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Araştırma Makalesi - Research Article

Effects of Thin Cell Layer Technique and Nutrient Media Contents of Regeneration and Browning Characteristics of the *Laurus nobilis L.* Explants

İnce Hücre Tabaka Tekniğinin ve Besin Ortamı İçeriklerinin *Laurus nobilis* L. Eksplantlarının Rejenerasyon ve Kararma Karakteristiklerine Etkileri

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ABSTRACT

The aim of this study is in order to investigate the effects of explant source, explant type and MS media composition containing different concentrations of sucrose, activated carbon and Coconut milk for regeneration and browning of *Laurus nobilis* L. using thin cell layer (TCL) culture system. A higher rate of callus (57.15%) and shoot (2%) regeneration and a lower rate of blackening were determined in transversely cut stem TCL explants compared to leaf explants. While 1.33% callus regeneration was achieved in leaf explants; shoot regeneration could not be achieved. While more callus regeneration (35.17%) was found in explants taken from the field, more shoot regeneration (1.5%) and lower rate of browning were obtained in explants taken from *in vitro*. In the sugar trials, the highest callus regeneration (40.83%) was defined in MS medium containing 30 g/L sucrose supplemented with 1 mg/L BAP, and the highest shoot regeneration (2.5%) was determined in MS medium containing 45 g/L and 60 g/L sucrose supplemented with 1 mg/L BAP. When explant type, explant source and nutrient media composition are considered together; the highest callus regeneration (100%) was obtained in field-sourced stem TCL explants cultured in medium containing 25 mL/L coconut milk and 1 mg/L BAP. The highest shoot regeneration (6.6%) was determined in *in vitro* stem TCL explants cultured in MS media containing 30, 45, 60 g/L sucrose and 1 mg/L BAP. The lowest percentage of browning (50%) was obtained from *in vitro* stem TCL explants cultured in MS medium containing 2 g/L activated carbon and 1 mg/L BAP.

Keywords*- browning; *callus; Laurus nobilis L.; regeneration; Thin Cell Layer

ÖZ

Bu çalışmanın amacı, İnce Hücre Tabaka (TCL) sistemi kullanılarak *Laurus nobilis* L. bitkisinin rejenerasyonu ve kararması üzerine eksplant kaynağı, eksplant tipi ve farklı konsantrasyonlarda sükroz, aktif karbon ve Hindistan cevizi sütü içeren MS ortamı kompozisyonunun etkilerini araştırmaktır. Enine kesilmiş sap TCL eksplantlarında yaprak eksplantlarına kıyasla daha yüksek oranda kallus (%57.15) ve sürgün (%2) rejenerasyonu ve daha düşük

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oranda kararma belirlenmiştir. Yaprak eksplantlarında %1.33 oranında kallus rejenerasyonu elde edilirken; sürgün rejenerasyonu elde edilememiştir. Arazideki dişi *Laurus nobilis* L. ağacından alınan eksplantlarda daha fazla kallus rejenerasyonu (%35,17) elde edilirken; *in vitro* koşullarda çimlendirilen tohumlardan alınan eksplantlarda daha yüksek oranda sürgün rejenerasyonu (%1,5) ve daha düşük oranda kararma belirlenmiştir. Şeker denemelerinde, en yüksek kallus rejenerasyonu (%40,83) 30 g/L sükroz içeren besin ortamında; en yüksek sürgün rejenerasyonu (%2,5) 45 g/L ve 60 g/L sükroz içeren besin ortamında elde edilmiştir. Eksplant kaynağı, eksplant tipi ve besin ortamı kompozisyonu interaksiyonu beraber incelendiğinde, en yüksek kallus rejenerasyonu (%100) 25 mL/L Hindistan cevizi sütü içeren besin ortamında kültüre alınan arazi kaynaklı sap eksplantlarında; en yüksek sürgün rejenerasyonu (%6,6) 30 g/L, 45 g/L ve 60 g/L sükroz içeren besin ortamında kültüre alınan *in vitro* kaynaklı sap TCL eksplantlarında; en düşük kararma yüzdesi (%50), 2 g/L aktif karbon içeren besin ortamında kültüre alınan *in vitro* kaynaklı sap TCL eksplantlarında elde edilmiştir.

Anahtar Kelimeler- kararma; kallus; Laurus nobilis L.; rejenerasyon, İnce Hücre Tabaka

I. INTRODUCTION

The homeland of the *Laurus nobilis* L. plant is stated as the Mediterranean countries in some sources, and Western Asia Minor (Anatolia) and the Balkans in some sources. There are approximately 45 genera and 1000 species in the Lauraceae family, and two species in the *Laurus* genus: *L. canariensis* Willd and *Laurus nobilis* L.. In the plant kingdom, *Laurus nobilis* L. (the laurel plant) is included in the vascular plants subkingdom, seed plants phylum, dicotyledons class, *Magnoliidae* subclass, angiosperms subsection, *Laurales* order, *Lauraceae* family and *Laurus* genus. *Laurus nobilis* has four subspecies: aurea, crispa and undula, which have wavy edges, and angustifolia, which has narrow leaves [1,2].

Laurus nobilis L., a plant of the scrub flora; it is a shrub-shaped plant that can withstand temperatures as low as 15 °C, is dioecious and evergreen, can grow up to 10-15 m tall and has dense branches [3,4]. Its fresh shoots are green and later turn red-black and are naked. Its leaves are fragrant. Its fruit is black in color, resembling an olive, and has a thin, oily wall apart from its chickpea-sized seeds. *Laurus nobilis* L. leaf is 5-10 cm long, its edges are slightly wavy, it has an elliptical structure and tapers towards both ends. The upper surface of the leaf is shiny dark green. Fresh *Laurus nobilis* L. leaves are light green in color and have thin veins. Fresh leaves have little aromatic smell [5].

