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Plant Regeneration through Somatic Embryogenesis from Calli Derived from Leaf Bases of *Laurus nobilis* L. (Lauraceae)

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Abstract

Somatic embryogenesis was induced in embryo culture on half MS medium supplemented with NAA (8 mg/l) as the sole plant growth regulator after incubation of the media in the refrigerator at 4°C for two weeks to promote callus induction and somatic embryogenesis in *Laurus nobilis*. Both embryogenetic calli and somatic embryos were induced in the above selected medium. Embryo growth and development were stimulated by separation of embryos successfully from embryo clusters and transferred onto fresh half MS. Among the selected explants, only leaf bases were found to respond actively to plant regeneration, especially in inducing callus formation and in sustaining faster callus growth. Root formation of regenerated plantlets tended to decrease with time on regeneration media. Overall, 75% of the plantlets derived from the callus survived in the greenhouse; and they all grew to phenotypically normal plants. This procedure will enable the use of regeneration tissue culture technology for germplasm conservation of *L. nobilis*, a plant of high medicinal and commercial value.

Introduction

The family Laureaceae consists of 32 genera and about 2000-2500 species of numerous aromatic and medicinal plants with showy flowers. The genus *Laurus*, comprises evergreen trees or large shrubs and are dioecious and is widely

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distributed from the temperate to the subarctic zone in the Northern Hemisphere (Abu-Dahab et al. 2014, Ozcan et al. 2010). Some of the plants are frequently cultivated in gardens, and widely used in floral arrangement for ornamental purposes. *L. nobilis* L. (Lauraceae), bay laurel, is a large, erect evergreen shrub with leathery leaves and small, pale greenish-yellow flowers occurring in dense clusters. The oval fruits are glossy black when ripe. Bay laurel is native to the Mediterranean region and its crops are found in areas with moderate and subtropical climate, including Jordan.

The dried leaves are used extensively as a spice in cooking worldwide, and the essential oil is generally used industrially as a flavor (Bauer and Garbe 1985). Several studies describe the medicinal value of the bay laurel as an integral part of traditional remedies in the Mediterranean countries (Qnais et al. 2012, Kivcak and Mert 2002, Saab et al. 2012, Al-Kalaldeh et al. 2010, Ozcan et al. 2010, Ramos et al. 2012, Barla et al. 2007). Like other *Laurus* species, the propagation of *L. nobilis* through seedlings is difficult due to poor fruit set and a very low germination rate (Hartmann 1997). Moreover, the small seed yield from wild trees is associated with difficulties of pollination and a considerable loss due to damage caused by birds. Although bay laurel is widely utilized, no alternative propagation procedure, e.g. via tissue culture is available. Accordingly, routine cultivation is restricted. In consequence, this species has become rare and is endangered due to over cutting (Chang et al. 2002). At present, the development of conventional methods of propagation by cutting, seeds, and layering are very slow and do not promise homogeneity.

Plant tissue culture could be a powerful alternative technique for conservation and propagation of plants, especially for those that are rare and difficult to propagate by conventional methods. In principle, there are two different approaches, either the direct regeneration of shoots from calli or the propagation on the basis of somatic embryos, derived from calli. Unfortunately, all attempts to induce shooting from *Laurus* calli failed. Accordingly, we focused on plant regeneration via somatic embryogenesis, reported already for various other species of *Laurus* (Ying-Chun and Chen 2009). The development of somatic embryos nearly replicates the process of zygotic embryo formation. Callus is usually derived from somatic tissues. The tissues used to initiate callus formation depends on plant species and which tissues are available for explant culture. The cells that give rise to callus and somatic embryos usually undergo rapid division or are partially undifferentiated such as meristematic tissue. In alfalfa, *Medicago truncatula*, however callus and somatic embryos are derived from mesophyll cells that undergo dedifferentiation (Wang et al. 2011). Plant growth regulators in the tissue culture medium can be manipulated to induce

