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Effects of different boron salt treatments on micropropagation and genetic stability in *in vitro* cultures of *Liquidambar orientalis* Miller

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ABSTRACT

In the present study, the effects of different boron salts on the micropropagation of *Liquidambar orientalis* Mill., a relict-endemic plant species, were investigated and genetic stability of micro-shoots was determined by ISSR marker technique. Especially in species with low salinity and drought tolerance, salt stress may cause physiological and molecular changes such as plant growth and development, increase in secondary metabolite content in response to stress, and somaclonal variation. In this context, three different concentrations of boric acid, sodium perborate, sodium metaborate and disodium octaborate salts were applied to meristems isolated from *in vitro* clonal propagated *L. orientalis* and the effects of these boron salts on meristem regeneration and development were evaluated. When compared to the control group samples in which no salt application was applied, the best regeneration percentage was determined as 1 mgL^{-1} disodium octaborate treatment with a value of 100%, while when the shoot forming capacity index was evaluated, 5 mgL^{-1} sodium perborate treatment with a value of 4.94 gave the best results. However, when compared with the mother plant, it was observed that all salt treatments caused somaclonal variation on genetic stability, and in the light of the analyzed data, the lowest 30% (5 mgL^{-1} disodium octaborate) and the highest 49% (1 mgL^{-1} boric acid) somaclonal variation were determined in all applications.

1. Introduction

Liquidambar orientalis Miller, which is known as Anatolian sweetgum tree is a relict endemic and medicinal-aromatic tree belonging to the Altingiaceae family (Figure 1a) and spreading in the southwestern regions of Turkiye [1, 2]. In the IUCN list of hazard categories, Anatolian Sweetgum is listed in the category of “Highly Threatened in the Medium-Term Future in Nature” [3]. In addition, this species was included in the noble hardwood group by EUFORGEN (European Forest Genetic Resources Program) in 2001 and accepted as a species to be protected throughout Europe. In addition, this plant species survives only in Fethiye, Marmaris and Köyceğiz districts as a natural forest area in the world, but it is a plant species that is in danger of extinction because of reasons such as climate and making room for agricultural production [4].

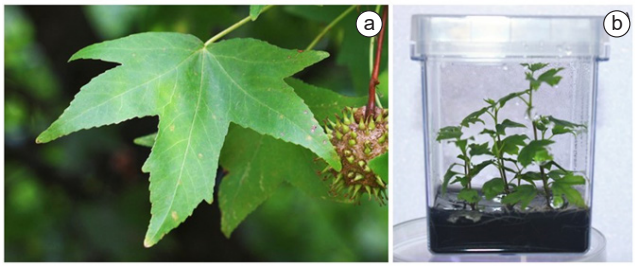

Figure 1. *L. orientalis* plant in natural population area (a); *in vitro* clonal propagated seedlings (b).

The main use of Anatolian sweetgum tree is its oil obtained from pathological balsam channels rather than wood production. Anatolian sweetgum oil, which has been used and traded for thousands of years, is an important source of raw materials for the cosmetics, pharmaceutical and chemical industries and is called "Turkish cytirax" in the world markets [5, 6].

Micropropagation is a tissue culture technique, and it is the name of the process of producing more than one plant with the same genetic characteristics as that plant from organs such as shoots, roots, and stems which capable of forming a new *in vitro* micro-shoots. It requires less material and is a faster production method compared to seed or cutting method production [7]. This technique, which has also been used in the commercial sector, is used in many plant species in park-garden crops, agriculture and forestry [8, 9].

L. orientalis is traditionally produced with seeds.

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Sometimes seed germination can get to two years. Regeneration percentages are generally low. In addition, germ planting has to be done seasonally. For this reason, the mass production is choosen commercially. Clonal propagation by semi-mature shoot cuttings is limited and it is very difficult to collect propagation material from tall trees [10]. Micropropagation is an alternative production model of Anatolian Sweetgum for commercial and conservation purposes [11]. Existing *ex situ* and *in situ* techniques used in the plant genetic resources preservation are reinforced by *in vitro* techniques [12]. Axillary and/or apical shoot meristem-based *in vitro* propagation is one of the beneficial technology used in preservation of the plant biodiversity [13].