The most commonly used parts of the plant are its leaves and fruits [6]. *Laurus nobilis* L. leaves, fresh or dried, are used as spices to add flavor to dishes [3]. In addition, since its leaves and fruits have aromatic and stimulating properties [6], the oils obtained from leaves are used in the cosmetic and pharmaceutical industries [3,7]. Essential oils obtained from fruits and seeds are used in the treatment of arthritis, psoriasis, eczema and herpes [7]. *Laurus nobilis* L. tree is also used as an ornamental plant. It can be planted to provide shade in gardens and parks or grown as a small tree. In addition, the branches of the tree are a source of wood used in fireplace fires [8]. 90% of the *Laurus nobilis* L. in the world is found in Turkey and spreads over an area of approximately 150 thousand hectares. Around 22 thousand tons of dried bay leaves are produced every year and approximately 1 million people are employed in this field. When we look at the foreign trade figures in Turkey, it is seen that the total laurel exports increased more than 3 times from 2005 to 2017, and as of 2017, dried laurel exports reached 40 million dollars ($ 36,058,749). Considering the exported by-products, the economic volume of bay laurel is estimated to be over 100 million dollars. Considering the high export potential of bay laurel and the diversity of its by-products, it is predicted that it will become an increasingly demanded product in the world [9].

Laurus nobilis L. areas are being destroyed due to excessive collection and faulty cultural practices. In addition, the production amount is not sufficient to meet the ever-increasing demands. Therefore, studies to protect, propagate and develop the *Laurus nobilis* L. are becoming more important day by day [9]. Like other *Laurus* species, propagation of *L. nobilis* by seedlings is difficult due to poor fruit set, very low germination and double dormancy in the bark [3]. In addition to these, there are restrictions such as the low yield of small seeds obtained from wild trees, pollination difficulties, and the fact that *Laurus nobilis* L. is a forest plant, harvesting the land in every 3 years. For all these reasons, there is a need for alternative propagation procedures such as plant tissue culture techniques that can clonally produce a large number of plants that are not affected by seasonal changes in a short time and in a narrow area [10].

Plant cell, tissue and organ cultures are very valuable tools that are used for basic and applied studies as well as for the commercial market. Plant cell, tissue and organ cultures are techniques designed for the growth and reproduction of plant materials. For this reason, various tissue culture methods are used in plant tissue cultures to protect endangered species and to produce species that are difficult to propagate [11]. Tissue culture techniques have different types of culture such as embryo culture, protoplast culture, meristem culture, anther and pollen

culture, ovary and ovule culture, callus culture and micropropagation [12]. Embryo culture is defined as the isolation of embryos from plant seeds and ovules and then culturing them in nutrient media. The most important issue in embryo culture is to determine a culture environment that supports the regular development of embryos cultured at different development periods. The technique in which protoplasts are cultured is called protoplast culture. When the cell wall of a plant cell is removed, the remaining part is the protoplast. Protoplasts can survive in isotonic environments, form a new cell wall, divide by mitosis, form new cell groups (microcallus) and then new plants. The culture of the meristematic dome smaller than 1 mm is called meristem culture. Meristem cultures are especially used to obtain virus-free plants. Anther culture is the separation of anthers containing immature pollen (microspores) from the buds and placing them in artificial nutrient media under *in vitro* conditions, where haploid embryos are obtained from immature pollen. The formation of haploid embryos and plants by culturing unfertilized ovaries or egg cells is called ovary or ovule cultures [13].

Micropropagation, one of the plant cell, tissue and organ culture techniques, is the process of propagating and rooting shoots obtained directly (organogenesis or somatic embryogenesis) or indirectly (callus, protoplast, etc.) from organized meristems and somatic cells that are not yet mature or have completed their maturation [13]. Callus, plant wound tissue, are cell masses without morphological order obtained by placing explants taken from the mother plant that have not lost their ability to divide into sterile nutrient media under *in vitro* conditions [14]. Callus can initiate from various plant parts such as pollen, embryo, petiole, root parts and internodes, where cells with the ability to divide are located [12]. Callus culture is a technique in which organ or tissue explants taken from plants are cultured in in vitro conditions, mostly in semi-solid nutrient media containing carbon sources and plant growth regulators, and unorganized cells are formed [11]. The process by which plant organs are derived from the callus mass is called indirect organogenesis [15]. In indirect organogenesis, plant regeneration is carried out by taking advantage of the pluripotent feature of the callus [16]. The production of buds or shoots directly from the tissue without the callus stage is called direct organogenesis [15]. In somatic embryogenesis, plant somatic cells undergo differentiation into embryonic stem cells and then form full plants through embryonic development, indicating that plant cells are totipotent thanks to the embryogenic callus. Somatic embryos can be produced directly from somatic cells or indirectly from embryonic callus. Under suitable conditions, somatic embryos can develop into shoots and roots. Callus occurs in both indirect organogenesis and indirect embryogenesis; however, the properties of the callus are different. Somatic embryogenesis induces embryogenic callus with totipotency and then into a somatic embryo, while organogenesis induces non-embryogenic callus with pluripotency. Furthermore, indirect organogenesis can cause genetic instability and somaclonal variance [16].