callus formation and subsequently changed to induce embryos to from the callus. The ratio of different plant growth regulators required inducing callus or embryo formation varies with the type of plant (George 1993). Somatic embryos are mainly produced *in vitro* and for laboratory purposes, using either solid or liquid nutrient media which contain plant growth regulators (PGR's). The main PGRs used are auxins but can contain cytokinin in a smaller amount (Zhang et al. 2005, von Arnold et al. 2005, George 1993). Shoots and roots are monopolar while somatic embryos are bipolar, allowing them to form a whole plant without culturing on multiple media types. Somatic embryogenesis has served as a model to understand the physiological and biochemical events that occur plant developmental processes as well as a component to biotechnological advancement (Tran Thi and Pleschka 2005, Toribio et al. 2004, Park et al. 2005, Quiroz-Figueroa et al. 2006). The first documentation of somatic embryogenesis was by Steward et al. (1958) and Reinert (1959) with carrot cell suspension cultures. As demonstrated in various monocotyledonous plants, the morphology of embryos, especially of the cotyledon, which forms the scutellum, varies depending on the species (Hartmann et al. 1997). For *Laurus* species, although much information on this issue are available, due to the high variation in induction of somatic embryogenesis, for each cell culture system, the corresponding details have to be elaborated (Radojevic et al. 1987, Laublin et al. 1991, Radojevic and Subotic 1992, Jehan et al. 1994, George 1993, Cuenca and Amo-Marco 2000, Frett 1986). The present study was aimed at to developing a valuable protocol for *L. nobilis* regeneration by somatic embryogenesis to contribute to the conservation of *L. nobilis* in Jordan as a part of a national strategy to conserve the wild genetic resources of the country. A corresponding approach was already successfully realized in Jordan for the monocotyledonous bulbous plants such as *Sternbergia clusiana* (Oran and Fattash 2005), *Narcissus tazetta* (Abu-Zahra and Oran 2009), *Iris nigricans* and other species of *Iris* (Al-Gabbiesh et al. 2006, 2007, Al-Gabbiesh 2005).

Matrials and Methods

Samples of growing *Laurus nobilis* L. used in this study were collected from the wild in Madaba and Hashemite University campus (Zarka, Jordan) during April 2012. Collected plants were identified by using standard descriptive references (Al-Eisawi 1986, Flora Palaestina 1986). To establish sufficient mother stock cultures, the endangered *L. nobilis* cuttings were also cultivated in the field.

All media used consisted of half MS solidified with 0.4% agar (Sigma-Aldrich) and supplemented with 1.5% sucrose. Media were adjusted to pH 5.7 and then stored at 4°C for two weeks to promote callus induction and somatic

embryogenesis. Concentrations of plant growth regulators (PGRs) are given in Table 1. Inoculated jars were sealed with Nesco film and incubated at 25°C applying a day/night rhythm 16 hrs/8 hrs, photon flux density was either 250 or 50 μmol m^2/s.

Table 1. Composition of media used in this study.

Components	Media
Half MS + 4.5 μM 2,4-D + 0.5 μM of Kn + 4.5 μM of NAA + 2.5 μM BAP.	Primary medium (PM)
Half MS + 4.5 μM BA + 1.5 μM IAA 0.45 μM 2,4- D + 4.5 μM NAA.	Secondary medium (SM)
Half MS + 4.5 μM of 2,4 –D + 0.5 μM Kn + 4.5 μM NAA + 200 mg/l proline.	Tertiary medium (TM)
Half MS + 4.0 μM BA + 0.45 mM BAP.	Regeneration medium (RM)
Half MS + 4.5 μM IBA.	Rooting medium (RoM)

Samples were rinsed with running tap water. Different explants (flower bases, leaf bases) were excised and soaked in 70% ethanol for 5 min. Subsequently, these explants were cut longitudinally into 3-5 mm segments, which were surface sterilized for 15 min in sodium hypochlorite (5% v/v) solution, supplemented with a few drops of Tween 80. Finally, the explants were rinsed five times with sterile distilled water for 15 minutes. Anthers used as explants were sterilized by soaking in 70% ethanol for one minute before transferring them on primary callus medium (PM). Cultures were maintained under dark conditions at 25°C.

Explants of roots, stem, flower bases, and leaf bases were transferred to fresh SM medium supplemented with 10 mg/l ascorbic acid to avoid browning in case of exudations. Callus cultures were maintained by sub-culturing on fresh SM media. After about four weeks, calli were transferred from PM to SM-medium and maintained by sub-culturing in the dark. After about three weeks, friable calli were transferred to Tertiary medium (TM), to induce embryogenesis. Frequencies of explants producing calli and of calli producing embryo structures were recorded. Callus initiation frequency was measured as the number of explants producing calli divided by the total number of explants cultured. Embryo structures frequency corresponds to the number of calli producing embryo structures divided by the total number of explants cultured. Data were evaluated using the arcsine square root transformation for statistical analysis. Data were analyzed as a complete random design with three replications.

