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Plant Regeneration through somatic embryogenesis from flower bases of Laurus nobilis L. (Lauraceae).

Ahmad H. Al-Gabbiesh[1], Ghabeish I.H [2]

*1. Department of Allied Medical Sciences, Zarka University College, Al-Balq'a Applied University, Salt-Jordan.
2. Dept. of Plant Production and Protection, Al-Balq'a Applied University, Al-Salt 19117, Jordan.

***Corresponding author email:** ahmad.gabesh@bau.edu.jo

ABSTRACT: A simple protocol has been developed for plant regeneration by somatic embryogenesis of Laurus nobilis L. (Lauraceae), newly recorded to the Flora of Jordan, laurel crop plants. Somatic embryogenesis was induced in zygotic embryo culture on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxy acetic acid (8 mg/L) as the sole plant growth regulator, where both embryogenesis calli and somatic embryos were induced. Separation of embryos from embryo clusters was necessary to enhance the frequency of germination. Germination was stimulated by separation of embryos successfully from embryo clusters and transfered onto fresh MS medium. Only flower bases were positively responding to plant regeneration than other explants, especially in inducing callus formation and in sustaining faster callus growth.
Keywords :Micrppropagation, Aseptic conditions, Callus, Murashige and Skoog Media, Somatic embryogenesis,

Abbreviations
MS: Murashige & Skoog - 2,4-D: 2,4-dichlorophenoxiacetic acid - BA:Benzyl adenine –IAA: Indole acetic acid – BAP: Benzyl amino purine - NAA: naphthalene acetic acid.

INTRODUCTION

Laurus are dioecious evergreen trees or large shrubs, with leathery aromatic leaves, and small yellow flowers followed on female plants by black berries.L. nobilis is a large, erect evergreen shrub with aromatic, narrowly ovate, leathery leaves useful in cooking. Flowers small, pale greenish-yellow, in dense clusters; fruit oval, glossy black when ripe. Bay laurel (Laurus nobilis L., Lauraceae) is native to the Mediterranean and laurel crops are distributed in areas with moderate and subtropical climate, The Laureaceae family which comprise 32 genera and about 2000-2500 species of numerous aromatic and medicinal plants with showy flowers. The genus Laurus is widely distributed from the temperate zone to the subarctic zone in the Nothern Hemisphere (Ozcan et al.,2010). These plants are commonly planted in gardens, and widely used in floral arrangement for ornamental purposes. Laurus nobilis L., also called bay laurel. L. nobilis is a species held in high esteem since ancient times. It was dedicated to Apollo, the ancient Greek god of light, and a symbol of peace and victory used to make wreaths for emperors, generals, and poets (Rohwer, 1993). Laurus nobilis L. native to Madaba and Zarka -Jordan as Mediterranean regions is also known as sweet bay, bay laurel, Grecian laurel, true bay, and bay (Figure 1). The dried leaves are used extensively in cooking, and the essential oil is generally used in the flavourings industry (Bauer and Garbe, 1985). Furthermore, the propagation of Laurus species through seedlings is known to be difficult due to a poor fruit set and a very low germination rate (Hartmann, 1997). However, this species has become very rare and endangered by overcutting (Chang et al. 2002). At present, the conventional methods of propagation by cutting, seeds, and layering are very slow and do not guarantee homogeneity. The small seed yield from wild trees is associated with difficulties of pollination and damage consumption by birds. Plant tissue culture is a powerful alternative technique for conservation and propagation of plants, especially for those that are rare and difficult to propagate by conventional methods. Plant regeneration via somatic embryogenesis has been reported in various species of Laurus (Ying-Chun Chen and Chen Chang, 2009). Somatic embryogenesis is an ideal system for the study of morphological, physiological, molecular and biochemical events occurring during the onset and development of embryogenesis in higher plants (Quiroz-Figueroa et al. 2006). In monocotyledonous plants, the morphology of embryos, especially of the cotyledon, also called the scutellum, varies depending on the species (Hartmann et al. 1997). For laurus species, detailed information concerning morphological characteristics of somatic embryos is very limited (Radojevic et al. 1987; Laublin et al. 1991; Radojevic and Subotic 1992; Jehan et al. 1994).The study conducted to develop a valuable protocol for Laurus nobilis L. regeneration by somatic embryogenesis as a

contribution to conservation of Laurus nobilis L. in Jordan as parts of a national strategy to conserve the wild genetic resources of the country, have been conducted in Jordan dealing with similar monocot bulbous plants such as Sternbergia clusiana (Oran and Fattash, 2005), Narcissus tazetta (Abu-Zahra and Oran, 2009) the black Iris (Iris nigricans and other species of Iris (Al-Gabbiesh et al., 2006, 2007).

MATERIALS AND METHODS

Micropropagation of Laurus nobilis L.