In the thin cell layer (TCL) culture system, longitudinal thin cell layer culture (lTLC) is obtained from different plant organs (stems, leaves, inflorescences, flower organs, apical region or embryo) by cutting them longitudinally when 1-2 mm remains, or transverse thin cell layer culture (tTCL) is obtained by cutting them transversely. While lTCLs are cut longitudinally and contain only one tissue type, such as a single layer of epidermal cells; now that TCLs are cut transversely, they contain a small number of cells from different tissue types [17,18]. Since the surface area of an explant in contact with the nutrient medium is greater in TCL explants compared to conventional explants, the components in the nutrient medium can reach the recipient cells of the explants more. This allows faster and more successful *in vitro* growth and morphogenesis to be observed in TCL explants [19]. Although they are much smaller than traditional explants, the regeneration potential of explants obtained with the TCL culture system is considerably higher than traditional explants. Factors such as explant type, morphological characteristics and source of the explant directly affect the regeneration potential [20]. TCL culture is also an effective method for inducing specific morphogenic pathways such as controlled somatic embryo production [18].

In addition to being used for in vitro micropropagation and synthetic seed production, the TCL technique is also successfully used in cryopreservation and genetic research [19]. It is also a very advantageous method for the propagation and protection of endangered and economically important plants [21,22]. One of the important advantages of the TCL technique is its ability to induce somatic embryogenesis, the formation of embryos from somatic cells. In this way, it is used as a highly efficient method for clonal propagation as it enables the production of many genetically identical plants from a single explant [20].

There are a number of factors that affect the regeneration capacity of TCL explants. The influence of parent plant and origin is an important factor, but other biotic factors such as genotype (species and variety), tissue or organ from which the explant is prepared, age, size and shape of the parent tissue or organ also affect regeneration success. While genotype determines the regeneration ability of plants through their genetic structure and characteristics, the selected tissue or organ of the explant also affects its regeneration potential. Younger tissues or organs from the parent plant may often have higher regeneration capacity. Besides, the regeneration of TCL explants is also related to the size and shape of the donor tissue, since tissues or organs of a certain size and shape may show better regeneration. Biotic factors such as microbial infections (fungi, bacteria or viruses) are also important. Therefore, it is important to ensure sterile working conditions. Additionally, growth hormones,

nutrients, light conditions and other physical environmental factors used in tissue culture may also affect regeneration capacity. Trial and error methods can be used to determine optimal regeneration conditions [18,20].

Within the scope of this study, the regeneration of L *Laurus nobilis* L., which is difficult to propagate by seed and vegetative propagation methods, was attempted under *in vitro* conditions. Due to the increasing demand and the excessive and destructive use of its natural distribution areas, alternative production techniques are needed for the production of this plant. At this point, it was decided to use the TCL culture technique, which offers a wide range of advantages compared to other plant tissue culture techniques. In addition, within the scope of this study, various experiments were carried out to determine the effect of explant source, TCL type and nutrient medium composition on callus and shoot regeneration. When the literature studies on the regeneration of the *Laurus nobilis* L. were examined so far, no study using the TCL technique was found. Therefore, this study is intended to be pioneering and guiding. When the data obtained from this study were evaluated, it was aimed to establish an effective regeneration protocol by determining the best explant source, TCL type and nutrient medium composition for callus and shoot regeneration in *Laurus nobilis* L.

II. MATERIAL AND METHOD

A. Plant Material

In this study seed, leaf and stem explants to be obtained from the female tree of *Laurus nobilis* L. at the Aegean Agricultural Research Institute were used as starting materials. After the seeds were germinated under *in vitro* conditions, the leaf and stem parts *in vitro* plantlets and also the leaf and stem parts taken from the female tree were cut transversely using the TCL technique and used for regeneration experiments (Figure 1).

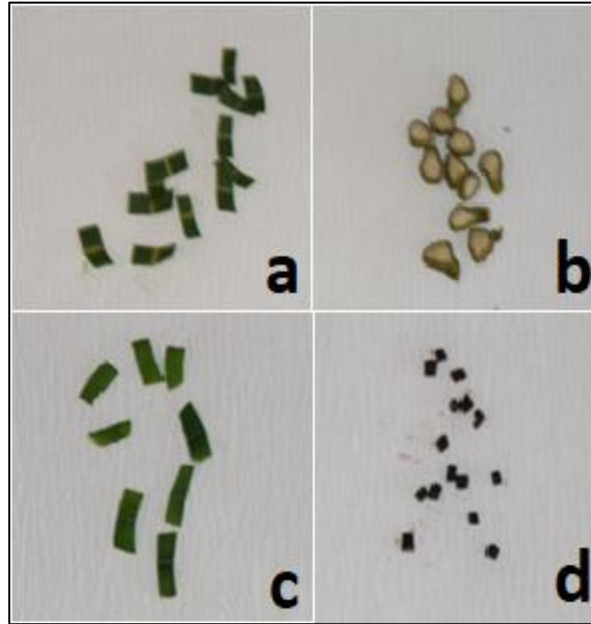

Figure 1. TCL explants a. leaf TCL explants taken from the female tree, b. stem TCL explants taken from the female tree, c. leaf TCL eksplants taken from *in vitro* plantlets, d. stem TCL explants taken from *in vitro* plantlets.

B. Sterilization

Seed, leaf and stem explants taken from the field were surface sterilized. For the sterilization of leaf explants, firstly, pre-washing (rinsing in water with detergent for 1 minute and waiting in running water for 10 minutes) and then sterilization in a laminar air flow cabinet (waiting in 0.1% $HgCl_2$ for 1 minute, then rinsing with sterile water 5 times) were carried out. For the sterilization of stem explants, the method of keeping them in 70% ethyl alcohol during 1 minute and in 25% bleach for 5 minutes in a laminar air flow cabinet and then rinsing with sterile water 5 times was used. For sterilization of seeds without pericarp, they were kept in 70% ethyl alcohol for 1 minute and 0.1% $HgCl_2$ for 5 minutes in a laminar flow cabinet and then rinsed with sterile water 5 times.

