Inhibition of browning problem during micropropagation of *Sideritis trojana* bornm., an endemic medicinal herb of Turkey

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**Abstract**

Micropropagation is used increasingly for ex situ conservation of endangered endemic plants and this in vitro technique provides mass propagation of medicinal plants. However during this process, stages of micropropagation are limited by serious problems. One of these problems is the explant browning which is related to the oxidation of phenolic compounds. In this research, several different methods have been used to eliminate the browning problem in tissue culture.

In the present research, inhibitory effects of different treatments such as Heller medium or adding activated charcoal, morpholine ethane sulfonic acid (MES) or ascorbic acid/citric acid combination to the medium, fast subculture passages or changing culture conditions to the dark were investigated against browning problem of *Sideritis trojana* Bornm. Adding a combination of 100 mg/l ascorbic acid and 50 mg/l citric acid to the Murashige and Skoog (MS) medium was found as the most effective treatment.

**Keywords**: *Sideritis trojana*, browning problem, phenolic compounds, micropropagation

**Introduction**

*In vitro* propagation techniques such as micropropagation provide *ex situ* conservation of rare and endangered plants. Clonal and mass propagation can be achieved in a relatively short time in small places. Then *in vitro* propagated plants can be transferred and adapted to their natural habitats [1]. In addition to that, micropropagation also can be used for obtaining plant secondary metabolites from medicinal plants [2,3].

Many plants, especially medicinal and aromatic plants, are naturally rich in polyphenolic compounds. When explants are cut and placed on the *in vitro* culture medium, these phenolic compounds are released from cut surface of the explants and oxidize to form phytotoxic products. As a result of this event, the media and explants turn brown and the explants are unable to grow further and eventually die [4,5,6]. In previous studies, several different methods such as antioxidants, activated charcoal, photoperiod, contents of media and plant growth regulators have been used to prevent the browning problem [7].

*Sideritis trojana* Bornm., of the family *Lamiaceae*, is an endemic species of Ida Mountain (Kaz Mountain), Canakkale, Turkey. This plant species has been marked as an endangered (EN) in Red Data Book of Turkish Plants [8] *Sideritis* species are generally known as mountain tea and widely used as herbal tea in folk medicine as a nervous system stimulant and as a carminative, anti-inflammatory, antispasmodic, analgesic, sedative, antitussive, stomachic, in the treatment of coughs due to colds and for curing gastrointestinal disorders [9,10].

Although there are some reports on tissue culture studies of *Sideritis* species [11,12,13,14], to date any *in vitro* studies have not been reported in *S. trojana*. The major problem in micropropagation of *S. trojana* has been the high mortality rate due to lethal browning of explants and medium caused by the phenolic compounds secretion [1]. The aim of the present
research was to determine the most effective treatment and inhibitory effects on browning of *S. trojana* cultures.

**Materials and Methods**

**Plant Material and Sterilization**

Seeds of *S. trojana* were collected from Ida Mountain, Turkey (coordinates 39° 41’ 30.2” N, 26° 52’ 30.1” E, altitude 1694 m.) [15,16] and soaked for 1 hour in sterile distilled water at 50°C, surface-sterilised with 2% sodium hypochloride and 0.1% (v/v) Tween-20 for 30 min, rinsed three times with sterile distilled water.

**In vitro Culture Conditions**

Sterile seeds were placed in petri plates, containing MS medium [17], with 3% (w/v) sucrose and 0.8% (w/v) agar (pH:5.75). Petri plates were incubated at 26±2°C and 16/8 photoperiod with 72 μmol m⁻² s⁻¹. Leaf explants were excised from twelve weeks old seedlings and were placed to the six different testing series with tree replicates in petri plates. MS basal medium was used for a control group. Others are as follows;

1) Heller medium [18] 2) MS medium supplemented with 0.1% (w/v) activated charcoal 3) MS medium supplemented with 1g/l MES 4) MS medium supplemented with 100 mg/l ascorbic acid and 50 mg/l citric acid combination 5) Fast subculture passages for once in a week 6) keeping in continuous darkness by covering the petri plates with foil for one week then transferring to 16/8 photoperiod were used for preventing browning problem.

MS basal salts and Heller medium supplemented with 3 % (w/v) sucrose and 0.8 % (w/v) agar, 3 mg/l K, 0.5 mg/l 2,4-D were used for *in vitro* experiments. The pH of medium was adjusted to 5.75 before adding agar, then autoclaved at 121°C for 15 min. All of the cultures except the dark treatment were kept in the growth chambers at 25 ± 2°C under 16/8 h photoperiod with 72 μmol m⁻² s⁻¹.

