New insights on carob floral biology

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Faculdade de Engenharia de Recursos Naturais

New insights on carob floral biology * Custódio,

7

Doutoramento em Ciências Biotecnológicas

LIST OF ERRORS

Page	*§*	*Line*	*How it reads*	*How it should be read*
Account		27	Nutricão Mineral das Plantas	Nutrição Mineral de las Plantas
Aknowl.	6	1	Dr. Maria-Amélia Loução	Prof. Dr. MA Martins-Loução
List of Abbrev.	column 2	8	double haploids	double haploid
		24	capillary	capillary gas chromatography
		27	capillary gas chromatography coupled to mass spectometry detection	headspace solid phase microextraction followed by capillary gas chromatography coupled to mass spectometry detection
Table of contents		2	List of abreviations	List of abbreviations
		46	Analysis of the volatiles of carob tree flower	Analysis of the volatiles of carob tree flowers
		12	Before «5.4. Conclusions» it should be read «5.3.3. Concentration of carbohydrates in pulp and seeds during fruit development ....152»	
Resumo	4	4	Para alem disso	Para além disso
	5	5	único açúcar solúvel	único açúcar insolúvel
			Keywords	Palavras chave
5	2	5	bissexual	bisexual
7	2	2	Bosh et al. 1996	Bosch et al. 1996
8	2	1	it does not nodulate	does not nodulate
8	4	5	Blondel an Aronson 1999	Blondel and Aronson 1999
11	1	2	and the same origin	and of the same origin
17	1	2	information for the male	information of the male
19	1	1	access	assess the seasonal
28	Fig. 2.2	caption	At the end of the caption it should be read (McCormick 2004)	
30	3	2	(Fig. 2.4)	(Figs. 2.3, 2.4 and 2.5.)
33	4	6	The average weight of 25	The average weight of 30
33	5	The sentence: «For the flowering ... were used» should not be read.		
38	4	4	epidermis with short, trichomes,	epidermis with short trichomes,
46	3	2	2.9D	2.9E
46	3	4	Plate 2.9D	Plate 2.9E
46	3	9	2.9E	2.9D
52	1	4	with and marginal placentation.	with a marginal placentation
63	Plate 2.4	Fig.B	Following the } it should be read “pi”	
85	6	1	Arthur and Pawliszyn	Arthur and Pawlizyn
92	Fig. 3.4	caption	by SPME/CGC-MSD	by HS-SPME-CGC/MSD.
94	Sub-title		3.3.2. Volatiles of isolated floral parts	3.3.2. Volatiles of isolated floral organs
96	Fig. 3.5	caption	by HS-SPME-GC/MS.	by HS-SPME-CGC/MSD.
99	1	7	(Tollsten and Bergstrom 1993)	(Tollsten and Bergström 1993)
103	1	7	from emitted by	from the emitted by
105	1	3	access	assess
119	2	3	between the sexual type	between the sexual types
120	1	7	in the opposite was true	the opposite was true
120	3	7	(Buwalda and Meekgins 1990)	(Buwalda and Meekings 1990)

Page	*§*	*Line*	*How it reads*	*How it should be read*
124	2	4	higher nutrient	lower or similar nutrient
137	2	1	carbohydrate	carbohydrates
140	2	4	(Fig. 5.3	(Fig. 5.3).
144	3	16	short-term	short-term sinks
145	2	11	sucrose content sucrose	sucrose content
149	1	2	which decreased from	with a plateau from
149	2	3	(Fig. 5.9)	(P<0.05, Fig. 5.10)
150	Fig. 5.9	caption	The sentence «and for different…upper case letters)» should not be read	
150	2	2	(Fig. 5.9)	(P<0.05, Fig. 5.9)
151	1	2	(Fig. 10).	(Fig. 5.9).
152	Fig. 5.10	caption	The sentence «and between sexual…upper case letters)» should not be read	
153	3	2	accessed	assessed
153	4	2	was where not	was not
154	Fig. 5.11	caption	Following «… (lower case letters), » it should be read « and for different sexual type and cultivars for the same organ and stage of fruit development (upper case letters)»	
169	1	4	leve	level
172	2	6	male and female	male and hermaphrodite
175	2	2	this pollen	that pollen
181	1	6	indunce	induce
182	2	1	that the both the	that both the
182	2	4	flower	flowers
182	4	2	induction androgenesis	induction of androgenesis
193	1	2	Following «Nitsch 1974, 1981» it should be read «Reinert and Bajaj 1977»	
203	2	1	multicelular	multicellular
204	2	7	dehidratation	dehydratation
205	1	2	pathay	pathway

Capa: Estampa de *Ceratonia siliqua* (Estampa T2. 100).
Edição: Jardim Botânico, Museu Nacional de História Natural.