C. Germination

A cut was made on the edge of the seed to break dormancy. Afterwards, the seeds were cultured in glass tubes containing 10 mL MS [23] nutrient medium supplemented with 1 mg/L BAP (30 g/L sucrose, 3 g/L gelrite, pH 5.8).

D. Regeneration

Both sterilized leaf and stem explants taken from female *Laurus nobilis* L. tree and also taken from seeds germinated under *in vitro* conditions were cut transversely at a thickness of 0.5 - 1 mm using the TCL system and cultured in the nutrient media containing with different amount of sucrose, activated carbon and Coconut milk specified in Table 1. Explants were maintained in a 16-hour light and 8-hour dark photoperiod, 24 ± 2 ºC and 3500 lux light intensity.

Table 1. Nutrient media codes and ingredients

Nutrient media codes	Amount of sucrose (g/L)	Amount of activated carbon (g/L)	Amount of Coconut Milk (mL/L)
B1	15 g/L	-	-
B2	30 g/L	-	-
B3	45 g/L	-	-
B4	60 g/L	-	-
B5	30 g/L	0.5 g/L	-
B6	30 g/L	1 g/L	-
B7	30 g/L	1.5 g/L	-
B8	30 g/L	2 g/L	-
B9	30 g/L	-	25 ml/L
B10	30 g/L	-	50 ml/L

*MS-based nutrient media containing 1 mg/L BAP were used (3g/L gelrite).

E. Statistical evaluation

The trials were carried out with 3 replications, with 10 explants in each trial, according to the randomized plot design. Minitab 17 Statistical Software (Minitab Inc, PA, USA) was used to evaluate the data obtained. Statistically, differences between applications were compared based on the Tukey test with an error limit of %5 and expressed with different letters.

III. RESULTS AND DISCUSSION

A. *Effect of explant type on callus regeneration, shoot regeneration and browning*

When the effect of explant type on callus and shoot regeneration is examined; a higher percentage (%) was defined in stem TCL explants compared to leaf TCL explants taken from field and *in vitro*. While 57.15% callus-forming explant percentage were obtained from stem TCL explants; in leaf TCL explants, this value was found as 1.33%. Considering the effect of explant type on shoot formation, shoot regeneration was not achieved in leaf TCL explants; 2% shoot regeneration rate was determined in stem TCL explants. Browning was observed at a rate of 100% in leaf TCL explants and 87.5% in stem TCL explants (Table 2).

Table 2. Effect of explant type on callus formation, shoot formation and browning

Explant type	Percentage of explants forming callus (%)	Percentage of explants forming shoot (%)	Browning percentage (%)
Stem	57.15**a**±3,91	2**a**±0.572	87.5**b**±3.15
Leaf	1.33**b**±0,556	0**b**±0	100**a**±0

*Tukey was applied for forming callus, Fisher was applied for forming shoot and for browning.

B. ***Effect of explant source on callus regeneration, shoot regeneration and browning***

When the explant source was considered in terms of callus formation, a higher rate of callus was obtained in TCL explants taken from the field compared to TCL explants taken from plants grown *in vitro*. While determining the percentage of callus-forming explants in TCL explants taken from the field as 35.17%; in TCL explants taken from *in vitro*, this value was found 23.32%. The highest percentage of shoot-forming explants (1.5%) was defined in TCL explants taken from *Laurus nobilis* L. seeds germinated *in vitro*. Very similar browning rates (%) of explants taken from the field and from *Laurus nobilis* L. plants germinated under i*n vitro* conditions were obtained (Table 3).

Table 3. Effect of explant source on callus regeneration, shoot regeneration and browning

Explant source	Percentage of explants forming callus (%)	Percentage of explants forming shoot (%)	Browning percentage (%)
Field	35.17**a**±5,21	0.5**a**±0.284	94.17**a**±2.44
In vitro	23.32**b**±3,70	1.5**a**±0.522	93.33**a**±2.30

*Tukey was applied for forming callus, Fisher wat applied for forming shoot and browning.

C. ***Effect of nutrient media on callus regeneration, shoot regeneration and browning***

The highest percentage of callus-forming explants (40.83%) was obtained in B2 (MS + 1 mg/L BAP + 30 g/L sucrose) nutrient medium. The highest shoot regeneration (2.5%) was defined in B3 (MS + 1 mg/L BAP + 45 g/L sucrose) and B4 (MS +1 mg/L BAP + 60 g/L sucrose) nutrient media. No shoot regeneration was observed on B5 (MS + 1 mg/L BAP+ 0.5 g/L activated carbon), B6 (MS + 1 mg/L BAP+ 1 g/L activated carbon), B7 (MS + 1 mg/L BAP+ 1.5 g/L activated carbon) and B8 (MS + 1 mg/L BAP + 1.2 g/L activated carbon) nutrient media. The lowest browning percentage was determined as 77.5% in B8 (MS + 1 mg/L BAP + 1.2 g/L activated carbon) nutrient medium. The highest percentage of browning explants (100%) was found in B1 (MS + 1 mg/L BAP + 15 g/L sucrose), B2 (MS + 1 mg/L BAP + 30 g/L sucrose), B4 (MS + 1 mg/L BAP + 60 g/L sucrose), B9 (MS + 1 mg/L BAP + 25 ml/L coconut water) and B10 (MS + 1 mg/L BAP + 50 ml/L coconut water) nutrient media (Table 4).

