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In Vitro Regeneration Potential of *Lotus maritimus* L. (Leguminosae)

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ABSTRACT

To investigate the regeneration potential of *Lotus maritimus* L., the stem, hypocotyl, cotyledon, root, and epicotyl were cultured in MS media containing different combinations of α-naphthalene acetic acid (NAA) concentrations (1, 2, and 4 mg L^{-1}) and cytokinin types [0 (control), 1 mg L^{-1} of 6-Benzilaminopurin (BAP), and 0.5 mg L^{-1} of kinetin]. The highest callus induction was achieved with the stem and epicotyl explants at 1 or 2 mg L^{-1} concentrations of NAA plus 0.5 mg L^{-1} of kinetin. The best plant regeneration values (3.56 and 3.20 shoots per callus) were determined with the epicotyl and hypocotyl cultured in MS media containing 1 mg L^{-1} of NAA. The obtained shoots were easily rooted in half-strength MS media containing 1 mg L-1 NAA and substantially adapted to the external environment.

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ÖZET

Lotus maritimus L.'nin rejenerasyon potansiyelini araştırmak için, *L. maritimus*'un tohumları *in vitro* koşullarda ekilmiş ve daha sonra kök, hipokotil, kotiledon, sap ve epikotil parçaları, farklı α-naphthalene acetic acid (NAA) konsantrasyonları (1, 2 ve 4 mg L^{-1}) ve sitokinin tiplerinin [0 (kontrol), 1 mg L^{-1} 6-Benzilaminopurin (BAP) ve 0.5 mg L^{-1} kinetin] kombinasyonlarını içeren MS ortamlarında kültüre alınmıştır. Sonuçlara göre, en yüksek kallus indüksiyonu, sap ve epikotil eksplantlarından 1 veya 2 mg L^{-1} NAA konsantrasyonu + 0.5 mg L^{-1} kinetin ile sağlanmıştır. En iyi bitki rejenerasyonu değerleri (3.56 ve 3.20 sürgün/kallus) ise epikotil ve hipokotil parçalarının 1 mg L^{-1} NAA içeren MS ortamında kültüre alınmasıyla elde edilmiştir. Oluşan sürgünler, 1 mg L^{-1} NAA içeren ½ MS ortamında kolayca köklendirilmiş ve büyük ölçüde dış ortama aktarılmıştır.

Araştırma Makalesi

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INTRODUCTION

Genus *Lotus* L. (Leguminosae) comprises between 125 and 180 species, which is worldwide spread except in extremely cold regions and the lowlands in tropical areas of Southeast Asia, South America, and Central America (Arambarri et al., 2005; Diaz et al., 2005; Sokoloff and Lock, 2005). Among the species, *Lotus corniculatus* L., *Lotus tenuis* Waldst. & Kit. ex Willd., and *Lotus uliginosus* Schkuhr. are widely cultivated (George, 2012). However, knowledge about the agricultural characteristics of other *Lotus* species remains limited.

Regarded as pioneer legumes in acidic and barren soils of cool and humid areas, *Lotus* species possess a non-bloating quality that encourages various livestock to graze upon pure stands of them (Diaz et al., 2005; Escaray et al., 2012). Among them, a less widely cultivated species, *L. maritimus* is also called *Tetragonolobus maritimus* Roth. (*syn.*), it grows in rocky areas, seashores, and coastal slopes of Europe, Africa, and the temperate regions of Asia (Roskov et al., 2005). Owing to its natural distribution, *L. maritimus* demonstrates a potential for forage production in the saline and acidic soils of a temperate and humid areas (Kavak, 2014).

On the other hand, improvement of breeding programs for *Lotus* species is slow and difficult due to their cross-pollinating and polyploid nature (Diaz et al., 2005). Furthermore, selecting and obtaining highly homozygous lines of *Lotus* species for breeding takes many years when classical breeding methods are applied. Tissue culture methods with

micropropagation allow the production of numerous genetically homogeneous and disease-free progenies from a single plant (Suman, 2017). Also, the success of genetic transformation depends upon high-frequency shoot regeneration. A major limitation in the application of tissue culture methods is that different genotypes have various regeneration potential, which necessitates determining the appropriate culture conditions for each genotype. Concerning *Lotus* species, regeneration studies have been performed with *L. tenuis* (Piccirilli et al., 1988; Espasandin et al., 2010), *Lotus pedunculatus* Cav. (Pupilli et al., 1990), *Lotus japonicus* (Regel) K. Larsen (Handberg and Stougaard, 1992; Lombari et al., 2003), *Lotus angustissimus* L. (Nenz et al., 1996), and *L. corniculatus* (Akashi et al., 1998; Nikolic et al., 2006; Orcen, 2013) but not with *L. maritimus*.