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New insights on carob floral biology

Luísa Margarida Batista Custódio

Dissertação apresentada à Universidade do Algarve para obtenção do Grau de Doutor em Ciências Biotecnológicas (Biotecnologia)

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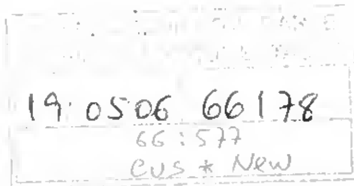
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Na presente dissertação incluem-se resultados que foram, ou serão, alvo de publicação em colaboração com A. Romano, A. Cavaco, M.A Martins-Loução, M.F. Carneiro, J.M.F Nogueira, P.J Correia, D. Pereira, H. Serra, N. Fernandes, P. Jesus e S. Gonçalves.

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The real voyage of discovery
consists not in seeking new landscapes,
But in having new eyes….

Marcel Proust

Research is formalized curiosity.
It is poking and prying with a purpose.

Zora Neale Hurston

To my parents and sister, for their unconditional support

To Rui, for being the one…

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List of Abbreviations

ANOVA	analysis of variance
ADPGPPase	ADP glucose pirophosphorylase
BA	N^6-benzyladenine
CoA	acetyl-coenzyme A
CWSP	cell wall structural polysaccharides
Da	Dalton
DAPI	4,6-diamidino-2-fenilindole.HCl
DH	double haploids
DMAPP	dimethylallyl pyrophosphate
DMSO	dimethylsulfoxide
DOXP/MEP	deoxyxylulose phosphate/methylerythritol
eV	electro Volts
FAA	formalin-aceto-alcohol
FDA	fluorescein diacetate test
Fig.	figura
FPP	farnesyl pyrophosphate
Fru-6-P	fructose-6-phosphate
Gal	galactose
GC	gas chromatography
GGPP	geranylgeranyl pyrophosphate
GM	galactomannans
GPP	geranyl pyrophosphate
g, mg, µg	gram, milligram, microgram
CGC	capillary
h, min, s	hour, minute, second
HPLC	high-performance liquid chromatography
HS-SPME-GCD/MSD	capillary gas chromatography coupled to mass spectrometry detection
H_2SO_4	sulphuric acid
ID	internal diameter
IPP	isopentenyl diphosphate
Kin	kinetin
l, ml, µl	liter, millilitre, microliter
m, cm, mm, nm, µm	meter, centimetre, millimetre, nanometer, micrometer
Man	mannose

Man-6-P	mannose-6-phosphate
MS	Murashige and Skoog medium
MS	mass spectometry
MSD	mass spectrometry detection
NADH	nicotinamide adenine dinucleotide
NSC	non-structural carbohydrates
RT	room temperature
SEM	scanning electron microscopy
SPME	solid phase microextraction
SPS	sucrose phosphate synthase
SPSS	statistical package for the social sciences
Suc	sucrose
Suc-6-P	sucrose-6-phosphate
TIC	total ion chromatogram
TDZ	thidiazuron
Torr	Non-SI unit of pressure; = 133.3223684 Pascals
Tween 20	polyoxyethylene sorbitan monolarate
UDP-gal	uridine diphosphate galactose
v/v	volume/volume
w/v	weight/volume
Zea	zeatin
2,4-D	2,4-dichlorophenoxyacetic acid
%	percentage

SUMMARY

The general purpose of this work was to provide new approaches for the understanding of some aspects of the floral biology of carob tree (*Ceratonia siliqua* L.),one of the most useful trees of the Mediterranean basin, particularly those related with the reproductive process.