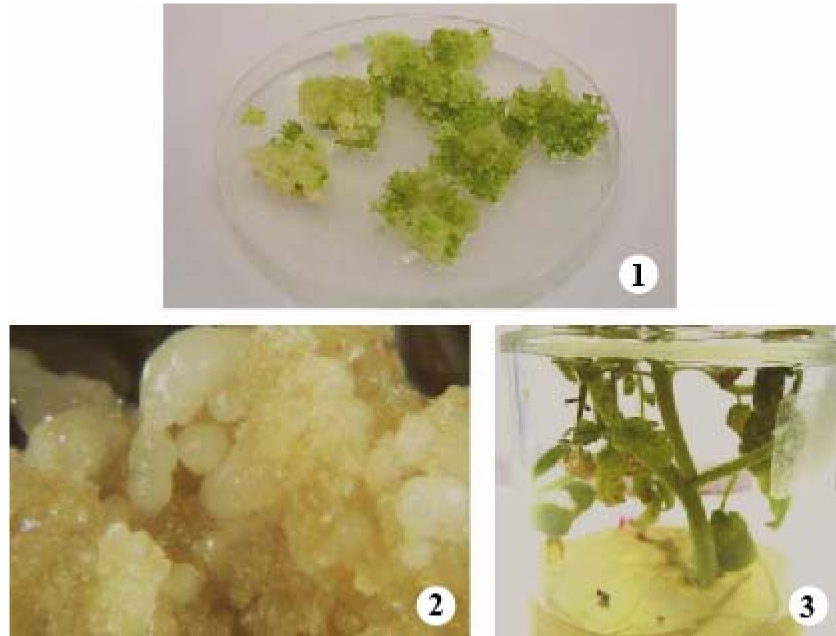
Results and Discussion

Somatic embryogenesis in calli from of *Laurus nobilis* L. in spite of substantial efforts to induce callus from different explants, *i.e.*, roots, stem, flower bases, leaf bases, only leaf bases were found to respond successfully to the culture conditions outlined above. This is in agreement with the findings of Lu and Vasil (1981). Overall, in this study, more than 550 leaf bases from *L. nobilis* had been inoculated, resulting in 344 calli, corresponding to a success rate of 62.5%. After about 3 - 4 weeks, all calli were transferred to SM medium for enhancing their growth and to produce sufficient material for further experiments. Apart from 10 initial samples, which were discarded due to contaminations, the remainder of all samples from sporadic calli developed within 3 - 4 weeks after transfer. Subsequently, all 334 calli were transferred to TM medium in order to induce somatic embryogenesis. Overall, somatic embryogenesis was induced in three cases, corresponding to a success rate of 0.9%. Four weeks after transfer to the TM medium, many somatic embryos had developed on the surface of the callus, derived from leaf base of *L. nobilis* (Fig. 2). Calli transferred to TM medium first produced numerous white globular like embryo- structures within two weeks. These structures grew rapidly, and new ones continued to appear on the callus surface. Within 3 more weeks, clusters of structures at various stages of development were found on the same callus (Fig. 3). These structures were similar to the somatic embryos described by Kaurinovic et al. (2010) and Souayah et al. (2002) for the same plant. The best embryo producing callus was sub-cultured by transferring regularly aliquots to RM medium after 3 - 4 weeks of development. This embryogenic callus was used for the regeneration experiments.

Reports are available outlining the regeneration of plantlets from somatic embryos of numerous plant species, *e.g.*, *Iris* species (Jehan et al.1994; Radojevic and Subotic 1992), *Panicum maximum* (Lu and Vasil 1981), ginger (Malamug et al. 1991), or *Sorghum bicolor* (Wemicke and Brettell 1980). The medium routinely used in this laboratory for regeneration was chosen (Table 1). Each callus was divided into five or six pieces and transferred to RM medium. After a few weeks somatic embryos developed and small green shoots were emerging. Every 3 - 4 weeks, the calli bearing the small plantlets were transferred to new RM medium. After about two months, the plantlets developed two to four pairs of about 6 cm long leaves. At this stage, they were transferred to ROM medium to induce rooting. After about five weeks the plantlets had formed many roots (Fig. 3) and were ready for acclimation for growing in soil.

