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Micropropagation of Carob Tree (*Ceratonia siliqua*): Somatic Embryogenesis from Immature Seeds

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***Abstract*— Somatic embryos of carob tree (*Ceratonia siliqua* L.) were induced from segments excised from immature seeds when cultured on Murashige and Skoog (M.S.) medium supplemented with a gradient of concentrations of 6-benzylaminopurine (BA). Roots and shoots were also formed in the same media. Besides culture media composition, the developmental stage of the explants showed a strong influence on somatic embryogenesis, with embryos and cotyledons providing high levels of induction; thus, elevated frequencies of development occurred on culture media containing various concentrations of BA. The presence of morphologically normal and abnormal somatic embryos was also observed. Upon transfer into full- and half-strength M.S. medium supplemented with sucrose (3%), plantlets were coming through embryos. Attempts to induce embryogenic callus from immature seeds of carob tree were successful. The resulting outgrowths were very high in embryogenetic potential from green immature seeds of carob tree and expants transferred into M.S. enriched with B.A. developed a large number of somatic embryos.**

***Index Terms*—carob, seeds, somatic embryogenesis.**

I. Introduction

Ceratonia siliqua L. (commonly known as carob tree) is a slowly growing, woody evergreen sclerophyll species, widespread as a native plant in the Mediterranean Basin; it is also distributed in Arabia and Oman, and has been introduced in California, Mexico and Australia [1]. *Ceratonia siliqua* L. (Leguminosae) is considered a phylogenetically primitive species of tropical origin that has been cultivated in the Mediterranean area since historic times [2]. According to the Plant List, the genus *Ceratonia* includes four species, among them *Ceratonia siliqua* is a morphologically distinct, very interesting, tolerant plant species. Also, *C. siliqua* is a significant component of the native Mediterranean vegetation and an important economic plant [3], which has been used for afforestation in semi-arid regions [4]. Carob pods with their rich in sugars pulp are a staple in the diet of farm animals and have been used by people in times of famine. As a food source, carob pods can be stored and transported long distances. Also, a kind of flour that has application in food industry is obtained from the seed endosperm of *C. siliqua* and is rich in galactomannans [5], [6]. Currently, the main interest is seed collection for gum extraction [7]. Carob trees are also useful as ornamentals, for natural windbreaks, landscaping, and urban horticulture. Although, carob tree has been neglected with respect to cultural practices, very useful information about this plant species can be found in articles written by scholars working in Cyprus [8], Greece [9]-[11], Italy [12], Israel [13], Portugal [14] and Spain [15]. The scientific name of carob tree (*Ceratonia siliqua* L.) derives from the Greek *keras* (*κεράτιον*, i.e. horn), and the Latin *siliqua*, alluding to the hardness and shape of the pod [16]. It was supposed that carob seeds were constant in weight [17], therefore carob has given its name to the carat and was particularly famous in this regard.

In the eastern Mediterranean, *C. siliqua* is subjected to a prolonged drought period, which immediately follows its main growth period and leaf development, during spring [18], [19]. Carob trees grow up to 10 m in height and exhibit broad semi-spherical crowns and thick, dark brownish trunks with rough barks and sturdy branches. The compound leaves of *C. siliqua* expand within a 3-month period (i.e. from March to May), then they ceased growing, whereas they are maintained on the stems and are exposed to the environmental conditions of the Mediterranean ecosystem for approximately twenty months [20]. Also, the leaves have a very thick single-layered upper epidermis, the cells of which contain phenolic compounds in their large vacuoles, and stomata are present only in the lower epidermis and arranged in clusters [21]. It was found that the root apices of deep tap roots of *C. siliqua* sustain water flux to the leaves [11], [22]. During flowering period of *C. siliqua*, in autumn, male, female and hermaphrodite flowers are generally expanded on different trees [23], [24].

In the Mediterranean region wild and naturalized carobs are distributed in more or less the same geographic and climatic belt as the cultivated trees of this species. Spontaneous carob stands are particularly common at low altitudes along the Mediterranean coast, Peloponnesus, Crete and the Aegean region in Greece, southwest Spain, southern Portugal, the Balearic Islands, southeast France, the shores of southern Italy including Sicily, the Adriatic coast of Croatia, Turkey, along the northern and southern ranges of the isle of Cyprus, in the islands of Malta, in the maritime belt of Lebanon and Israel, the north and south of Morocco and the coastline in Tunisia. However, the distribution of the evergreen *C. siliqua* in the Mediterranean region, is restricted by winter cold stress [25]. In some areas along the shores of the Mediterranean Sea, wild carobs occupy places not disturbed by cultivation. Wild carobs are reproduced by seeds, whereas cultivated varieties are propagated as clones. The three main fruit traits that distinguish domesticated carobs from their wild relatives are the bean size, the amount of pulp and the sugar content; in fact, the pod features together with productivity and environmental adaptation seem to have been the most important selection criteria. Propagation of seedlings and

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mature tissues of *C. siliqua*, through the formation and conversion of somatic embryos has been carried out with promising results [26]-[28].