Table 4. Effect of nutrient media on callus regeneration, shoot regeneration and browning

Nutrient media code	Percentage of explants forming callus (%)	Percentage of explants forming shoot (%)	Browning percentage (%)
B1	26.67**ab**±8.01	0.83**a**±0.833	100**a**±0
B2	40.83**a**±11.8	1.67**a**±1.12	100**a**±0
B3	19.17**b**±6.68	2.50**a**±1.79	97.5**ab**±2.5
B4	17.5**b**±7.99	2.50**a**±1.31	100**a**±0
B5	29.92**ab**±11.4	0**a**±0	86.67**bc**±7
B6	31.67**ab**±10.3	0**a**±0	85.83**bc**±7.83
B7	33.33**ab**±10.7	0**a**±0	90**abc**±7.18
B8	36.67**ab**±11.6	0**a**±0	77.5**c**±8.89
B9	32.50**ab**±12.5	1.67**a**±1.12	100**a**±0
B10	24.17**ab**±11.3	0.83**a**±0.833	100**a**±0

*Tukey was applied for forming callus, Fisher was applied for forming shoot and browning.

D. ***Effect of explant type, explant source and nutrient medium interaction on callus regeneration, shoot regeneration and browning***

Regarding the explant type*explant source*nutrient medium interaction on callus formation, the highest percentage of callus-forming explants (100%) was obtained in the stem TCL explants of the female *Laurus nobilis* L. tree in the field cultured in B9 (MS + 1 mg/L BAP + 25 mL/L coconut milk) nutrient medium; the lowest callus-forming explant percentages (0%) were obtained both *in vitro* and in field leaf TCL explants. The highest shoot formation percentage (6.66%) was found in the stem TCL explants from *in vitro* cultured in B2 (MS + 1 mg/L BAP + 30 g/L sucrose), B3 (MS + 1 mg/L BAP + 45 g/L sucrose) and B4 (MS + 1 mg/L BAP + 60 g/L sucrose).

No shoot regeneration was observed in leaf explants taken from *in vitro* and the field. The lowest percentage of browning explants (50%) was defined from the stem TCL explants of the female *Laurus nobilis* L. tree in the field cultured in B8 (MS + 1 mg/L BAP + 2 g/L activated carbon) nutrient medium (Table 5).

Table 5. Effect of explant type, explant source and nutrient medium interaction on callus regeneration, shoot regeneration and browning

Explant source	Explant type	Nutrient media	Percentage of explants forming callus (%)	Percentage of explants forming shoot (%)	Browning percentage (%)
IN VITRO	STEM	B1	23.33**efg**±6.67	3.33**ab**±3.33	100**a**±0
		B2	63.33**abcdef**±3.33	6.67**a**±3.33	100**a**±0
		B3	40**cdefg**±5.77	6.67**a**±6.67	90**ab**±10
		B4	26.67**defg**±6.67	6.67**a**±6.67	100**a**±0
		B5	46.33**bcdefg**±27.5	0**b**±0	83.33**abcd**±16.7
		B6	63.33**abcdef**±13.3	0**b**±0	70**bcde**±15.3
		B7	66.67**abcdef**±20.3	0**b**±0	73.33**bcde**±26,7
		B8	70**abcde**±5.77	0**b**±0	50**e**±0
		B9	30**defg**±10	3.33**ab**±3.33	100**a**±0
		B10	10**g**±0	3.33**ab**±3.33	100**a**±0
	LEAF	B1	16.67**fg**±3.33	0**b**±0	100**a**±0
		B2	6.67**g**±3.33	0**b**±0	100**a**±0
		B3	0**g**±0	0**b**±0	100**a**±0
		B4	0**g**±0	0**b**±0	100**a**±0
		B5	0**g**±0	0**b**±0	100**a**±0
		B6	0**g**±0	0**b**±0	100**a**±0
		B7	3.33**g**±3.33	0**b**±0	100**a**±0
		B8	0**g**±0	0**b**±0	100**a**±0
		B9	0**g**±0	0**b**±0	100**a**±0
		B10	0**g**±0	0**b**±0	100**a**±0
FIELD	STEM	B1	66.67**abcdef**±12	0**b**±0	100**a**±0
		B2	93.33**ab**±3.33	0**b**±0	100**a**±0
		B3	36.67**cdefg**±14.5	3.33**ab**±3.33	100**a**±0
		B4	43.33**bcdefg**±26	3.33**ab**±3.33	100**a**±0
		B5	73.33**abcde**±12	0**b**±0	63.33**cde**±18.6
		B6	63.33**abcdef**±12	0**b**±0	73.33**bcde**±26.7
		B7	63.33**abcdef**±8.82	0**b**±0	86.67**abc**±13.3
		B8	76.67**abcd**±14.5	0**b**±0	60**de**±26.5
		B9	100**a**±0	3.33**ab**±3.33	100**a**±0
		B10	86.67**abc**±13.3	0**b**±0	100**a**±0
	LEAF	B1	0**g**±0	0**b**±0	100**a**±0
		B2	0**g**±0	0**b**±0	100**a**±0
		B3	0**g**±0	0**b**±0	100**a**±0
		B4	0**g**±0	0**b**±0	100**a**±0
		B5	0**g**±0	0**b**±0	100**a**±0
		B6	0**g**±0	0**b**±0	100**a**±0
		B7	0**g**±0	0**b**±0	100**a**±0
		B8	0**g**±0	0**b**±0	100**a**±0
		B9	0**g**±0	0**b**±0	100**a**±0
		B10	0**g**±0	0**b**±0	100**a**±0

*Tukey was applied for forming callus, Fisher was applied for forming shoot and browning.