In conclusion, embryogenic calli derived from the young leaves of L. nobilis L. were induced on (4.5 μM 2,4-D, 0.5 μM Kn, 4.5 μM NAA and 200 mg/l of

proline)-supplemented medium. These calli were cultured on plant growth regulators of (4.5 µM BA, 1.5 µM IAA, 0.45 µM 2,4- D and 4.5 µM NAA). Thereafter, Somatic embryos formed on the surface of the calli regenerated into



Figs 1-3: 1. Callus from leaf bases of *L. nobilis* cultured on SM medium after transfer from PM medium. 2. Process of somatic embryogenesis in a callus derived from the leaf base of *L. nobilis*. Numerous somatic embryos developed on the surface of the callus four weeks after transfer to TM medium. 3. A regenerated plant of *L. nobilis* after four weeks of growth in ROM medium.

healthy plantlets on the (half MS, 4.0 µM of BA, 0.45 mM of BAP). Secondary somatic embryos formed into calli from the base of the regenerants. Thus, a somatic embryogenesis system for *L. nobilis* was successfully established to multiply this sparingly available medicinal species economically in an efficient manner.

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References

Abu-Dahab R, Kasabri V and **Afifi F** (2014) Evaluation of the Volatile Oil Composition and Antiproliferative Activity of *Laurus nobilis* L. (Lauraceae) on breast cancer cell line models- Rec. Nat. Prod. **8**(2): 136-147.

Al-Eisawi DM (1986) Studies on the flora of Jordan 12. Monocotyledon new to Jordan, with notes on some interesting species- Kew Bull. **41**(2): 349-357.

Al-Gabbiesh AH, Hassawi D and **Afifi F** (2006) *In vitro* propagation of endangered *Iris* species. J. Biol. Sci. **6**(6): 1035-1040.

Al-Gabbiesh AH, Hassawi D, Taha M and **Afifi F** (2007) Effect of increasing concentrations of quinine on the volatile compounds in cultured tissues of *Iris atrofusca* Baker and *Iris petrana* Dinsmore- Biot. **6**(2): 218-224.

Al-Gabbiesh AH (2005) Calli propagation and genetic analysis of Iris species grown in Jordan and the effect of quinine on the produced calli. MSc Thesis, Al-Bal. App. Uni., Salt, Jordan, pp. 20-25.

Al-Kalaldeh JZ, Abu-Dahab R and **Afifi FU** (2010) Volatile oil composition and antiproliferative activity of *Laurus nobilis*, *Origanum syriacum*, *Origanum vulgare* and *Salvia triloba* against human breast adenocarcinoma cells. Nutr. Res., **30**: 271-8.

Bauer K and **Garbe D** (1985) Common fragrance and flavor materials, preparation, properties and uses. VCH. Weinheim, Germany.

Barla A, Topcu G, Oksuz S, Tumen G and **Kingston DGI** (2007) Identification of cytotoxic sesquiterpenes from *Laurus nobilis* L. Food Chem. **104**: 1478-84.

Chang SH, Ho CK and **Tsay JY** (2002) *In vitro* culture of *Cinnanomum kanehiraeHay*- Taiwan J. For. Sci. **17**(4): 491-501. [in Chinese with English abstract].

Cuenca S and **Amo-Marco JB** (2000) *In vitro* propagation of two Spanish endemic species of *Salvia* through bud proliferation. *In vitro* Cell. Dev. Biol. Plant. **36**: 225-229.

Frett JJ (1986) Tissue culture propagation of *Salvia gregii* Hort. Scie. **21**: 859.

Hartmann TH, Kester DE, Davie FTJ and **Geneve RL** (1997) Plant Propagation: Principles and Practices (6th edition) - Englewood Cliffs, NJ: Prentice-Hall.

Jehan HD, Courtois C, Ehret K, Lerch K and **Petiard V** (1994) Plant regeneration of *Iris pallida* Lam. and *Iris germanica* L. via somatic embryogenesis from leaves, apices and young flowers- Plant Cell Rep., **13**: 671-675.

George EF (1993) Plant propagation by tissue culture. Part.1. The technology- Exegetics Ltd., Edington. 335-354.

Kivcak B and **Mert T** (2002) Preliminary evaluation of cytotoxic properties of *Laurus nobilis* leaf extracts. Fitoterapia. **73**: 242-3.

Laublin G, Hargurdeep S and **Mario C** (1991) *In vitro* plant regeneration via somatic embryogenesis from root culture of some rhizomatous irises. Plant Cell Tiss. and Org. Cult. **27**: 15-21.

Lu C and Vasil IK (1981) Somatic embryogenesis and plant regeneration from leaf tissues of *Panicum maximum* Jacq. Theor. Appl. Genet. **59**: 275-280.

Kaurinovic B, Popovic M and **Vlaisavljevic S** (2010) *In vitro* and *in vivo* effects of *Laurus nobilis* L. leaf extracts. Molecules **15**: 3378-3390.