The purpose of this work was to study the formation of somatic embryos from immature seeds of carob (*Ceratonia siliqua* L.); this comprehensive information would allow us to improve understanding of micropropagation via the functionality of this species. To the best of our knowledge *in vitro* micropropagation techniques using green, immature carob seeds have not been hitherto reported.

II. Materials and Methods

Green immature seeds (5–8 mm in length) from stands of wild, adult carob trees, grown under field conditions in Peloponnesian territory of Greece (latitude 37°09′20′′, longitude 21°55′36′′, altitude 300 m), were collected during spring. The immature seeds used in the experiments were the source of explants also used for *in vitro* culture establishment of *C. siliqua*. All the plant tissues used in this study were surface-sterilized by immersion in 70% ethanol for 1 min, and in 2% (w/v) sodium hypochlorite for 10 min, followed by duplicate rinses (5min each) in sterile distilled water. To induce the formation of calli, various, different tissues were cultured on full-strength and half-strength Murashige and Skoog (M.S.) micronutrients medium [29] containing a gradient of 6-benzylaminopurine (BA) concentrations (i.e. 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 μM). Also, in the medium that was solidified with agar, sucrose 3% was added and the pH was adjusted to 5.6 prior to autoclaving (120 °C, 20 min). In the beginning, immature embryos and cotyledons of *C. siliqua* were placed in the culture media and remained in a growth cabinet at 25±1 °C and 16 h photoperiod (500 μmol cm^{-2} s^{-1} PAR), for 15 days; then selected explants were transferred in the growth cabinet for 25, 45 and 50 days.

III. Results and Discussion

With regard to explants formed by immature seeds collected from the wild tree, 5-6 months after the flowering of *C. siliqua*, the development of somatic embryos reached approximately 40%, 20%, 80%, 50% and 12% in growth media containing 1.0, 2.5, 5.0, 10.0 and 25.0 μM of BA, respectively. Also, somatic embryos have been observed on the surface of swollen hypocotyls of the zygotic embryos and on cotyledons (direct somatic embryogenesis), as well as on the surface of calli, which had been formed by the same explants (indirect somatic embryogenesis) Multiple somatic embryos have been frequently developed per explant, in the M.S. medium supplemented with 5 μM BA, which 'sprouted' together like 'bouquets' (Fig. 1).

The observed co-existence of multiple, individual somatic embryos in the same explant (Fig. 2) was accompanied by either regularly developed or anomalous somatic embryos, possessing different development, i.e. globular, heart-shaped, torpedo and cotyledon-stage types have bssn observed; this is in agreement with earlier results [30]-[34]. In addition, multiple primary and secondary somatic embryos have been produced. In particular, the observed formation of non-synchronized somatic embryogenesis in the same explant, either directly or indirectly, from calli demonstrates different reactions of various cell-types of each explant to the same stimuli. For example, somatic embryos did develop from explants grown in M.S. medium enriched with other plant-growth regulators, i.e. IBA (indole-3-butyric acid), NAA (a-naphthaleneacetic acid) and 2.4-D (2,4-dichlorophenoxyacetic acid), and but in substantially smaller percentages, in comparison with those formed in M.S. enriched with BA.

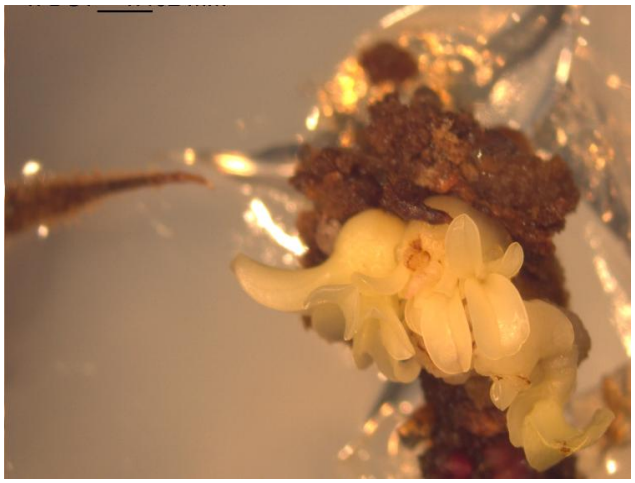

Fig. 1. Multiple somatic embryos of carob tree sprouting together like 'bouquets" from explant cultured in M.S. supplemented with 5 μM BA (black bar scale: 1.5 mm).