It was observed that the androecium of functional male flowers consists of a group of 4-7 stamens, inserted periphericaly around the receptacle. Each filament is partly surrounded by a sepal and has dorsifixed introrse versatile anthers. The gynoecium of functional female flowers is apocarpous with a superior unilocular ovary inserted in a central depression in the nectarial disk. Hermaphrodite flowers are a combination of male and female types, having both the androecium and the gynoecium. During the development of flowers six stages were identified. Anthers of both male and hermaphrodite flowers are tetrasporangiate and dithecal with microsporangia positioned laterally that open longitudinally when mature. The microsporogenesis is sucessive and results in binucleate pollen. It seems that macrogametogenesis is from the *Polygonum*-type. Ovule formation and macrosporogenesis occur during flower developmental stages 0, I and II, while megagametogenesis begins in stage III.

More than twenty-five compounds were identified in the headspace of whole fresh flowers and isolated floral parts by HS-SPME-CGC/MSD. The female flowers were much less fragrant than the male ones and flowers in stages III and IV showed higher abundances. Linalool and its derivates were the dominant volatiles.

Differences in nutrient concentrations between vegetative and reproductive organs are influenced not only by the phenological stages, but also by the tree sexual type. Flowers of female trees showed a lower nutrient concentration compared to males or hermaphrodites. Moreover, the female trees were able to allocate more nutrients to leaves than males.

The dynamics of partitioning and allocation of non structural carbohydrates seems to be very complex, and dependent on the sexual type and female cultivar. Glucose was the main soluble sugar present in leaves, flowers and fruits of carob tree. Females accumulated a higher level of total soluble sugars in the pulp in stage III of fruit development. Galactomannan was the only insoluble sugar present in seeds, and there were no differences in its content, between the sexual types and female cultivars.

A cryopreservation protocol for pollen was developed and the best results were obtained after a pre-treatment with 0.5 M sucrose, or with glycerol (2.5%) + DMSO (2.5%), and a storage period of 5 months. To obtain embryogenic *calli,* anthers from flowers in developmental stage I should be used, and cultured on MS medium supplemented with 0.5 mg l^{-1} 2,4-D + 4 mg l^{-1} TDZ.

Keywords: Anther culture, carbohydrates, *Ceratonia siliqua* L., cryopreservation, HS-SPME-CGC/MSD, macrosporogenesis, microsporogenesis, nutrients, trioecy, volatiles.

RESUMO

O objectivo geral deste trabalho foi o estudo de diferentes aspectos da biologia floral da alfarrobeira (*Ceratonia siliqua* L.), uma das árvores mais importantes da região Mediterrânica, particularmente os relacionados com o processo reprodutivo.

Foi observado que o androceu das flores masculinas funcionais é composto por um grupo de 4-7 estames, inseridos na periferia do receptáculo. Cada filamento é parcialmente rodeado por uma sépala, e possui anteras dorsifixas. O gineceu das flores femininas funcionais é apocárpico, e possui um ovário unilocular superior, inserido numa depressão central no disco nectarial. As flores hermafroditas são uma combinação dos tipos anteriores, e possuem o androceu e o gineceu. Durante o desenvolvimento das flores foram identificadas seis fases. As anteras das flores masculinas e hermafroditas possuem duas tecas com quatro microsporângios posicionados lateralmente, que abrem longitudinalmente quando estão maduros. O processo de microsporogénese é sucessivo, e resulta na formação de grãos de pólen binucleados. O processo de macrogametogénese parece ser do tipo *Polygonum*. A formação dos óvulos e a macrosporogénese ocorrem durante as fases 0, I e II de desenvolvimento da flor, enquanto que a megagametogénese se inicia na fase III.

Mais de vinte e cinco compostos voláteis foram identificados *in vivo* no aroma de flores inteiras e partes florais isoladas, por HS-SPME-CGC/MSD. As flores femininas possuem menos compostos voláteis do que as masculinas, e as flores nas fases de desenvolvimento III e IV apresentam maiores abundâncias. Os compostos voláteis dominantes foram o linalool e seus derivados.

As diferenças na concentração de nutrientes entre os orgãos vegetativos e reprodutivos são influenciadas não só pelo estado fenológico, mas também pelo tipo sexual da árvore. As flores femininas exibem uma menor concentração de nutrientes do que as flores masculinas e hermafroditas. Para alem disso, as árvores femininas apresentam uma maior capacidade de translocação de nutrientes para as folhas, comparativamente com as masculinas.