Callus and shoot regeneration achieved and browning observed in experiments using different 10 nutrient media, 2 explant types and 2 explant sources are given in Figure 2, Figure 3 and Figure 4.

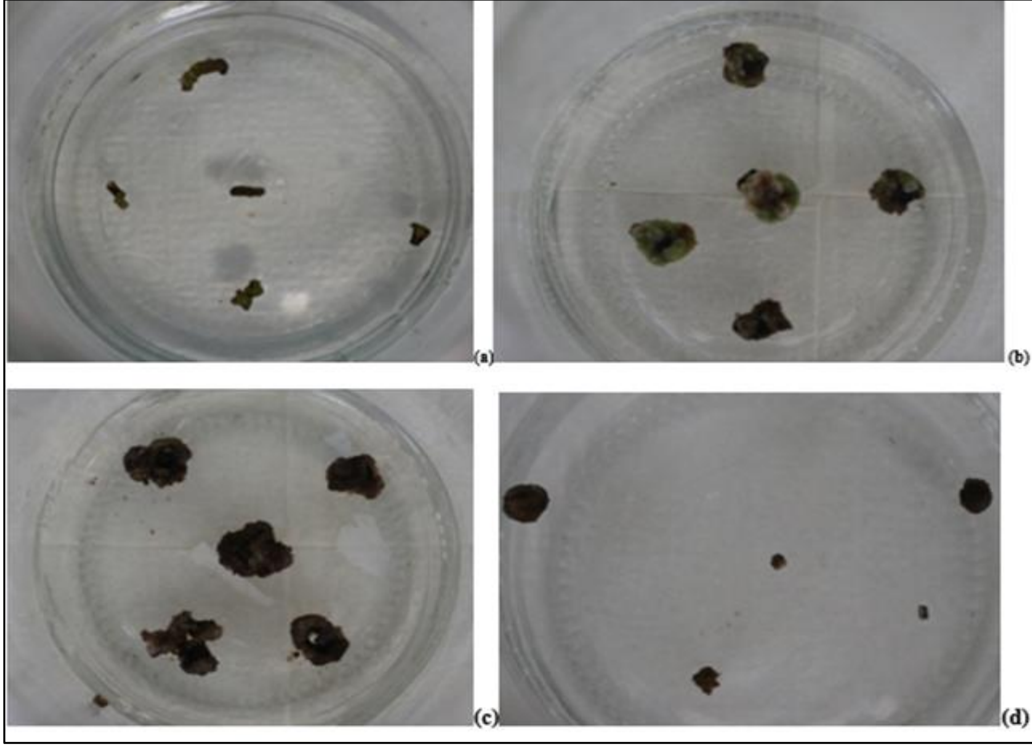

Figure 2. Calli regeneration obtained in the trials: a. Calli formed on explants belonging to the *in vitro* sourced leaf explant*B2 nutrient medium, b. Calli formed on explants belonging to the field sourced stem explant*B2 nutrient medium, c. Calli formed on explants belonging to the field sourced stem explant*B10 nutrient medium, d. Calli formed on explants of *in vitro* derived stem explant*B2 nutrient medium.

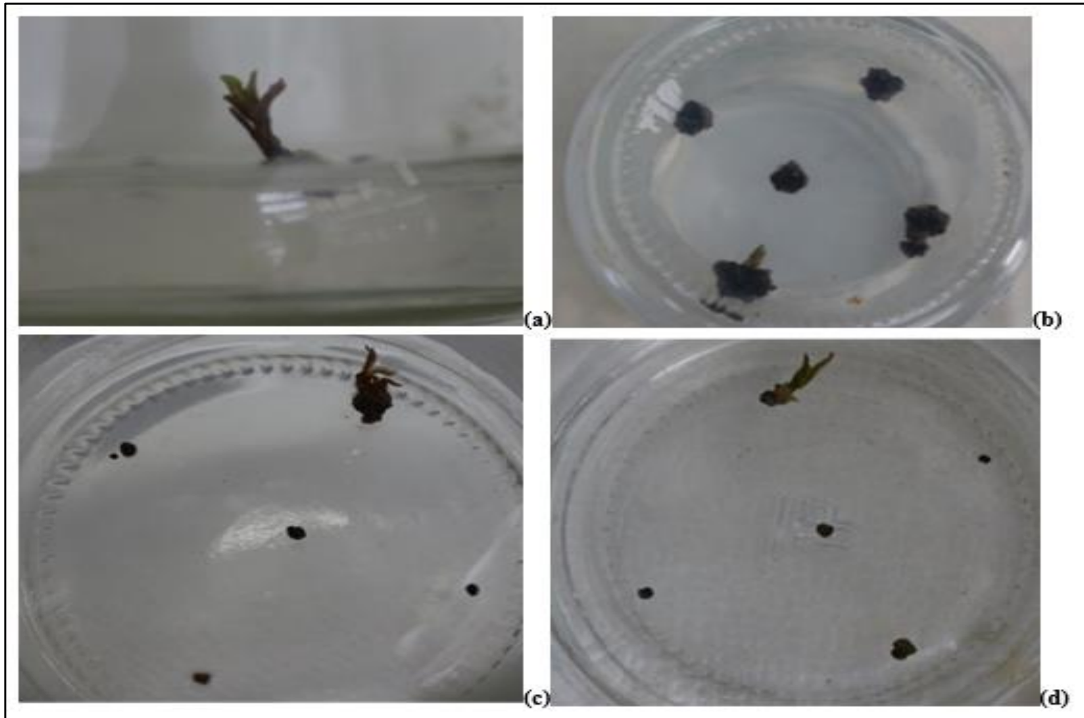

Figure 3. Shoot regeneration achieved in the trials: a. Shoots formed in explants of *in vitro* sourced stem explant*B4 nutrient medium, b. Shoots formed on explants belonging to the in vitro sourced stem explant*B9 nutrient medium, c.Shoots formed in explants belonging to the field derived stem explant*B10 nutrient medium, d.Shoot formation on explants of *in vitro* sourced stem explant*B9 nutrient medium.