Although boron salts and other salinity stresses and their influence on seed germination, shoot growth and development have been studied in the literature [14, 15], there are still some uncertainties regarding the response of plants to the combined stress due to boron salts and other salinity stresses. Irrigation with water having high concentration of boron and other salts in cereals [15] or naturally occurring high levels of salinity and boron are usually found in arid and semi-arid areas [16]. At present, there are limited studies on the effects of boron toxicity and salt stress on plant growth and development and the responses of plants to these stresses, and there is no consensus on the reciprocal relationships between boron toxicity and salinity stress. In this context, the present study aimed to examine the effects of different boron salts on regeneration and development of micro-shoot in *in vitro* growing liquidamber plants.

The another aim of the current work is to analyze the influences of different boron salt treatments *in vitro* on genetic stability at the molecular level. For this purpose, inter simple sequence repeat (ISSR) method, one of the PCR-based molecular marker techniques, was used. This technique was preferred in the present study due to its advantages such as yielding results with a small number of samples, being the dominant marker, primer design without the need for sequence information, high level of allele variations, fast and low cost.

2. Materials and Methods

2.1. Plant Samples and in vitro Culture Establishment

The seeds of *L. orientalis* were collected from the natural sweetgum plantation located within the borders of Muğla Province Köyceğiz District Toparlar District (36°59'28.63"N, 28°38'49.07"E). The seeds were disinfected by the seed surface sterilization method (70% ethyl alcohol for five minutes, 10% hydrogen peroxide for five minutes, two times commercial bleach for ten minutes and, sterile distilled water until rinsed) developed by Kaya et al. [17] and transferred to woody plant medium (WPM) medium [18] that does not contain any growth regulators.

2.2. Boron Salt Treatments

Four different boron salts (disodium octaborate, sodium metaborate, boric acid and sodium perborate) at three different concentrations (1, 3 and 5 mgL^{-1}) were separately combined with WPM nutrient medium supplemented with 1 mgL^{-1} benzyl adenine, 7 gL^{-1} (pH 5.8) and meristems cut from *in vitro* clonal propagated *L. orientalis* (Figure 1b) were transferred to these nutrient mediums separately. Ten meristems were used for each application, and each parameter was repeated at least three times. Each sample was incubated for 4 weeks in standard culture conditions (16/8-h photoperiod, 25±2 °C, 50 $\mu mol^{-1}m^{-2}s^{-1}$ with white cool fluorescent light).

Each salt treatment was evaluated separately. Regeneration percentages, shoot numbers per meristem and stem lengths of meristems grown in nutrient media containing four different boron salts in three different concentrations were recorded separately for each repetition, and the data were then statistically analyzed. The rootstock plant without any salt application was used as the control group. Shoot Forming Capacity (SFC) index was calculated by Eq.1 [19]. Where AVN is average shoot number derived from each regenerating meristem and RMP is regenerating meristem percentage.

$$SFC = \frac{(AVNxRMP)}{100} \quad (1)$$

2.2.1. Determination of genetic stability

After four weeks of incubation, the remaining *in vitro* material after data collection was individually packaged and stored at -20°C for use in genetic stability analysis. The CTAB-based method developed by Doyle and Doyle [20] was used for the isolation of genomic DNA from the samples. The genomic DNA was quantified spectrophotometrically and stored at -20°C to be used as a template in molecular analysis. Using total genomic DNA as template, PCR was performed with five primers giving the best band profile. Reactions were performed using 0.4 mM dNTP, 2.5 mM $MgCl_2$, 40 ng primer, 50 ng genomic DNA and 2 units TaqPolymerase in a 25 µl total reaction volume [21]. PCR products were migrated on a 1.5% agarose gel and the band profiles were visualized under UV. 1 kb and 100 bp DNA Ladders were used as guides and were coded as “1” if there is a band and “0” if there is no band.” was analyzed using Eq.2. Where HB represents homologous bands and nHB stands for non-homologous bands.

$$Similarity\ (\%) = HB/HB + nHB \quad (2)$$

2.3. Statistical Analysis

The nonparametric data statistical analysis was achieved via SPSS (IBM SPSS Statistics 24.0).