Fig. 2. Somatic embryos developed over the surface of the callus form a cotyledon-explant cultured in M.S. supllemented with 2.5 μM BA, and remained in darkness for 45 days.

In immature seeds collected 5 months after the onset of blossoming and cut on the sagittal axis, in darkness, the development of calli, as well as the appearance of budding and somatic embryogenesis was observed (Table 1), whereas, neither blastogenesis nor rhizogenesis were observed. The somatic embryos were transferred to either a full-strength or a half-strength M.S., in which they grew, developing stems, leaves and roots (Fig. 3). With regard to the whole embryos, developed from cotyledons of immature seeds (approximately 5 mm in length), 5.5 months after the onset of anthesis in the darkness, the development of calli and the appearance of budding in a very high percentage of embryos (50% to 90%) and somatic embryogenesis in a lower percentage has been observed. However, neither blastogenesis nor rhizogenesis were observed. Somatic embryogenesis was apparent in 20% to 60% of the explants,

depending on the concentration of BA (Table 1). With regard to the sections of embryos and cotyledons formed by immature seeds (5-8 mm in length), 6 months after the onset of anthesis in the darkness, the appearance of budding, rhizogenesis and somatic embryogenesis have been observed. Calli did develop, when the concentration of 10 μM BA was added to the medium and the percentage of outgrowths and somatic embryogenesis was 90%. With regard to the explants from calli and swollen tissues from cotyledons and parts of embryos from immature seeds (5-8 mm in length), 6 months after the onset of anthesis, lower percentages have been detected; it is worth mentioning that in the above mentioned cases, the tissues were cultivated in M.S. medium for 25, 45 and 50 days, in the darkness. Somatic embryogenesis was observed in those cases in which the explants were cultivated for 45 days and a greater quantity displayed after 50 days cultivation in M.S., in the darkness; this was observed in 50% of the explants exposed to 1 μM BA and 3% sucrose. In M.S. medium containing 5 μM BA and 3% sucrose, the somatic embryogenesis reached 20% after 45 days, and in 2.5 μM BA was substantially higher, i.e. 80% after 50 days.

Table 1. Formation of calli, budding, roots and somatic embyos from tissues of carob immature seeds (5-6 month old); explants were additionally cultured in M.S. media, in the darkness for 25, 45 and 50 days (d), respectively.

Time after blossom (months)	BA (μM)	calli (%)	budding (%)	rhizo genesis (%)	somatic embryo genesis (%)
	seeds	cut	along	sagittal axis	
5.0	1.0	50	50		50
5.0	2.5	32	61		40
5.0	5.0	30	60		51
5.0	10.0	31	50		52
5.0	25.0	52	52		54
	embryos	with	cotyledons		
5.5	1.0	40	30		45
5.5	2.5	80	46		38
5.5	5.0	78	68		22
5.5	10.0	90	80		59
	parts of	embryos	and	cotyledons	
6.0	1.0		32	26	30
6.0	2.5		18	30	18
6.0	5.0		29	15	29
6.0	10.0	4	12		10
6.0	25.0		10	28	
6.0+25 d	1.0	18		20	
6.0+25 d	2.5	70		19	
6.0+25 d	5.0		21	22	
6.0+25 d	10.0	55		10	
6.0+25 d	25.0			12	
6.0+45 d	1.0	31	22	61	50
6.0+45 d	5.0	38	30	20	20
6.0+45 d	10.0	78	19		
6.0+45 d	25.0	91		5	
6.0+50 d	2.5		90		85
6.0+50 d	10.0		72		73

Fig. 3. Roots, stems and leaves developed from somatic embryos of *C. siliqua* cultured into half- (left) and full-strength M.S. (right).

IV. Conclusion

Immature seeds isolated from pods of *C. siliqua* were used as a source of explants for callogenesis and somatic embryogenesis. Suitable developmental stages of immature seed explants, and *in vitro* culture conditions for the production of translucent calli were identified. The bud induction and multiplication was greatly influenced by the addition of various concentrations of the cytokinin 6-benzylaminopurine (BA). It appears that BA supplemented into the culture medium was a prerequisite for callogenesis. Both the developmental stage of the explant and the BA concentration had significant effects on the percentage of calli production. Immature seeds gave rise to calli when cultured on media containing BA. Embryogenic calli consisted of a white-translucent mass, proliferating all around the explants, gave rise to somatic embryos. On day-45, somatic embryos were observed as globular structures. On day-50, complete somatic embryos were initiated. The study reflects a better understanding of the stabilization of techniques for *in vitro* micropropagated tissues from immature seeds *C. siliqua*.

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