Romanian Biotechnological Letters *Vol. 16, No. 6, 2011*
Copyright © 2011 University of Bucharest Printed in Romania. All rights reserved
ORIGINAL PAPER

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

Received for publication, March 5, 2011
Accepted, May 28, 2011

NURŞEN ÇÖRDÜK[1] AND CÜNEYT AKI[1]
[1] *Çanakkale Onsekiz Mart University, Faculty of Science and Arts, Department of Biology, 17020, Canakkale,Turkey. Tel: 90286 218 00 18- 2399, fax: 902862180533, e-mail: cuneytaki@comu.edu.tr*

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), Çanakkale, 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 39º 41′ 30.2″ N, 26º 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).

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 occurre 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].

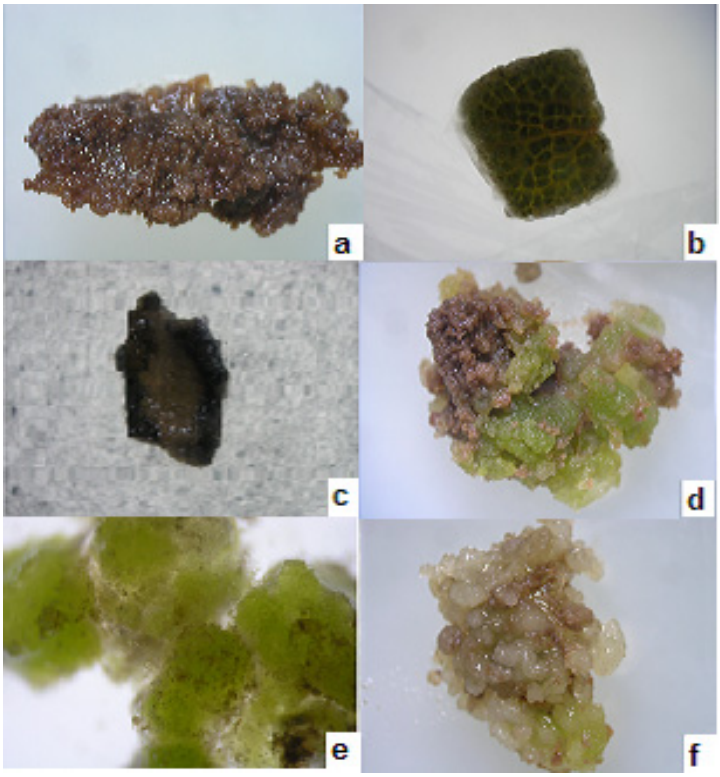

Figure 1. Explants and callus tissue cultured on MS medium without any treatment (a), leaf explant cultured on Heller medium (b), leaf explant cultured on MS medium supplemented with 0.1 % (w/v) activated charcoal (c), callus tissue cultured on MS medium supplemented with 1 g/l MES (d), callus tissue cultured on MS medium supplemented with 100 mg/l ascorbic acid, 50 mg/l citric acid combination (e), callus tissue cultured on MS medium in dark conditions (f).

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 (Figure2).

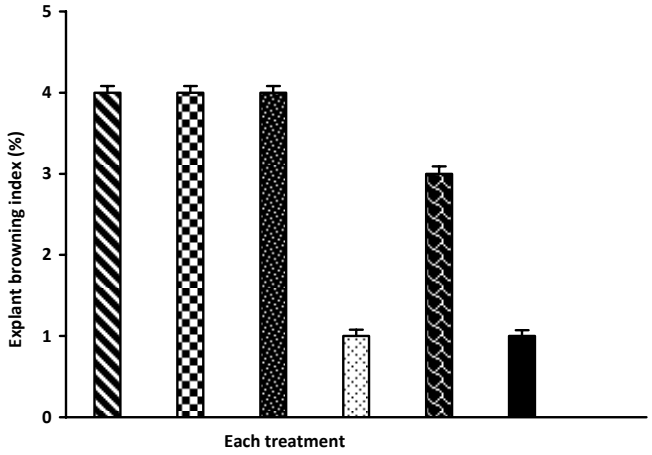

Figure 2. Browning percentage of *S. trojana* leaf explants cultured on MS medium without any treatment (▨), Heller medium (▩), MS medium supplemented with 0.1 % (w/v) activated charcoal (▩), cultured on MS medium in dark conditions (▨), MS medium supplemented with 1g/l MES (▩)MS medium supplemented with 100 mg/l ascorbic acid, 50 mg/l citric acid combination (■).

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 *Sideritis trojana*. Then we can focus on transferring these plantlets to their natural habitats for conservation of genetic sources.

Acknowledgements

This research was supported by The Scientific and Technological Research Council of Turkey (Project No: 107T125).