Therefore, the aim of the study was to determine the *in vitro* regeneration potential of *L. maritimus* using different explants in different combinations of NAA concentrations and types of cytokinin.

MATERIAL and METHODS

Naturally distributed around the Meşelik Campus of Eskişehir Osmangazi University, *L. maritimus* L. was used as the study's primary material. As Heyn (1970) has observed, the stem of the species is habitually erect and covered sparsely or densely with hairs, the plant stands 10–35 cm tall (Fig. 1a). Its leaflets are ciliate, asymmetric, and apiculate (Fig. 1b), and its flowers are single and the corolla is golden yellow or sulfur colors (Fig. 1c). Beyond that, its fruits are slightly straight and have four short membranous wings (Fig. 1d).

The experiment was conducted in a three-factor arrangement with a completely randomized design involving three replications. The first factor was the explant type (stem, epicotyl, hypocotyl, root, and cotyledon) of *L. maritimus*; the second was NAA concentrations (1, 2, and 4 mg L^{-1}); and the third was type of cytokinins [0 (control), 1 mg L^{-1} BAP, and 0.5 mg L^{-1} kinetin].

The fruits of *L. maritimus* were collected from its natural habitat during the years of 2016 and 2017. The seeds were extracted manually from the fruits after naturally drying and stored at +4-6 °C until their use in *in vitro* studies.

The hard-coated seeds were abraded with sandpaper to break dormancy before surface sterilization, during which, the seeds were kept in 95% alcohol for 1 min, then surface sterilization was performed in 25% commercial bleach (sodium hypochlorite: 4.6%) for 25 min. After sterilization, the seeds were rinsed 3 or 4 times with sterile distilled water and planted in growth tubes containing half-strength MS media (Murashige and Skoog, 1962). Cotyledon, epicotyl, hypocotyl, and root explants were cultured within 10 d and stem explant within 30 d following the germination of seeds. Five explants were placed in each culture vessel and transferred to a dark condition in the growth chamber with a temperature of 25±0.5 °C and 60% humidity.

Figure 1. Morphological characteristics of *L. maritimus*; (a) general view with erect stem, (b) leaf, (c) single flower with golden yellow corolla, (d) fruits.

Şekil 1. L. maritimus'un morfolojik özellikleri; (a) dik gövdenin genel görünümü, (b) yaprak, (c) sarı renkli corolla ile tek çiçek, (d) meyveler.

The explants were evaluated for callus induction after 20–30 d. After callus formation, the cultures were transferred to 18±0.5 °C at night and 25±0.5 °C during the day with an artificially illuminated condition for 10–15 d. All shoots from regenerated calluses were counted for each treatment, and the regeneration percentage was calculated. Regenerated shoots were cut and transferred to half-strength MS media containing with 1 mg L^{-1} of NAA for rooting. The shoots were easily rooted within 25–30 d, and rooted plantlets transferred to pots acclimated successfully.

The obtained data were subjected to an analysis of variance in Statistical Package for Social Sciences (SPSS) version 16.0. Arcsin √x transformation was applied to callus induction values beforehand. In the case of any significant difference among means, Duncan's multiple range test was performed.

RESULTS and DISCUSSION

Callus formation was induced in the dark 20–30 d after initiation of the culturing. Whereas the stems produced hard and compact calluses (Fig. 2a), both soft and hard calluses were obtained from the cotyledon,

hypocotyl, and epicotyl explants (Fig. 2b). Calluses were induced in the roots after swelling, although the roots quickly turned brown (Fig. 2c). At the same time, shoots formed on calluses after approximately 3 or 4 weeks in the dark. Chlorophyll formation commenced within 1 or 2 weeks after the cultures were transferred to the light (Fig. 2d). The shoots were rooted in half-strength MS media containing 1 mg L^{-1} of NAA (Fig. 2e) and readily acclimated to the external environment (Fig. 2f).

The effect of all three factors and their interactions on callus formation and plant regeneration was significant at 1% except for the NAA concentrations × cytokinin types interaction concerning plant regeneration (Table1). Percentages of callus induction ranged from 46.66 to 66.15% in root and stem explants, respectively. Similar but not statistically significant results regarding callus induction were obtained with the stems and hypocotyls. Plant regeneration varied from 0.00 to 1.16 (shoots per callus) in the roots and stems, respectively, and the stems, hypocotyls, and epicotyls exhibited similar values in terms of plant regeneration. The highest callus induction and plant regeneration were obtained from NAA concentrations of 2 and 1 mg L^{-1}, respectively. The best callus induction occurred with MS media containing 0.5 mg L^{-1} of kinetin, whereas control applications achieved better plant regeneration.