A dinâmica de partição e translocação de hidratos de carbono não estruturais parece ser bastante complexa, e dependente do tipo sexual da árvore e da cultivar feminina. A glucose foi o principal açúcar solúvel detectado nas folhas, flores e frutos. Foi observada uma maior acumulação de açúcares solúveis totais na polpa, em frutos na fase de desenvolvimento III. As galactomananas foram o único açúcar solúvel detectado nas sementes, e não foram observadas diferenças na sua concentração, entre tipos sexuais e cultivares femininas.

Foi desenvolvido um protocolo de criopreservação de pólen, e os melhores resultados foram obtidos após um pré-tratamento com 0.5 M sacarose, ou com glicerol (2.5%) + DMSO (2.5%), após um período de conservação de 5 meses. Para a obtenção de *calli* embriogénico devem ser utilizadas anteras isoladas de flores na fase de desenvolvimento I, e inoculadas em meio basal MS suplementado com 5 mg l^{-1} 2,4-D + 4 mg l^{-1} TDZ.

Keywords: *Ceratonia siliqua* L., criopreservação, cultura de anteras, hidratos de carbono, HS-SPME-CGC/MSD, macrosporogénese, microsporogénese, nutrientes, trioicia, voláteis.

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CHAPTER I

GENERAL INTRODUCTION

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1.1. THE CAROB TREE (*CERATONIA SILIQUA* L.)

'It is often stated that only a few staple crops produce the majority of the food supply. This might be correct, but the important contribution of many minor species should not be underestimated. Agricultural research has traditionally focused on these staples, while relatively little attention has been given to minor crops, particularly by scientists in developed countries. Therefore, such crops have generally failed to attract significant research funding. However, many of these neglected crops are adapted to various marginal growing conditions, such as arid places with very poor soils. Furthermore, many crops considered neglected at a global level are staples at a national or a regional level, contributing considerably to the economics of that region. The limited information available on many important and frequently basic aspects of neglected and underutilized crops hinders their development and their sustainable conservation.'

In: Batlle and Tous 1997

The carob tree (*Ceratonia siliqua* L.) has been grown since antiquity in most countries of the Mediterranean basin, usually in mild and dry places with poor soils. All over this area, carob is a conspicuous constituent of the Mediterranean sclerophyllous 'maquis' vegetation (Zohary 2002). The 'maquis' is today's Mediterranean forest, representing a semi-natural vegetation which is either a '*stage in the degradation of the Mediterranean sclerophyllous evergreen forest (a degradation almost entirely due to human intervention) or one of the stages in the gradual evolution of the vegetation towards that forest*' (Talhouk et al. 2005). The value of carob was recognised by the ancient Greeks, who brought it from its native Middle East to Greece and Italy, and by the Arabs, who disseminated it along the North African coast into Spain and Portugal (Batlle and Tous 1997).

The scientific name of carob (*Ceratonia siliqua* L.) derives from the Greek *keras* and the Latin *siliqua*, in allusion to the hardness and shape of the pod (Batlle and Tous 1997). The common name originates from the Hebrew *kharuv*, from which are derived the Arabic *kharrub* and later *algarrobo* or *garrofero* in Spanish and *alfarrobeira* in Portuguese (Batlle and Tous 1997). The carob is also known as St. John's bread or locust bean, in reference to the presumed use of its 'locusts' as food by St. John the Baptist, and from that derives *Johannisbrotbaum* in German. Jewellers used its uniform seed as a unit of weight, the carat (200 mg) (Batlle and Tous 1997).