Malamug JJF, Inden H and **Asahira T** (1991) Plantlet regeneration and propagation from ginger callus. Sci. Hortic. **48**: 89-97.

Oran SA and Fattash IF (2005) *In vitro* propagation of an endangered medicinal bulbous plant *Sternbergia clusiana* Ker-Gawler (Amaryllidaceae). J. Hort. Sci. and Biot. **80**(4):399-402.

Ozcan B, Esen M, Sangun M.K, Coleri A and **Caliskan M** (2010) Effective antibacterial and antioxidant properties of methanolic extract of *Laurus nobilis* seed oil. J. Environ. Biol. **31**: 637-41.

Park SY, Ahn JK, Lee WY, Murthy HN and **Paek KY** (2005) Mass production of *Eleutherococcus koreanum* plantlets via somatic embryogenesis from root cultures and accumulation of eleutherosides in regenerants. Plant Sci. **168**:1221–1225.

Qnais EY, Abdulla FA, Kaddumi EG and **Abdullah SS** (2012) Antidiarrheal activity of *Laurus nobilis L*. leaf extract in rats. J. Med. Food. **15**: 51-7.

Quiroz-Figueroa FR, Rojas-Herrera R, Galaz-Avalos RM and **Loyola- Vargas VM** (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tiss. & Org. Cult. **86**: 285-301.

Radojevic L and **Subotic A** (1992) Plant regeneration of *Iris setosa* Pall. through somatic embryogenesis and organogenesis. J. Plant Physiol. **139**: 690-696.

Radojevic LJ, Sokic O and **Tucic B** (1987) Somatic embryogenesis in tissue culture of iris (*Iris pumila* L.). Acta Hort. **212**: 719-723.

Ramos C, Teixeira B, Batisa I, Matos O, Neng NR, Nogueira JMF, Nunes ML and **Marque A** (2012) Antioxidant and antibacterial activity of essential oil and extracts of bay laurel *Laurus nobilis* Linnaeus (Lauraceae) from Portugal- Nat. Prod. Res. **26**: 518-29.

Reinert J (1959) Uber die kontrolle der morphogenese und die induktion von adventivembryonen an gew- ebekulturen aus karotten. Planta **53**: 318–333.

Saab AM, Tundis R, Loizzo MR, Lampronti I, Borgatti M, Gmbari R, Menichini F, Esseily F and **Menichini F** (2012) Antioxidant and antiproliferative activity of *Laurus nobilis* L. (Lauraceae) leaves and seed essential oils against K562 human chronic myelogenous leukemia cells. Nat. Prod. Res. **26**:1741-5.

Souayah N, Khouja ML, Khaldi A, Rejeb MN and **Bouzid S** (2002) Breeding improvement of *Laurus nobilis* L. by conventional and *in vitro* propagation techniques. J of Herbs, Spices and Medicinal Plants **9**(2-3): 101-105.

Steward FC, Mapes MO and **Smlth J** (1958) Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am. J. Bot. **45**: 693-703.

Toribio M, Fernaadez C, Celestino C, Martinez MT, Sanjose MC and **Vieitez AM** (2004) Somatic embryogenesis in mature *Quercus roburtrees*. Plant Cell Tiss. & Org. Cult., **76**: 283-287.

Tran Thi L and **Pleschka E** (2005) Somatic embryogenesis of some *Daucus* species influenced by ABA. J. Appl. Bot. Food Qual. **79**: 1-4.

Von Arnold S, Bozhkov P, Clapham D, Dyachok J, Filonova L, Ho¨Gberg KA, Ingouff M and **Wiweger M** (2005) Propagation of Norway spruce via somatic embryogenesis- Plant Cell Tiss. & Org. Cult. **81**: 323-329.

Wang XD, Nolan KE, Irwanto RR, Sheahan MB and **Rose RJ** (2011) Ontogeny of embryogenic callus in *Medicago truncatula*: the fate of the pluripotent and totipotent stem cells. Annals of Botany **107**(4): 599-609.

Wernicke W and **Brettell R** (1980) Somatic embryogenesis from *Sorghum bicolor* leaves. Nature (London). **287**: 138.

Ying-Chun Chen and **Chen Chang** (2009) plant regeneration through somatic embryogenesis from young leaves of *Cinnamomum kanehirae* Hayata. Taiwan J ~~for~~ Sci., **24**(2): 117-125.

Zhang CG, Li W, Mao YF, Zhao DL, Dong W and **Guo GQ** (2005) Endogenous hormonal levels in *Scutellaria baicalensis* calli induced by thidiazuron Russ. J. Plant Physiol. **52**: 345-351.