In other researches, the highest callus induction or regeneration have been obtained from the hypocotyls of *L. angustissimus* (Nenz et al., 1996), the leaves of *L. tenuis* (Piccirilli et al., 1988, Espasandin et al., 2010), and the hypocotyls, cotyledon (Akashi et al., 1998), and shoot tips (Orcen, 2013) of *L. corniculatus*. In an early work, Piccirilli et al. (1988) reported that low NAA concentrations were efficient on callus induction and regeneration in *L. tenuis*. Since then, the positive effect of kinetin on the callus induction of *Lotus* species has been reported by Pupilli et al. (1990), Handberg and Stougaard (1992), and Nenz et al. (1996). In contrast to the study presented here, a high rate of regeneration has been achieved by adding low concentrations of BAP to the media for some *Lotus* species (Akashi et al., 1998, Lombari et al., 2003, Nikolic et al., 2006).

Callus induction occurred in the range of 0–100% depending on the explant types, NAA concentrations, and cytokinin types used (Table 2). The highest callus induction was obtained from 1 or 2 mg L^{-1} of NAA concentrations with combined 0 or 0.5 mg L^{-1} of kinetin for all explant types. Additionally, epicotyl and cotyledon produced 100% callus induction with 4 mg L^{-1} of NAA plus 0.5 mg L^{-1} of kinetin. However, no callus was derived from NAA combined with 1 mg L^{-1} BAP except in stem explants.

Reporting similar findings, Piccirilli et al. (1988) observed satisfactory callus induction with the hypocotyl explant of *L. tenuis* by using an equal ratio (1:1) of NAA and kinetin. In addition, Pupilli et al. (1990) reported that the best callus induction with the

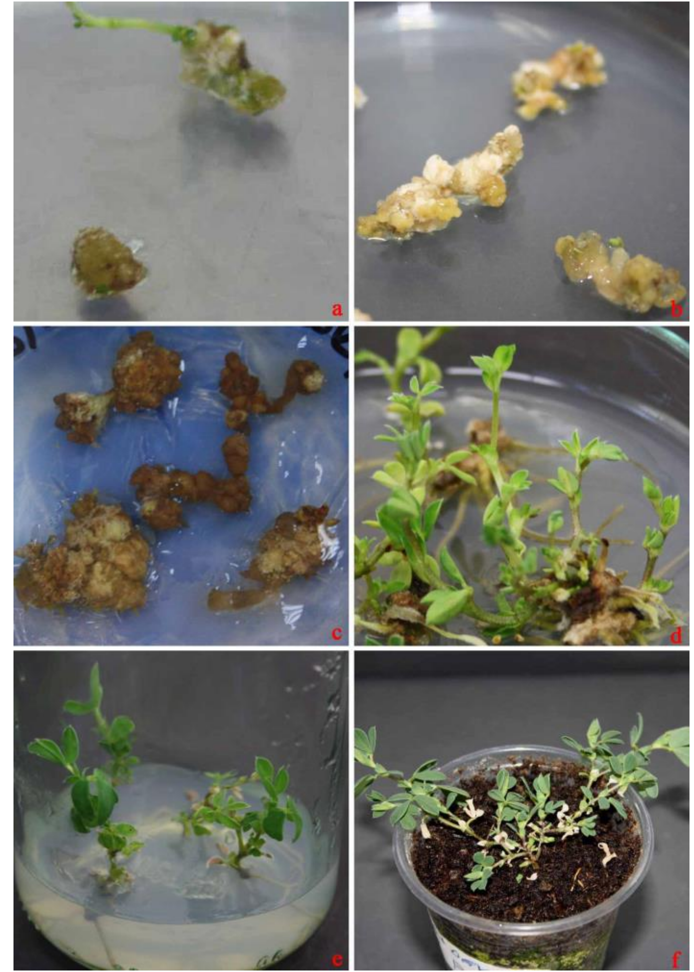

Figure 2. An efficient regeneration system in *L. maritimus*; (a) formation of compact calli from the stem in MS medium containing 2 mg L^{-1} of NAA after 20-30 d culture initiation, (b) induction of soft and compact calli from hypocotyl and epicotyl explants, (c) turn of root-derived calluses to brown, (d) chlorophyll and shoot formation on calli two weeks later in the illuminated condition, (e) rooting of shoots in half-strength MS media containing 1 mg L^{-1} of NAA, (f) acclimatization of rooted shoots to the external condition.