1.1.1. *Botanical description*

Systematically, carob can be characterised as follows (Cronquist 1981):

Division: *Angiospermae*
Class: *Dycotyledones*
Order: *Rosales*
Family: *Fabaceae* (= *Leguminosae*)[1]
Sub-family: *Caesalpinioideae*
Genus: *Ceratonia*
Species: *Ceratonia siliqua* L.
Tribe: *Cassiae*

The genus *Ceratonia* belongs to the family *Leguminosae* (syn. *Fabaceae*), and is generally placed in the tribe *Cassiae* of the subfamily *Caesalpinioideae* (Batlle and Tous 1997, Zohary 2002, Ramón-Laca and Mabberley 2004). Some authors are inclined to remove carob to a separate tribe from the other *Cassiae* since it exhibits some unique features, such as the unique sepal position (the median saggital being in the adaxial side in a pentamerous flower) and the proeminent floral disk (Irwin and Barneby 1981, Hillcoat et al. 1980, Tucker 1992). Carob lacks the showy corolla, zygomorphic symmetry, porate stamens, partially unidirectional floral organogeny, and the many convergent floral specializations such as heterostameny found among the three large genera of *Cassiae*, *Senna*, *Chamaecrista*, and *Cassia* s. str. (Irwin and Baneby 1981, 1982, Tucker 1992). Furthermore, according to Goldblatt (1981) it has a different chromosome number (n= 12) from the other *Cassiae* (base number n= 14), what may suggest that it might be an aneuploid. The large number of plastic or variable characteristics of Ceratonia suggests primitiveness rather than specialization, and therefore it does not have the characteristics needed to resemble an ancestor to the derived *Cassiae* (Tucker 1992).

[1] *Fabaceae (= Leguminosae, Legume or bean family) (Judd et al. 1999). Three subgroups are generally recognized within Fabaceae: Caesalpinioidae, Mimosoideae and Faboideae (= Papilionoideae) (Judd et al. 1999). In most classifications (Polhill et al. 1981) these are considered subfamilies, but they are sometimes treated as separate families (Cronquist 1981).*

Carob tree is a small to medium-sized evergreen and thermophilous tree that may grow up to 20 m in height under ideal climatic conditions (Catarino 1993, Batlle and Tous 1997). Carob is also a xerophytic species well adapted to the ecological conditions of the Mediterranean area, characterized by a long dry season, with negligible rainfall and a high evaporative demand. Like in other Mediterranean species with long-lived leaves and an extended flowering season, in carob tree mechanisms have evolved that optimize water, carbon and nitrogen use for reproduction (Correia and Martins-Loução 1995).

Despite the stressful conditions associated with the Mediterranean climate, the foliage of field-grown carob trees generally remains green and apparently turgid as soil dries during summer, and several studies have shown that carob trees can withstand drought by displaying an avoidance strategy in face of water shortage (Nunes et al. 1989, 1992, Rhizopoulou and Davies 1991, Ramalho et al. 2000, Correia et al. 2001). Those strategies include: 1) the ability to explore water in deep soil; 2) the existence of a strong stomata control and 3) the possibility of osmotic adjustment and maintaining turgor above zero in a broad range of low osmotic water content (Ramalho et al. 2000). Furthermore, leaflets exhibits nastic movements, which contributes to a reduction of the radiant energy that is absorbed by leaf, and the shedding of leaves or even branches can sometimes occur in extreme summer drought associated to high temperature, which favours the maintenance of water in the remaining parts of the plant (Ramalho et al. 2000).

Carob has a broad semispherical crown and a thick trunk with brown rough bark and sturdy branches, and has an extensive root system which penetrates deeply the soil. Leaves are 10-20 cm long, sclerophyllous, alternate and pinnate, with or without a terminal leaflet. Leaflets are 3-7 cm long, ovate to elliptic, in 4-10 normally opposite pairs, coriaceous, dark green, shiny above and pale green beneath. The leaves have a very thick single-layered upper epidermis, with cells containing phenolic compounds in the large vacuoles (Mitrakos 1988).

Carob is a polygamo-dioecious or monoecious species (Tucker 1992). Individual carob trees may be male (with inflorescences carrying only staminate flowers), female (with inflorescences carrying only pistilate flowers) and hermaphrodite (with inflorescences carrying staminate flowers and others with pistilate flowers, or with inflorescences carrying hermaphrodite flowers), which made some authors to consider trioecy as one of the most outstanding biological features of this species (Retana et al.

1994). Furthermore, inflorescences can be polygamous, and there is a high plasticity in inflorescence and flower characteristics (Haselberg 1986, Tucker 1992, Batlle and Tous 1997).