Discrete data were subjected to ANOVA to compare means followed by the least significant difference test at P≤0.05.

3. Results and Discussion

3.1. Influences of Boron Salts on Meristem Regeneration and Shoot Development

When compared the control group with the samples incubated in WPM nutrient medium containing four different boron salts at different concentrations, the best results were obtained from 5 mgL^{-1} sodium perborate (Figure 2).

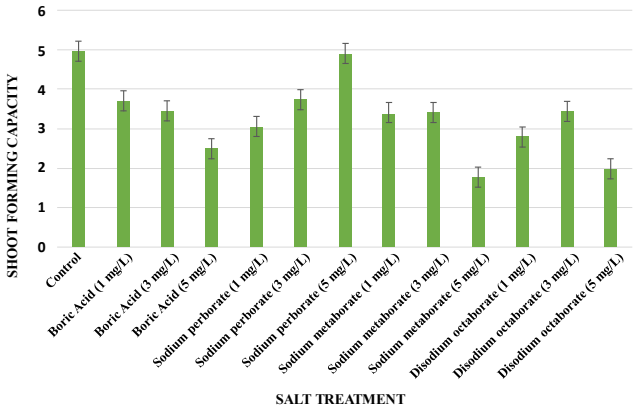

Figure 2. Calculated after salt application, meristem body forming capacities index.

However, the meristems cut from *L. orientalis* micro-shoots (Figure 3a) gave the best regeneration percentage (Figure 3b, Figure 4) in nutrient medium containing 1 mgL^{-1} disodium octaborate, while the maximum shoot numbers from per meristem were obtained from the application of 3 mgL^{-1} boric acid (Figure 5).

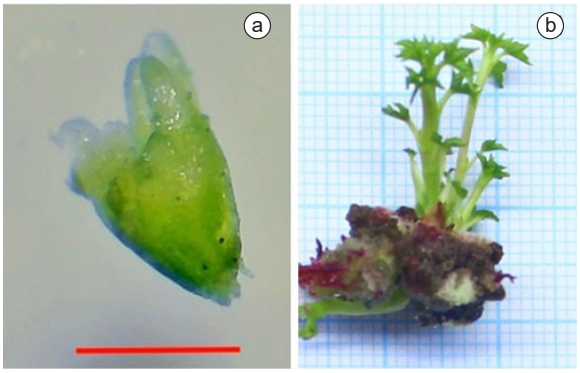

Figure 3. Meristem cut from *L. orientalis* micro-shoots (a), meristem grown in WPM nutrient medium containing 1 mgL^{-1} disodium octaborate (b), size bar 0.34 mm.

In vitro propagation of woody plants has some limitations due to genetic alteration in the process of development and aging of cultures. The *in vitro* cultures are the widely used method for multiplication of important species of woody plants [22] and cytokinin and/

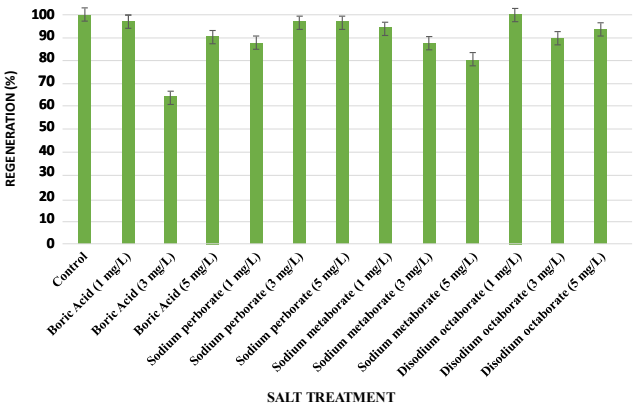

Figure 4. Meristem regeneration rates after different boron salt application.

or cytokinin and auxin growth regulators in optimized ratios are used for culture initiation. Nutrient media that do not contain plant growth regulators or contain auxin-like plant growth regulators for rooting [23] and indole butyric acid (IBA) are generally used to stimulate the roots of woody plants [24].

