 mg/L	0
3 mg/L	0

Different letters within a column are significantly different, Values in parenthesis indicate arcsine transformed % values

Figure 1. Micropropagation stages, a) Donor plant, b) Node explants including leaves, c) Explants cultured in the test tubes, d) Proliferation of the explants, e) Propagated plantlets, f) Plantlets in rooting medium, g) Acclimatization stage, h) Whole plantlets ı) Acclimatized and survival plantlets

There has been many reports published about the Turkish medicinal and aromatic species represented in Lamiaceae family such as *Origanum* sp. (Avtar and Çölgeçen 2019; Abdallah et al. 2017; Korkor et al. 2017; Sevindik et al. 2017; El Beyrouthy et al. 2015; Hussein et al. 2014; Yıldırım et al. 2013; Yashodabai et al. 2011; Özkum et al. 2007; Arafeh et al. 2006; El-Gengaihi et al. 2006; Goleniowski et al. 2003; Iyer and Pai 2000; Iconomou-Petrovich and Nianiou-Obeidat 1998; Iyer and Pai 1998; Sajina et al. 1996), *Sideritis* sp. (Sevindik et al. 2019; Yavuz 2016; Shtereva et al. 2015; Faria et al. 1998; Sánchez-Gras and Segura 1997; Garcia-Granados et al.1994; Sánchez-Gras and Segura 1988), but only a few report were published about *in vitro* propagation of *Lamium* sp. Most of the studies were focus on micropropagation of *Lamium album* (Dimitrova et al. 2010, 2011; Stefanova et al. 2011). In this study we investigate the effect of different combinations of BAP and GA_3 on node explants of *L. garganicum.* The plants cultured on PGR free MS medium did not regenerate but when significant effects of the BAP were observed on micropropagation of node explants cultured on MS medium with BAP as indicated in the Table 1. Additionally, in the other reports of the *in vitro* propagation of the Lamiaceae family members such as mentha (Maity et al. 2011), *Ocmium bacilicum* (Dode et al. 2003) and *Origanum sipyleum* (Sevindik et al. 2017) BAP was used as an efficient cytokinin. In our study, high BAP concentrations showed efficient micropropagation and multiplication with the combination of GA_3. Dimitrova et al. (2010) investigated the effect of auxin on micropropagation of *Lamium album* and obtained the highest shoot length on MS medium supplemented with 0.5 mg/L IBA. Additionally, Dimitrova et al. (2011) reported the effect of BAP+IBA combinations for micropropagation of *L. album*. They reported

that 0.8 mg/L BAP in culture medium promote the shoot development. In our study, BAP combined with GA_3 showed similar results on MS medium including 2 mg/L BA +2 mg/L GA_3. Effect of the BA and GA_3 concentrations was studied on different species for micropropagation such as *Alyssum dudley*i Adıgüzel & R.D. Tütüncü et al. (2018) obtained efficient micropropagation on MS basal medium supplied with 0.5 mg/L BA + 0.2 mg/L GA_3 for *Alyssum dudleyi.* Moreover, researchers indicated that nodes are important explant source for clonal propagation of the due to its high regeneration performance.

Low auxin concentrations with or without cytokinin induces rooting. In this study efficient rooting was obtained on MS medium including 0.5 mg/L IBA. Sevindik et al. (2017) obtained 62.5% rooting from *O. sipyleum* on MS medium including 1.5 or 2.5 mgL^{-1} IBA. Tütüncü et al. (2018) used different concentrations of IBA for root induction of the *Alyssum dudleyi* and they reported that efficient rooting was obtained at 1 mg/L IBA (20%). Dimitrova et al. 2010 used IBA both micropropagation and rooting in *Lamium album* and mean root number were determined as (5.4±1.2) on MS medium including 0.7 mg/L IBA. In some *in vitro* studies of the shrubs, rooting is observed spontaneously on MS medium without any plant growth regulator (Goleniowski et al. 2003). Also BA and GA combinations were used efficiently for the shoot regeneration of different plants at different family (Çeğil and Çürük 2019).

Acclimatization is very crutial and problematical stage of the *in vitro* micropropagation due to the major loss of regenerated plantlets. Another important problem is the desiccation of the acclimatized plants. To prevent this major loss attached to the sudden change of the environmental conditions, different applications were integrated to the acclimatization stage to increase the number of acclimatized plant number such as high humidity in the greenhouses. In this study, 80% of the plants easily acclimatized to the pots into the greenhouse due to the strong root formations, cultivation conditions (high humidity, regular irrigation) of healthy plantlets.

Conclusion

Tissue culture techniques is an efficient method for clonal and mass propagation for *L. garganicum.* BA and GA_3 combination highly effective for shoot multiplication and micropropagation. Although auxin concentrations induce callus formation, low dosage of the IBA without any cytokinin or gibberellin provide rooting. This report is an efficient protocol for in vitro propagation of *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum.*

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