Plant materials

Laurus nobilis L. species used in this study were collected from the wild in Madaba and Hashemite University campus (Al-Hashemia, Zarka, Jordan)
during April 2012. This species represent aromatic and medicinal plants from the Lauraceae family. They were identified by Prof. Sawsan Oran, plant taxonomist, and by using descriptive references (Al-Eisawi, 1986; Flora Palaestina, 1986).To establish enough mother stock cultures from the threatened Laurus nobilis L., cuttings were cultivated in the field.

Media preparation

The name of media and concentrations of growth regulators used in this study are adopted by Ahmad Husni Al-Gabbiesh and presented in Table 1. Stock solutions of MS basal medium (inorganic and organic elements are shown in Table 2) and stock solutions of growth regulators were also prepared. All the stock solutions were stored in the refrigerator. To prepare one liter of medium, inorganic and organic solutions, sucrose, and agar were mixed as recommended and then sterilized by autoclave at 121 °C and 15 psi for 20 minutes. Then, the medium was poured into 9-cm Petri dishes (about 25-30 ml in each); after solidification, Petri dishes were kept in refrigerator until using.

Callus culture system

The collected samples were washed with running tap water. Different explants (flower bases, leaf bases) were excised and soaked in 70% ethanol for 1-2 minutes. These explants were then longitudinally sectioned into 5-6mm segments. The small segments were surface sterilized for 15 minutes with 5% (v/v) sodium hypochlorite solution, supplemented with 1% (v/v) Tween 20 tand finally rinsed three times with sterile distilled water for 10 minutes. Anthers that also used as explants were sterilized by soaking in 70% ethanol for one minute. Sterilized explants were transferred to the surface of callus medium (CM).Cultures were maintained under dark conditions at 27+1 °C. Some explants (flower bases and leaf bases) were transferred to fresh (CM) medium because of browning that caused by phenolic exudations. Callus cultures were maintained by sub-culturing on fresh CM as needed. Calli that formed were transferred after about four weeks from initiation to callus medium (CM) and maintained in dark with continuous sub-culturing as needed. After about three weeks, friable callus were transferred from CM to embryogenesis medium (EM), where embryogenesis was observed. The following data were recorded: frequency of explants producing calli, frequency of calli producing embryo-like structures. Callus initiation frequency was measured as the number of explants producing calli divided by the total number of explants cultured, embryo-like structures frequency as the number of calli producing embryo-like structures divided by the total number of explants cultured. All the data were transformed using the (arcsine %) 1/2 transformation for statistical analysis. Data were analyzed as a complete random design with three replications.

RESULTS AND DISCUSSION

Micropropagation of Laurus nobilis L.

In spite of substantial efforts to induce callus from different explants (flower bases, leaf bases), only flower bases were found to respond positively to the culture conditions than other explants, especially in inducing callus formation and in sustaining faster callus growth. These results are in agreement with Jehan et al., (1994) who reported that flower pieces were the best for somatic embryogenesis. More than 550 flower bases from l. nobilis were cultured on CM. Sporadic callus initiation was observed in cultures within 3-4 weeks (Figure 4). The frequencies of studied characters are reported in (Table 3). The results showed different responses to callusing. Calli fragments that were transferred to EM produced white embryo-like globular structures within two weeks (Figure 5). These structures grew rapidly and new ones continued to appear on the callus surface, as well as on previously formed structures. Within 3 more weeks, clusters of structures at various stages of development could be found on the same callus (Figure 6). These structures were similar to the somatic embryos described by Reuther (1977) on Iris species, and by Souayah et al., (2002) and Kaurinovic et al., (2010) with L. nobilis and Jehan et al., (1994) with I. pallida and I. germanica, and by Radojevic and Subotic (1992) with I. setosa.

Table 1. Name of media used in this study.

Components	Media
MS medium + 4.5 µM 2,4-D + 0.5 µM Kinetin + 4.5 µM NAA + 2.5 µM BAP	Callus Medium (CM)
MS medium + 4.5 µM BA + 1.5 µM IAA 0.45 µM 2,4- D + 4.5 µM NAA	Callus Growth Medium (CGM)
MS medium + 4.5 µM 2,4 –D + 0.5 µM Kinetin + 4.5 µM NAA + 200mg/l proline	Embryogenesis Medium (EM)

Table 2. Nutritional components of Murashige and Skoog medium.

Components	(mg/l)
Macronutrients	
NH4NO3	1650
CaCl2.H2O	440
MgSO4.7H2O	370
KNO3	1900
KH2PO4	170
Micronutrients	
H3BO3	6.20
COCl2.6H2O	0.025
CuSO4.5H2O	0.025
Na2EDTA.2H2O	37.30
FeSO4.7H2O	27.80
MnSO4.7H2O	22.30
Na2MoO4.2H2O	0.25
KI	0.83
ZnSO4.7H2O	8.60
Organic Compounds and Vitamins	
Sucrose	30000.0
Myo-inositol	100.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Agar	8000
PH	5.8

Table 3 . Frequency (%) for two characters of one . Laurus nobilis L.