References

1. ÇÖRDÜK, N. AKI, C.(2010). Conservation of Genetic Sources of *Digitalis trojana* Ivan. and *Side ritis trojana* Bornm.by *In Vitro* Culture. The First International Symposium on Biology of Rare and Endemic Plant Species, Muğla,31-32.
2. BENSON, E.E. DANAHER, J.E. PIMBLEY, I. M., ANDERSON, CT. WAKE, J.E. DALEY, S. AND ADAMS, L.K. (2000). In vitro micropropagation of Primula scotica: a rare Scottish plant Biodiversity and Coservation. 9: 711–726.
3. ROUT, G.R. SAMANTARAY, S. AND DAS, P. (2000). In vitro manipulation and propagation of medicinal plants. Biotechnol. Adv. 18: 91-120.
4. BHOJWANİ, S.S. AND RAZDAN, M.K. (1996). Plant Tissue Culture: Theory and Practice, a Revised Edition.Studies in Plant Science Elsevier. 483-536.
5. ALİYU, O. M. (2005). Application of tissue culture to cashew (Anacardium occidentale) breeding: an appraisal. African J. Biotechnology, 4(13): 1485–1489
6. POUDYAL, B.K. DU, G. ZHANG Y. LİU, J. AND SHI, Q. (2008). Studies on browning problem and phenols content on shoots of Yali, Aikansui and Abbe Fetel pears for in vitro culture. Front. Agric. China 2008, 2(3): 321–330.
7. ELLİALTIOĞLU, Ş. (1999). Doku Kültürü Yoluyla Vegetatif Çoğaltmada Doku Kararması Sorunu, Nedenleri ve Çözüm Yolları. Biyoteknoloji. Kükem Dergisi, 24(1): 37-47.
8. EKİM, T. KOYUNCU, M. VURAL, M. DUMAN, H. AYTAÇ, Z. AND ADIGÜZEL, N. (2000). Red Data Book of Turkish Plants (Pteridophyta and Spermatophyta). Foundation for Turkish Nature Conservation and Van Centinential University Press, Ankara.
9. AYDIN, S. ÖZTÜRK, Y. BEIS, R. AND BAŞER, K.H.C. (1996). Investigation of Origanum onites, *Sideritis congesta,* and *Satureja cuneifolia* essential oils for analgesic activity. Phytotherapy Re search. 10, 342-344.
10. BAŞER, K.H.C. TÜMEN, G. ÇAKIR, H. AND KAYA, A. (1997). Balıkesir Kazdağı Yöresinde Çay Olarak Kullanılan Bitkiler Üzerine Morfolojik, Anatomik ve Palinolojik Çalışmalar, XI. Biyoloji Kongresi, Fırat Üniversitesi. Pp: 53-71.
11. GARCİA-GRANADOS, A.,Ü MARTİNEZ, A. ONORATO, M.E. PARRA. A. RECONDO, M.B. RİVAS, F. ARREBOLA, M.L. and Socorro, O. (1994). Products with biological activity obtained from *in vitro* micropropagated *Sideritis foetens.* Phtyochemistry. 35(3): 645-650.
12. FARİA, J.L.C. KOSTENYUK, I. AND SEGURA, J. (1998). Isolation, culture and plant regeneration from protoplasts of *Sideritis angustifolia.* Journal of Plant Physiology. 153 (1-2): 251-254.
13. ERDAĞ, B.B AND YÜREKLİ, A.K. (2000).Callus induction and shoot regeneration of *Sideritis sipylea* Boiss. (Lamiaceae).Bulletin of Pure and Applied Sciences.19B(1):41-46.
14. UÇAR, E. AND TURGUT, K. (2009). Bazı Dağ Çayı (Sideritis) Türlerinin *in vitro* Çoğaltımı. Akdeniz Üniversitesi Ziraat Fakültesi Dergisi. 22(1): 51–57
15. AKI, C. AND ÇÖRDÜK, N. (2008). Callogenesis of Kazdagi Endemic Plant, *Sideritis trojana*. Proceedings of the 30th Scientific Conference of Hellenic Association for Biological Sciences, p.12, Thessaloniki.
16. TEZCAN, M. VLACHONASIOS, K. AND AKI, C. (2010). DNA Barcoding Study on *Sideritis trojana* Bornm. An Endemic Medicinal Plant of Ida Mountain Turkiye. Fresenius Environmental Bulletin. Fresenius Environmental Bulletin. 19(7): 1352-1355.

17. MURASHIGE, T. AND SKOOG, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-497.
18. HELLER, R. (1953). Recherces sur la nutrition minerale des tissus vegetaux cultives in vitro, Ann. Sci. Nat. Bot., Biol. Vege.14, 1-223.
19. YANG, H. ZHOU, C. WU, F. AND CHENG J. (2010). Effect of nitric oxide on browning and lignification of peeled bamboo shoots. Postharvest Biology and Technology. 57, 72–76.
20. THOMAS, T.D. (2008). The role of activated charcoal in plant tissue culture. Biotechnology Advances. 26, 618–631.
21. MANSUROĞLU, S. AND GÜREL, E. (2001). Mikroçoğaltım. Doku Kültürü: Temel Laboratuar Teknikleri, Bitki Biyoteknolojisi, Doku Kültürü: Temel Laboratuvar Teknikleri. Ed; Babaoğlu, M. Gürel, E. and Özcan, S. Cilt 1, Selçuk Üniversitesi Vakfı Yayınları, Pp: 262-281.
22. POUDYAL, B. K. DU, G. ZHANG, Y. LIU, J. AND SHI Q. (2008). Studies on browning problem and phenols content on shoots of Yali, Aikansui and Abbe Fetel pears for *in vitro* culture. Front. Agric. China, 2(3): 321–330.