Flowers are small and numerous, 6-12 mm long, spirally arranged along the inflorescence axis in catlin-like racemes borne on spurs from old wood or in the trunk (cauliflory). The flowers are usually pentamerous with the calix placed on a short pedicel, and are unusual in lacking petals (Tucker 1992). The calix is disk-shapped, reddish green and has nectarines (Tucker 1992). The flowers are initially bissexual, but usually one sex is suppressed during late development of functional male or female flowers (Tucker 1992). Pollen grains are spheroidal and tetracolpate (Fergusson 1980), with a diameter of 28-29 µm at the poles and 25-28 µm at the equator (Fergusson 1980).

The fruits are indehiscent pods, enlongated, compressed, straight or curved, thicken at the sutures, 10-30 cm long, 1.5-3.5 cm wide and about 1 cm thick, with blunt or subacute apex (Batlle and Tous 1997). Pods are brown with a wrinkled surface and are leathery and ripe. The pulp comprises an outher leathery layer (pericarp) and softer inner region (mesocarp). Seeds are hard and numerous, compressed ovate-oblong, 8-10 mm long, 7-8 mm wide and 3-5 mm thick (Batlle and Tous 1997), and occur transversally in the pod, separated by the mesocarp.

Fruit growth follows a typical sigmoidal curve (Stephenson 1981), with an initial period of slow growth (stage I), a middle period of linear growth (stage II) and a final period of declining growth (stage III) (Fig. 1.1). However, the initial period is especially long in carob because in Mediterranean areas winter cold starts at the end of the carob blooming period, when flowers have wilted and fruit initiation is just starting (Bosch et al. 1996).

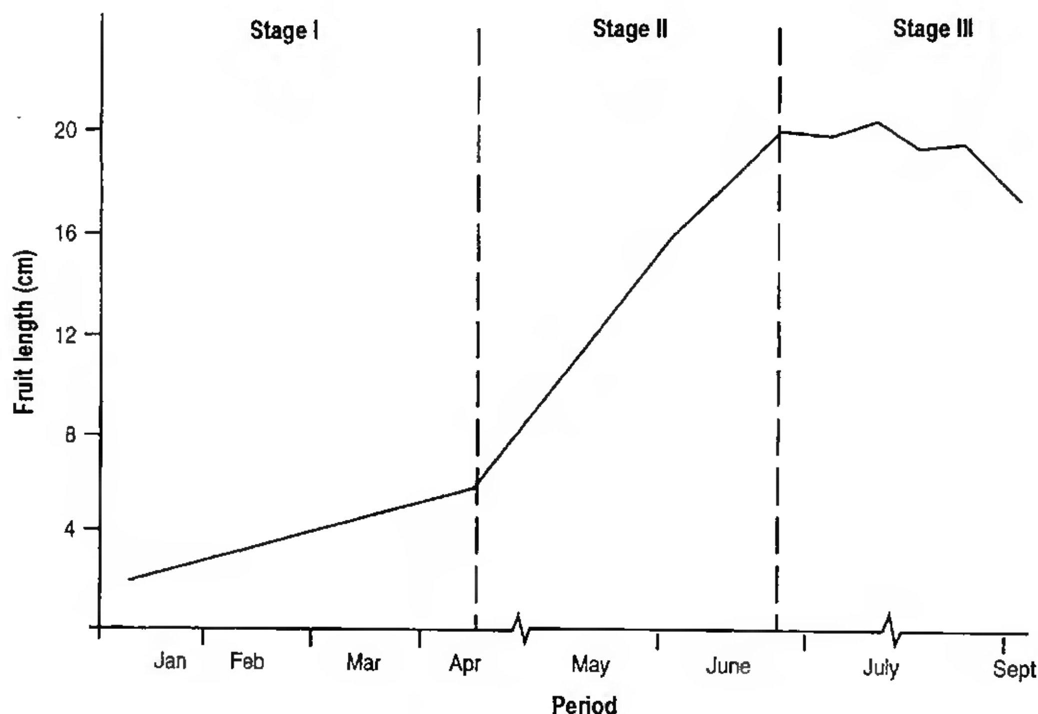

Figure 1.1. Stages of carob fruit development (Batlle and Tous 1997).