Şekil 2. L. maritumus da etkili bir rejenerasyon sistemi; (a) kültür başlangıcından 20-30 gün sonra 2 mg L^{-1} NAA içeren MS ortamında sap parçalarından kompakt kallusların oluşumu, (b) hipokotil ve epikotil parçalarından yumuşak ve kompakt kallusların oluşumu, (c) köklerden elde edilen kallusların kahverengiye dönmesi, (d) ışıklı ortamda 2 hafta sonra klorofil ve sürgün oluşumu, (e) 1 mg L^{-1} NAA içeren yarı kuvvetli MS ortamında sürgünlerin köklendirilmesi, (f) köklü sürgünlerin dış koşullara alıştırılması.

Table 1. Analysis of variance, mean values and differences between mean values of *L. maritimus* explants cultured at various NAA concentrations and cytokinin types.

Çizelge 1. L. maritimus eksplantlarının farklı NAA konsantrasyonları ve sitokin tiplerinde kültürlenmesiyle oluşan varyans analiz sonuçları, ortalama değerler ve ortalama değerler arasındaki farklar.

Factors *(Faktörler)*	Callus induction (%) *(Kallus oluşumu)*	Plant regeneration (shoots per callus) *(Bitki rejenerasyonu)*
Explant types *(Eksplant tipleri)*		
Stem *(Sap)*	66.15[a†]	1.16[a]
Epicotyl *(Epikotil)*	61.48[a]	0.84[a]
Hypocotil *(Hipokotil)*	52.59[b]	0.96[a]
Root *(Kök)*	46.66[b]	0.00[b]
Cotyledon *(Kotiledon)*	50.37[b]	0.07[b]
NAA concentrations (mg L^{-1}) *(NAA konsantrasyonları)*		
1	63.18[a]	0.95[a]
2	67.55[a]	0.74[a]
4	35.55[b]	0.12[b]
Cytokinin types *(Stokinin tipleri)*		
0 (control)	74.66[b]	0.96[a]
1 mg L^{-1} BAP	7.55[c]	0.34[b]
0.5 mg L^{-1} Kınetın	84.54[a]	0.54[b]
Analysis of variance *(Varyans analizi)*		
Explant types (Et)	*	*
NAA concentrations (Nac)	*	*
Cytokinin types (C)	*	*
EtxNac	*	*
EtxC	*	*
NacxC	*	ns
EtxNacxC	*	*

*: significant level of %1, ns: non-significant. †: letters show different groups at 5% level.

Table 2. The effect of different NAA concentrations and cytokinin types on callus induction in different explant types (%).

Çizelge 2. Farklı eksplant tiplerinde farklı NAA konsantrasyonlarının ve sitokinin tiplerinin kallus indüksiyonu üzerine etkisi (%).

		Cytokinin types *(Stokinin tipleri)*		
Explant types *(Eksplant tipleri)*	NAA concentrations (mg L^{-1}) *(NAA konsantrasyonları)*	0 (control)	1 mg L^{-1} BAP	0.5 mg L^{-1} Kinetin
Stem *(Sap)*	1	86.66 [a-c]*	53.33 [e-g]	80.00 [b-f]
	2	100.0 [a]	60.00 [d-g]	100.0 [a]
	4	86.00 [a-c]	0.00 [ı]	33.33 [gh]
Epicotyl *(Epikotil)*	1	93.33 [ab]	0.00 [ı]	100.0 [a]
	2	80.00 [a-e]	0.00 [ı]	100.0 [a]
	4	80.00 [a-e]	0.00 [ı]	100.0 [a]
Hypocotyl *(Hipokotil)*	1	80.00 [a-e]	0.00 [ı]	100.0 [a]
	2	93.33 [ab]	0.00 [ı]	86.66 [a-d]
	4	46.66 [fg]	0.00 [ı]	66.66 [c-f]
Root *(Kök)*	1	100.0 [a]	0.00 [ı]	100.0 [a]
	2	100.0 [a]	0.00 [ı]	100.0 [a]
	4	20.00 [hı]	0.00 [ı]	0.00 [ı]
Cotyledon *(Kotiledon)*	1	60.00 [d-g]	0.00 [ı]	100.0 [a]
	2	93.33 [ab]	0.00 [ı]	100.0 [a]
	4	0.00 [ı]	0.00 [ı]	100.0 [a]

* Letters show different groups at 5% level.

leaf and cotyledon explants of *L. pedunculatus* in media containing 2 mg L^{-1} of 2,4-D plus 0.25 mg L^{-1} of kinetin, and similar to the results presented here, callus was not induced by adding BAP in the presence of 2,4-D and NAA. In another study, equal ratios of 2,4-D and kinetin-containing media facilitated effective

callus induction in the hypocotyl, leaf, stem, root, and cotyledon explants of *L. angustissimus* L. (Nenz et al.,1996).