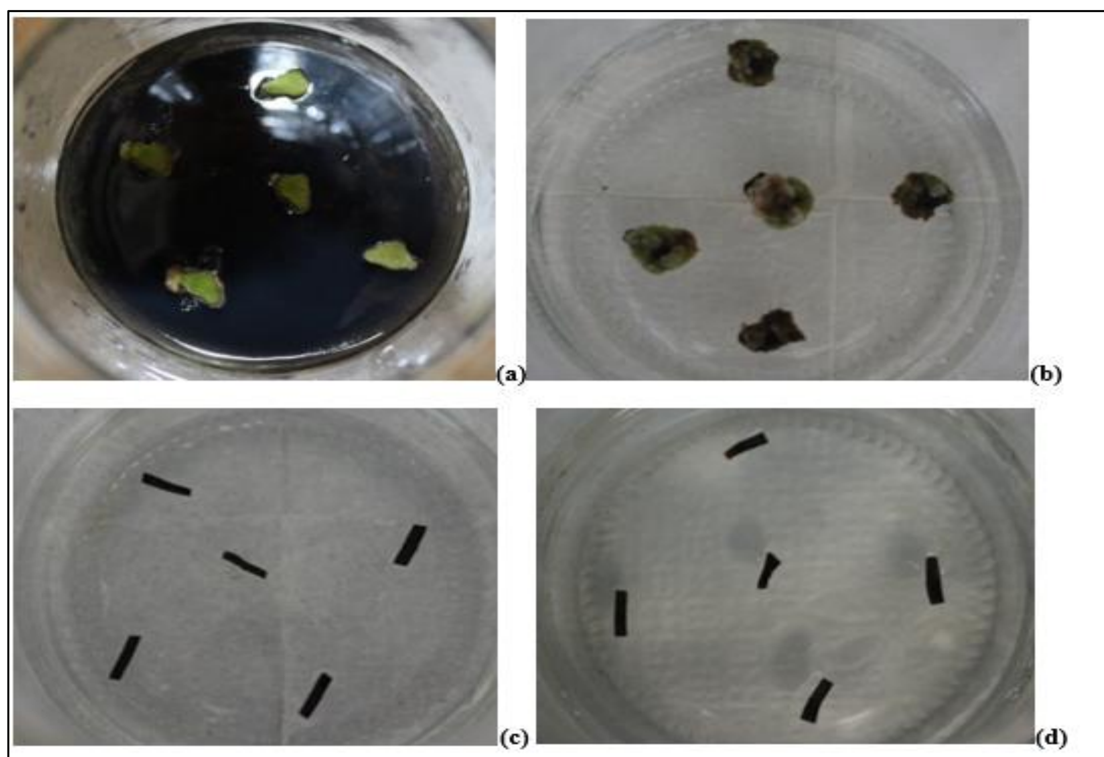

Figure 4. Browning situation observed in the trials: a. No browning explants belonging to the field-derived stem explant*B8 nutrient medium, b. Explants belonging to the field-derived stem explant*B9 nutrient medium, c. Explants belonging to the field-derived leaf explant*B1 nutrient medium, d. Explants of *in vitro* sourced leaf explant*B4 nutrient medium.

IV. DISCUSSION AND CONCULUSION

In this study, an attempt was made to determine the most suitable explant source, explant type and nutrient medium combination for the propagation of the bay plant, which is difficult to propagate by traditional methods, with alternative production techniques. In this context, leaf and stem explants taken both from the field and from seeds germinated under *in vitro* conditions were cut and cultured using the TCL technique. Since 1973, TCL explants have been used to check the organogenesis potential of a wide variety of plants. This is because the TCL system contains a wide range of advantages: higher regeneration potential compared to traditional *in vitro* methods [24], more effective transport of media components to target cells, early morphogenesis [25], cells in the TCL system have more contact with the nutrients and growth regulators in the culture medium than all explants [26], and micropropagation is easier and more efficient than other micropropagation techniques [27].

In this research, callus regeneration was obtained higher in stem TCL explants compared to leaf TCL explants. While 57.15% callus regeneration was achieved in stem TCL explants; this rate was achieved as 1.33% in leaf TCL explants. Küçükrecep and Tekdal carried out a study aiming to successfully create callus regeneration from the leaf and stem explants of selected Turkish genotypes of Common Bean. According to the results, the highest callus ratio was obtained stem explants [28]. In the study conducted by Mahood et al., a higher rate of callus regeneration was obtained in stem explants of *Gazania rigens* plant compared to leaf explants [29]. These results support our findings obtained in our study. Callus regeneration (100%) was achieved in stem TCL explants of female *Laurus nobilis* L. tree cultured in MS nutrient medium supplemented with 1 mg L^{-1} BAP and containing 25 mL L^{-1} coconut milk. Many studies have reported the growth-promoting effects of coconut milk. The growth-stimulating effects of coconut milk are due to its content of free amino acids, reduced nitrogen, myo-inositol and sorbitol, as well as unidentified substances [30]. George's study, revealed that coconut milk was useful for inducing the development of both callus and suspension cultures and at the initiation of morphogenesis [31]. Stanica et al. used modified MS + NAA 0.01 mg/L + BA 0.3 mg/L + ascorbic acid 250 mg/L + adenine sulfate 1 mg/L and DKW + IBA 0.1 mg/L as the basic nutrient medium in the tissue culture study conducted on *Laurus nobilis* L. The best result was achieved as 62% rate of callus development in the MS nutrient medium with 1 mg/L BA [32]. The data obtained in our study is higher than the literature.