Micropropagation of woody plant species plays an important role in improving forest yields by supplying seedlings of high trading value. They also play an important role in inducing mineral nutrition, organogenic responses. In the current study, the different boron salt effects on the organogenesis of meristems from *L. orientalis* micro-shoots grown *in vitro* were evaluated. The culture media supplemented with different boron salts at different concentrations affected the *in vitro* organogenic control of *L. orientalis* differently. Boron-induced callus formation in explants let the initation of well-developed micro-shoots that could be used for development of buds. Similarly, Brondani et al. [25] evaluated the calcium and boron effects on the nodal segments regeneration from *Eucalyptus grandis* micro-shoots. The Murashige and Skoog (MS) medium supplemented with different concentration of calcium and boron [26] was modified to induce regenerative responses in 45-day-old *E. grandis* nodal explants, and after 60 days, dry weight, fresh weight, fresh and dry

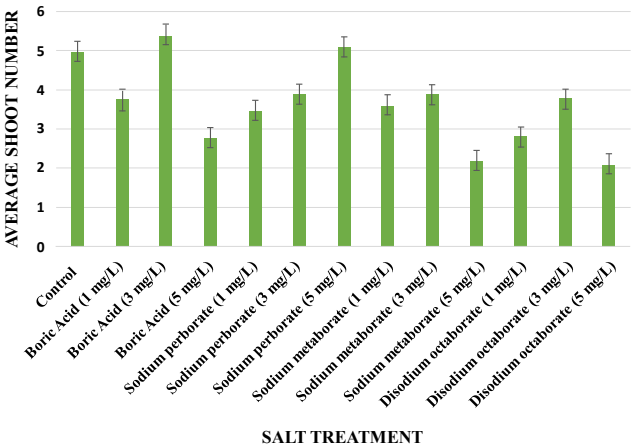

Figure 5. The shoot numbers obtained per meristem.

weight percentages, relative dry weight, fresh weight, fresh and dry weight accumulated by the explants, water content and relative substance content were evaluated. The culture medium supplemented with different concentrations of calcium and boron were found to affect the *in vitro* organogenic control of *E. grandis*.

It is known that the physical and superstructure features of cell walls are influenced by boron shortage [27]. In addition, when plants are developed with insufficient boron, boron is mainly accumulated in the cell wall [28]. Boron is not needed in large quantities by plants, but can cause critical plant development problems if not provided at essential amounts. Boron differs from other microelements in that it does not have chlorosis due to its shortage; however, it has similar toxicity effects as other microelements. Boron, along with calcium, is used in plant cell wall formation and is required for plant cell division. Other roles of boron include carbohydrate metabolism sugar translocation, potassium transport to the stoma, nitrogen metabolism, pollen germination, regulation of hormone levels and formation of certain proteins, regular functioning and growth of the apical meristems, membrane function and structure, and nucleic acid synthesis [29, 30]. In this study, four different boron salts (boric acid, sodium metaborate, sodium perborate, and disodium octaborate) at three different concentrations were used as a boron microelement source, and compared to the control group 3 mgL^{-1} boric acid and 5 mgL^{-1} sodium perborate applications, an increase was observed in the number of stems, while the application of 5 mgL^{-1} disodium octaborate was also found to be effective on stem elongation. When boric acid levels in plant cells are insufficient, plant cells take up borate salts and boric acid as undissociated boric acid via active transport. On the other hand, higher soil concentrations of it are occured passive diffusion. Boron and its salts are moved to the leaves via xylem, where the water vaporizes and the compounds are deposited on the leaves and left behind. In the phloem, boric acid and its salts are immobile and move little to other parts such as stem and fruit [31]. Boron is a necessary microelement for higher plant species, with the levels of interspecies variations requested for maximum plant development [32]. Studies on boron show that boron plays an important role in cell wall cross-linking, including complexation with specific pectin ingredient [28, 33].