**Statistics**

Browning of explants was calculated according to the browning index. Surface browning of explants were assessed by measuring the extent of the total browned area on each explants of the 10 in each replicate according to a 5 grade scale: 0, none; 1, browning area <10%; 2, browning area 10–25%; 3, browning area 25–50%; 4, browning area >50%. The browning index was calculated as ∑ (browning level×number of explants with that browning level)/(total number of explants) described by Yang et al. [19].

**Results and Discussion**

When leaf explants of *S. trojana* were placed on MS basal medium, callus with meristemoids was induced within 2 weeks of culture. But the production of phenols caused necrosis and callus turned brown and eventually died (Figure 1a). The browning problem as a result of phenolic compounds secretion which inhibits micropropagation was tried to be solved by applying six different treatments in *Sideritis trojana* Bornm plant species.

**The Effects of Medium Ingredients**

Our results showed that Heller medium was not effective for reducing the phenols. Besides, the Heller medium contains fewer nutrients than MS medium and was not suitable to induce shoot regeneration in *S. trojana* (Figure 1b).
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Similar results were recorded when the explants were cultured on the MS medium supplemented with 0.1% (w/v) activated charcoal (Figure 1c). Shoot regeneration did not occur in this medium. Activated charcoal is often used in plant tissue culture for the adsorption of inhibitory substances in the culture medium, drastically decreasing the phenolic oxidation or brown exudate accumulation [20]. But it was reported that activated charcoal may also absorb plant growth regulators. These results are quite similar with the report on *in vitro* propagation of *Sideritis strica*, *Sideritis perfoliata* and *Sideritis erythrantha*. Various explants such as leaf, node, internode of these plants were cultured on MS medium containing 0.3% (w/v) activated charcoal, with different concentrations and combinations of NAA and BAP. Regeneration was not obtained from these explants because of the explant browning and contaminations originated from the explants [14].

The addition 1 g/l MES to MS media was also used to overcome the browning problem. MES could be used to inhibit browning problem as a pH effectors [21]. Our results showed that it was more effective than using Heller medium or MS medium supplemented with 0.1% activated charcoal (Figure 1d).

Among all these treatments, addition of antioxidants (100 mg/l ascorbic acid and 50 mg/l citric acid) to the medium was more effective on solving the browning problem of this medicinal plant (Figure 1e). Addition of antioxidants to the culture medium is recommended in most of reports. In one report, addition of 100 mg/l ascorbic acid to MS medium was found as one of the best methods to control explants browning of *Pyrus bretschneideri*. As a result of this treatment, the browning problem occurred only in 8% of explants [22].

*The Effects of Culture Conditions*

In addition to the effects of medium ingredients, different changes of the physical factors were applied to overcome browning problems. Generally, fast subculture passages can eliminate phenolic secretion accumulation in the medium and prevent its penetration in the explants. In our research, fast subculture passages for once in a week were not effective for inhibition of browning problem.

Keeping cultures initially in the dark may also help to reduce the browning problem. According to our results, pre-culture at continuous dark was also effective to reduce the browning problem (Figure 1f). But callus did not turn green when transferred to a 16/8 photoperiod, callus tissue was not able to do photosynthesis and, therefore, was not able to regenerate shoots. In the study on browning problem in explants of *Pyrus bretschneideri*, the effect of dark treatment was determined on browning problem. For this, shoot tip, second node and other nodes of pear were cultured in continuous darkness for 48, 96 and 144 hrs. During dark treatments, all shoot tips in all treatments died. It was reported that only 96 hours dark treatment of other nodes was the best treatment to control browning and also it was discussed that the browning problem was not possible to be controlled by dark treatment alone in the shoot tip of *P. bretschneideri* [22].
In conclusion, many plants are naturally rich in polyphenolic compounds that are commonly regarded as inhibitory agents. In most of the cases, when these plants are cultured in vitro, the culture medium turns brown. Finally, the addition of antioxidants to medium was more effective to reduce the browning of *S. trojana* explants (Figure 2).
The individual treatments were not enough effective and so the combination of different treatments may be used to overcome browning problem completely. After overcoming this main problem, the next step will be the achievement of effective regeneration and micropropagation of \textit{Sideritis trojana}. Then we can focus on transferring these plantlets to their natural habitats for conservation of genetic sources.

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**References**