Species	No. of cultured flower bases	% of flower bases producing calli	% of flower bases producing embryo-like structures
Laurus nobilis	550	62.52	0.780

Table 4. Mean squares and degree of freedom (df) from analysis of variance for the frequency (%) of two characters for the flower bases of one Laurus nobilis L.

Source of variation	df	% produced calli	% produced embryo-like structures
Treatment	2	2083.964**	40.808**
Error	6	-	-

** Significant at P=0.01.

Figure 1. Laurus nobilis L. growing in natural habitat/Jordan (Photographed by Ahmad, Al-Gabbiesh).

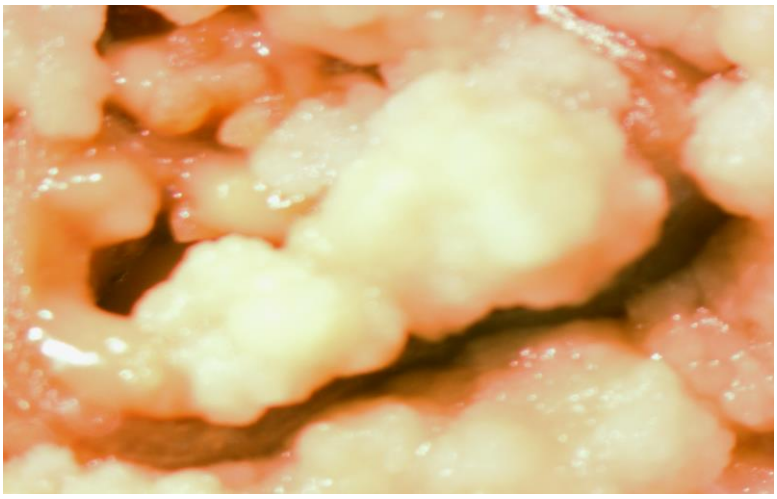

Figure 2. Callus formation from flower base of Laurus nobilis L. on callus medium.

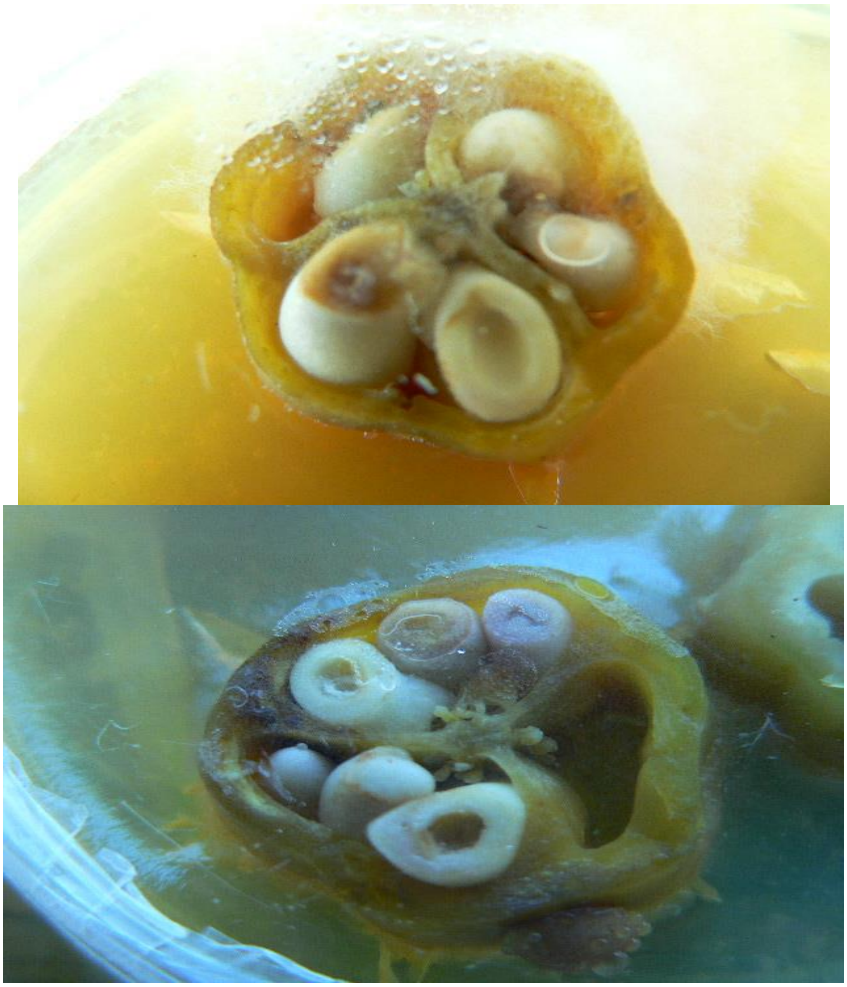

Figure 3. Embryo-like structure formation from flower base of Laurus nobilis L. after transferring callus to embryogenesis medium for 2 weeks.

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