The study of the functions of borax and boric acid on *Vicia faba* and other plant species has shown the effect of boron in plant nutrition [34]. The first large scale work of the functions of boron on plant development was achieved where fifty plant species were chosen and they were grown in sand cultures with normal nutrient solutions at different boron concentrations. The best plant growth, low concentrations that damage the plant, deficiency symptoms, trace boron levels were recorded for each species. The vast majority of plants showed best development up to 5 mgL^{-1} of boron levels and approximately one third of them grown at trace boron levels were shown morphological deficiency symptoms. It was concluded that the useful and harmful effects of boron salts overlap across plant species and therefore, he divided into the three broad categories of them sensitive, semi-tolerant and tolerant [35].

3.2. Effects of Boron Salts on Genetic Stability

It has been reported that phenotypic and genetic variations occur in *in vitro* cultures due to environmental conditions, prolongation of the subculture period, culture age, and nutrient media components [36]. The possibility of genetic variations induced by *in vitro* propagation processes requires exclusive consideration when the goal is to replicate special germplasms or to preserve genotypes. Therefore, it is especially critical to evaluate the genetic variation of main regenerants of *L. orientalis*, both because it is a relict endemic species and because of its medicinal aromatic properties. Molecular marker systems that can determine variations at the DNA level are progressively being used to access the genetic compatibility of micropropagated species with the parent material.

ISSR marker system includes the use of single simple sequence repeat (SSR) sequences to initiate the polymerase chain reaction (PCR) thus amplifying sequences between contiguous, but oppositely directional microsatellite regions [37]. ISSR markers have been successfully used in the determination of genetic stability in cauliflower [38], almond [39], banana [40] and *Swertia chirayita* [41] lines. Furthermore, ISSR marker system suggest many advantages, particularly in identifying somaclonal variation, a high degree of sensitivity, dominant representation of polymorphic genetic alleles and reproducibility. In the current thesis study, ISSR markers were used to evaluate the genetic stability of *in vitro* propagated *L. orientalis* microshoots with different concentrations of boron salt applied (Figure 6), and the current work is the first report on

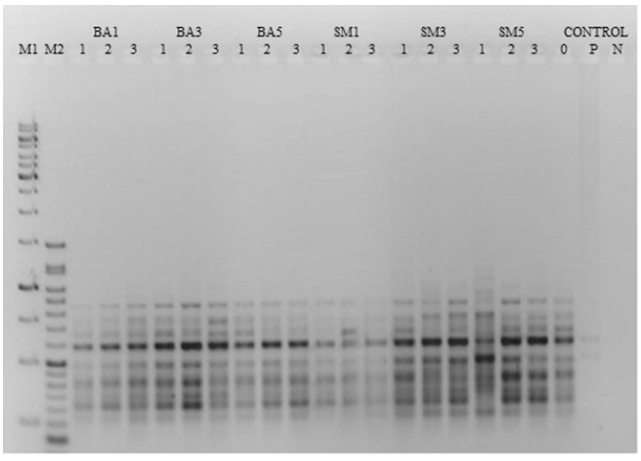

Figure 6. PCR band profiles obtained from ISSR 1 primer with DNA from the samples after application of three different concentrations of boric acid and sodium metaborate. M1: 1 kb gene ruler, M2: 100 bp gene ruler; BA boric acid (BA1: 1 mgL^{-1}; BA3: 3 mgL^{-1}; BA5: 5 mgL^{-1}); SM: sodium metaborate (SM1: 1 mgL^{-1}; SM3: 3 mgL^{-1}; SM5: 5 mgL^{-1}); Control 0: Control with no application; Control P: positive control of different plant; Control N: negative control without DNA template.

the determination of genetic variation as a result of application of different concentrations of boron salt in *in vitro* micro-shoots.