While 2% average of shoot regeneration acording to two explant sources was observed in the cultivated stem TCL explants which found to be more successful since no shoot regeneration occurred in the leaf explants. Shoot regeneration percentages of TCL explants taken from *in vitro* plants were higher than those of TCL explants

taken from field. No shoot formation occurred in leaf TCL explants taken both *in vitro* and in the field. This is thought to be due to the fact that lTCL explants contain a single type of tissue, while tTCL explants contain a small number of cells in different tissue types [19]. In this research, all nutrient media were supplemented with 1 mg/L BAP. Blakesley and Constantine stated that BAP is a shoot-stimulating cytokinin that is widely used in many plant species, and emphasized that when used in combination, the ratios of cytokinin and auxin in combination are of critical importance in stimulating shoot formation [32]. Cytokinines are one of the growth regulators widely used in plant tissue culture technology. Cytokinines cause a variety of physiological effects when administered at low concentrations as high molecular weight nitrogenous bases. For example, they promote axillary bud growth and stimulate the division and differentiation processes of plant cells [34]. Mechanisms such as cell division and callus formation can be stimulated by plant growth regulators [35].

In this study, the highest shoot regeneration (6.6%) was obtained *in vitro* stem TCL explants cultured in MS nutrient media containing 30, 45 and 60 g L^{-1} sucrose supplemented with 1 mg L^{-1} BAP. Nhut et al. found that in their study established with stem transverse TCL explants of *Lilium longiflorum* plant, a higher rate of shoot regeneration was obtained in nutrient media containing 30 and 40 g L^{-1} sucrose compared to nutrient media containing less concentration of sucrose. Shoot regeneration efficiency was obtained at a lower rate in nutrient media containing 10 and 20 g L^{-1} sucrose [36]. In the study by Gauchan, the effect of various concentrations of different sugars on root and shoot formation in maize was reported that all concentrations of maltose and sucrose showed a good growth response in shoot and root [37]. Zulfiqar et al., as a result of their study on the avocado plant, reported that there are some factors that determine the success of *in vitro* plant regeneration, and that the ontogeny of the mother plant, explant source (apical or axillary), the location of the explant in the mother plant, its metabolic state and genotype are effective on the regeneration potential [38]. Ismail et al. found that young explants were more likely to form shoots than mature explants [39]. The findings obtained in this study support the results obtained in our study.

Oxidation of phenolic compounds originating from the cut surface of explants in the nutrient medium can cause significant problems in the culture of some plant species. This situation causes browning of the nutrient medium and toxic effects in the tissues, negatively affecting the development and differentiation abilities of the explants. To prevent the nutrient medium and tissues from browning, phenolic compounds must be removed from the environment. For this purpose, phenolic compounds are removed from the environment by using phenolic adsorbents such as activated carbon or polyvinylpyrrolidone (PVP). These chemicals adsorb phenolic compounds and neutralize them [40]. In plant tissue culture studies, it has been shown that activated carbon can promote the irreversible adsorption of inhibitory compounds in the culture medium and significantly reduce the levels of toxic metabolites, phenolic exudation, and brown exudate accumulation [41]. In this study, it was determined that adding 2 mg/L activated carbon to the nutrient medium reduced the browning percentage (77.5%). In a study conducted in Tunisia, Souayah et al. stated that the best shoot development in the propagation of *Laurus nobilis* L. by tissue culture was with the addition of BAP + GA_3 (1g/L) to 1/3 MS medium. He stated that activated carbon improves shoot, root and callus development. In various species of the family Lauraceae, one of the main problems is high levels of tannins and phenolic compounds that leach into the environment, where they undergo oxidation and interfere with growth. Therefore, the role of activated carbon appears to be essential in the process due to its ability to neutralize the reaction of tannins [42].

Laurus nobilis L. is an evergreen tree from the Lauraceae family, belonging to the Mediterranean climate, with shiny dark green leaves and small greenish yellow flowers, containing aromatic, valuable essential oils. It is widely used in ornamental plants, cosmetics and food fields. In addition to the fact that *Laurus nobilis* L. seeds have a double dormancy and low germination rate, alternative production techniques such as plant tissue culture techniques are needed for the production of the *Laurus nobilis* L. due to reasons such as excessive collection and destruction of *Laurus nobilis* L. areas due to faulty cultural processes. By using plant tissue culture techniques, the problems encountered in traditional *Laurus nobilis* L. production can be eliminated in a short time and regardless of the season, and it becomes possible to obtain a large number of plants. This research is a pioneering study, as there has been no study conducted using the "Thin Cell Layer (TCL) Culture System" on the plant *Laurus nobilis* L. to date.

According to the results of this study, important findings were obtained when examined in terms of callus and shoot formation, and it was determined that the *in vitro* regeneration potential of stem TCL explants of *Laurus nobilis* L. plant was higher compared to leaf explants. TCL explants taken from female *Laurus nobilis* L. trees in the field were found to have a higher tendency to form callus, whereas TCL explants belonging to plants grown *in vitro* were more convenient in order to form shoots. It has been determined that adding activated carbon to the nutrient medium reduces browning. With the important findings to be obtained, it is obvious that TCL technique will be addressed more comprehensively in this plant species.

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