Among other results, no shoot regeneration occurred without callus induction, and plant regeneration ranged from 0.0 to 3.56 shoots per callus (Table 3). The highest plant regeneration was obtained by culturing epicotyl explants in a concentration of 1 mg L^{-1} of NAA; however, that value did not achieve a significant difference compared with plant regeneration values obtained with hypocotyls and stems cultured in 1 mg L^{-1} of NAA and 1 or 2 mg L^{-1} of NAA plus 1 mg L^{-1} of BAP, respectively. With 4 mg L^{-1} of NAA, the explants produced no shoots except in the stems with the control and 0.5 mg L^{-1} kinetin.

Contrary to those findings, the addition of BAP or kinetin to the culture media for different *Lotus* species positively affected shoot regeneration. Akashi et al. (1998) reported that morphogenic callus forming shoots from both cotyledon and hypocotyl explants were obtained at doses of 0.5 mg L^{-1} of BAP with 0, 0.01, or 0.1 mg L^{-1} of NAA. Piccirilli et al. (1988) indicated that leaf explants produced a higher rate of shoots than cotyledon and hypocotyl explants at a concentration of 1 mg L^{-1} of NAA with 0.5 mg L^{-1} of BAP. According to Nenz et al. (1996), hypocotyl explants provided a high rate of shoot regeneration in media containing 1 mg L^{-1} of NAA with 0.25 kinetin or 0.25 BAP.

Table 3. The effect of different NAA concentrations and cytokinin types on plant regeneration in different explant types (shoots per callus).

Çizelge 3. Farklı NAA konsantrasyonlarının ve sitokinin tiplerinin farklı eksplant tiplerinde bitki rejenerasyonuna etkisi (kallus başına sürgünler).

Explant types *(Eksplant tipleri)*	NAA concentrations (mg L^{-1}) *(NAA konsantrasyonları)*	Cytokinin types *(Stokinin tipleri)* 0 (control)	1 mg L^{-1} BAP	0.5 mg L^{-1} kinetin
Stem *(Sap)*	1	0.66^{ef*}	2.53^{a-d}	0.65^{ef}
	2	2.00^{b-d}	2.66^{a-c}	0.00^{f}
	4	1.50^{c-d}	0.00^{f}	0.33^{ef}
Epicotyl *(Epikotil)*	1	3.56^{a}	0.00^{f}	1.33^{c-f}
	2	1.46^{c-e}	0.00^{f}	1.20^{d-f}
	4	0.00^{f}	0.00^{f}	0.00^{f}
Hypocotyl *(Hipokotil)*	1	3.20^{ab}	0.00^{f}	2.00^{b-d}
	2	1.33^{c-f}	0.00^{f}	2.15^{b-d}
	4	0.00^{f}	0.00^{f}	0.00^{f}
Root *(Kök)*	1	0.00^{f}	0.00^{f}	0.00^{f}
	2	0.00^{f}	0.00^{f}	0.00^{f}
	4	0.00^{f}	0.00^{f}	0.00^{f}
Cotyledon *(Kotiledon)*	1	0.33^{ef}	0.00^{f}	0.00^{f}
	2	0.33^{ef}	0.00^{f}	0.00^{f}
	4	0.00^{f}	0.00^{f}	0.00^{f}

* Letters show different groups at 5% level.

CONCLUSION

In this study, the *in vitro* regeneration potential of *L. maritimus* was investigated for the first time. The best explants in terms of both callus induction and plant regeneration were the stem, epicotyl, and hypocotyl, and plant regeneration was adversely affected with an NAA concentration after 2 mg L^{-1}. Whereas 1 or 2 mg L^{-1} of NAA plus 0.5 mg L^{-1} of kinetin achieved greater callus induction, 1 or 2 mg L^{-1} of NAA concentrations achieved greater plant regeneration. In addition, plant regeneration was negatively affected when BAP was added to the media in all explant types except stem. As a result, the highest plant regeneration of the species can be achieved by culturing epicotyl or hypocotyl explants on media containing 1 mg L^{-1} of NAA. That method allows the proliferation of *L. maritimus* genotypes with superior qualities in pure lines in an extremely short time. Nevertheless, other explant types, media, and growth regulators should be investigated to shed additional light on the regeneration potential of *L. maritimus*.

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Statement of Conflict of Interest

Author has declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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