Salt stress due to combined effect of different minerals in the soil is within the important abiotic stresses that inhibit yield production worldwide [42], and the total area affected by salt stress is estimated to be ~80 mha [43]. Saltiness has profound influence on general plant morphology and physiology in different ways, ranging from metabolic, anatomic and physiological changes to molecular degradation [44, 45]. *L. orientalis* trees have moderate tolerance to various adverse environmental stresses, while their tolerance to salinity and drought may be low. *L. orientalis* is an economically important medicinal and aromatic plant that grows in wet and temperate regions. While sweetgum produces a limited number of shoots with traditional methods, *in vitro* tissue cultures are an excellent application for mass seedling production, but it is very critical to preserve the genetic stability of these and similar medicinal aromatic plants in *in vitro* propagated micro-shoots. The present study aimed to investigate the effects of different boron salts on the micropropagation of the plant in order to achieve genetically stable mass propagation in sweetgum and to produce genetically stable plants on a commercial scale. In ISSR PCR reaction analyzes using DNAs isolated from shoots of sweetgum plants after *in vitro* regeneration using meristem tissues as explant source, the lowest (30.77%) after 5 mgL^{-1} disodium octaborate application, and the lowest after 1 mgL^{-1} boric acid application. Polymorphism was determined with the highest rates (49.45%). Propagation of plants by tissue culture often undergoes genetic alterations among regenerated plant species, most likely due to stresses applied during micropropagation [46]. These alterations contain point mutations, chromosomal rearrangements and methylated DNA [47, 48]. The somaclonal variation is mostly due to genetic and/or epigenetic alterations during *in vitro* propagation of plants [49].

Somaclonal or culture-induced alterations have long-term been recognized as a source of beneficial or undesirable effects in plants cultured *in vitro*. Some of these undesirable effects contain anatomical and morphological features, secondary metabolite production, environmental stress tolerance, and other critical features of interest to the plant producer [50]. Many factors potentially responsible for somaclonal variation have been identified in the literature, such as desiccation, injury, inappropriate nutrient supply, and osmotic stress [51]. During micropropagation, micro-shoots are exposed to these stress conditions, which introduces both epigenetic and genetic alterations in the resulting multiplicated shoots [52]. Genetic changes persist in following generations in the form of somatic recombination, chromosome rearrangements, addition of transposons, point mutations and ploidy variation. Epigenetic changes consist of histone modifications and DNA methylation. The molecular markers have commonly been used to determine genetic alteration between source material and obtained somaclons in different plant species. In our study, three different concentrations of four different boron salts were tested for regeneration and micro-shoot development from meristem tissue. In woody species that are difficult to propagate *in vitro*, such as *L. orientalis*, some limitations are observed, such as necrosis formation on shoot tips, suppression of growth and development, suppressing *in vitro* culture. The cell development and *in vitro* regeneration of plants are an asexual process involving only mitotic cell divisions. In this context, the emergence of random spontaneous and uncontrolled alterations while *in vitro* clonal propagation is a critical limitation [53]. In such cases, *in vitro* cultures are treated by changing the concentration and/or variety of growth regulators, organic components, various amino acids, macro and micro elements in the nutrient medium composition. During these applications, application pressure from time to time, changing salt concentration and/or stress effects depending on the type cause somaclonal variations.

4. Conclusions

The tolerant species withstand a high concentrations of boron with little effect, and the sensitive species react strongly to too much or too little boron. In the light of the results obtained from our study, it can be said that it is in the semi-tolerant category for *L. orientalis*. There are many studies in the literature showing that various variations are noticed in plants propagated by *in vitro* tissue culture techniques [54, 55]. These variations, which are spontaneous in nature or caused by environmental factors, are stimulated by different stress conditions and biochemical compounds in *in vitro* cultures. Variation occurs from differentiated cells during embryogenesis and organogenesis using multiple or single cells, and also *in vitro* cultural condition induces the regulation of pre-existing variation expressions. The most of these variations are a big problem for seedling producers. However, these alterations are promising for the future studies focusing on plant quality.

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