1.1.2. *Reproductive biology and growth cycle*

Many basic aspects of carob reproductive biology, such as floral biology, pollination compatibility between different cultivars, flowering and fruiting phenology remains largely unknown (Batlle and Tous 1997). However, some progress has been made by diverse authors (Tucker 1992, Retana et al. 1994, Bosch et al. 1996, Ortiz et al. 1996, Arista et al. 1999, Ortiz et al. 1999).

It is difficult to find carobs of the three sexual types in naturalised populations of the same area. Hermaphrodite individuals are sparser in the southern coastal belt of Spain and in the Algarve area of Portugal and more frequent in the eastern coastal belt of Spain (Zohary 2002). There are many different forms of males, but the main types observed in Portugal are often locally named after their anther colour as 'Red' and 'Yellow'.

Carob is the only Mediterranean tree with the main flowering season in autumn (September-November) with a peak in October (Martins-Loução and Brito de Carvalho 1989, Retana et al. 1994, Batlle and Tous 1997), instead of spring and similar to many

truly tropical plants (Batlle and Tous 1997). However, both time and length of the flowering period is conditioned by the local climatic conditions, as in most fruit and nut trees (Batlle and Tous 1997). Carob is mainly (70-85%) insect pollinated by bees, flies, wasps and moths (Retana et al. 1994, Ortiz et al. 1996, Zohary 2002). Flowers of the three sexual types secrete nectar, and its volume and sugar content is higher in female flowers than in male ones (Ortiz et al. 1996). Male and hermaphrodite flowers emit a semen-like odour that attracts insects (Batlle and Tous 1997).

The production of a low percentage of fruits per raceme is a constant characteristic in carob (Bosh et al. 1996, Arista et al. 1999, Ortiz et al. 1999). This may be caused by a deficient pollination (Arista et al. 1999) or by various features of carob floral biology, such as the racemes being caulogeneous and the female flowers less fragrant than the male ones (Ortiz et al. 1996). Furthermore, it has been suggested that the variations in flowering intensity and pod yields are likely to be more influenced by endogenous factors related to alternate bearing, which usually occurs in carob (Batlle and Tous 1997) than by climatic conditions (Haselberg 1986).

During the growth cycle of carob tree, four distinct phases can be identified: 1) from January to April, where growth is mainly vegetative with the formation of new leaves. In this phase the fruit is in development stage I; 2) from April to mid June, fruit abortion takes place and the fruits remaining in the tree enter an active period of growth (stage II); 3) starting from mid June the fruits enter in developmental stage III, reach their final size in the beginning of summer drought, and ripe in early August Thus, development of flowers starts when fruits are still maturing on the trees; and 4) from October to December the flowering period of carob ends, and the fruit initiation is just starting. With cold weather, fruit growth completely stops and fruit shed is low. Shedding of carob flowers and young fruits occurs mainly from October to December, slows down during January-February and rarely occurs from June to early August (Bosch et al. 1996). As in most Mediterranean trees the vegetative growth slows below 10°C, and thus, it seems that this species enters some kind of 'light' dormancy, at least in most cool latitudes (Batlle and Tous 1997). However in some very warm places and under favourable conditions, carob grows without becoming dormant either in winter or summer (Liphschitz and Lev-Yadun 1988).

During spring and summer, several phenological events occur simultaneously in carob: branch and leaf growth, enlargement of fruits initiated during the previous season

(Martins-Loução and Brito de Carvalho 1989), and probably, floral induction (Correia 1996). Flower buds and first inflorescences appear in late June, and a clear blooming occurs in late August and September, when pollination occurs. Fruit enlargement begins in April and fruit set is established just after pollination, being most pods ready to be harvested in August.

Although being a legume carob, like most *Caesalpinioideae*, it does not nodulate, being unable to fix atmospheric nitrogen (Martins-Loução 1985). Arbuscular mycorrhizal (AM) fungi have been shown to colonize carob roots, but no ectomycorrhizal association was found (Martins-Loução et al. 1996).

1.1.3. *Origin, centres of diversity and distribution*

Carob has been cultivated for thousands of years as a forage crop on a wide variety of soils in Asian, European and North African countries along the coast of the Mediterranean Sea. However, the centre of origin of this species is still not clear.

The carob is thought to have originated either in the eastern Mediterranean or in the Arabian Peninsula (Batlle and Tous 1997, Zohary 2002). The Mediterranean basin is considered at least as one of its domestication centres, as carob was planted extensively in warmer parts of both the southern and eastern shores of the Mediterranean, as part of the existing sylvo-pastoral systems (Blondel an Aronson 1999, Talhouk et al. 2005). However, there are authors that advocate that this fruit crop is not native here, but had its origin far south of this region (Zohary 2002). That place of origin was indicated by Schweinfurth (1896, *in* Zohary 2002), based on two facts: 1) tolerance of carob to hot climate, its flowering period (autumn) and its cauliflorous habit, all common adaptations in tropical plants; and 2) reports of the occurrence of stands of carob in Yemen (Deflers 1889 *in* Zohary 2002). On the basis of that information Schweinfurth (1896 *in* Zohary 2002) concluded that carob was introduced into the Mediterranean basin from the south, already as a cultivated plant, and became extensively naturalised there. This assumption was accepted by other botanists (Zohary 1973, 2002). However, both paleontological and archaeological findings clearly indicate that carob is native to the East Mediterranean basin, from the southern coast of Asia Minor to Syria (Catarino 1993, Zohary 2002,

Ramón-Laca 2004). Nevertheless, it is still not known the status of the wild-growing carobs in the West Mediterranean area (Batlle and Tous 1997, Zohary 2002). It remains unclear whether the carob existed in the West Mediterranean before domestication, or whether the wild-growing populations that occur today in the West Mediterranean resulted from the introduction of the carob culture into this area, followed by a subsequent wide-scale naturalisation (Batlle and Tous 1997).

For many years, the genus *Ceratonia* was considered to be monospecific, comprising only *C. siliqua*. However, Hillcoat et al. (1980) described a second species, *C. oreothauma* Hillcoat, Kewis and Verdc., which is restricted to Oman and north of Somalia. Two subspecies were distinguished: subsp. *somalensis*, native to the North of Somalia and subsp. *oreothauma*, native to Arabia (Oman). *C. oreothauma* is morphologically distinct from *C. siliqua*. Moreover, it has slightly smaller pollen grains than *C. siliqua*, and they are tricolpate rather than tetracolpate (Fergusson 1980). Since tetracolporate pollen grains are more evolved than tricolpate ones, *C. oreothauma* was suggested to be the wild ancestor of the cultivated *C. siliqua* (Hillcoat et al. 1980). Considering the current climatic conditions in the Mediterranean, the thermophilous nature of the carob tree should indeed be considered as a clear indication of an exotic origin (Batlle and Tous 1997, Zohary 2002).

Due to its interesting morphophysiological characteristics, such as resistance to drought and salinity, adaptation to poor soils, and minimal cultural requirements, carob has been introduced into the whole Mediterranean basin and other Mediterranean-like regions (Batlle and Tous 1997, Zohary 2002) (Fig. 1.2). Nowadays, wild and naturalized carobs in the Mediterranean region are distributed in more or less the same geographic and climatic belt as the cultivated cultivars (Batlle and Tous 1997, Zohary 2002). Wild-growing carobs are particularly common at low altitudes along the Spanish Mediterranean coast, Southwest Spain, Southern Portugal, the Balearic Islands, Southeast France, the shores of Southern Italy including Sicily, the Adriatic coast of Croatia, the Aegean region in Greece and Turkey, along the Northern and Southern ranges 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 (Batlle and Tous 1997, Zohary 2002). Those wild-growing trees are commonly associated with other Mediterranean thermophilous species such as *Pistacia lentiscus*, *Olea europaea* subsp. *oleaster*, *Myrtus communis* and *Juniperus phoenicea* (Zohary 2002). In Lebanon, carob was included recently in a national list of priority forest genetic

resources as a target for conservation and management, due to the neglected status of the semi-natural populations (Talhouk et al. 2005).

Figure 1.2. World distribution and centres of origin of carob (Batlle and Tous 1997).

Worldwide, the total area of carob production is 200 000 ha, from which 148 000 ha (74%) are located in Spain, Italy, Portugal and Greece (Batlle and Tous 1997). In Portugal, the distribution of carob overlaps with the areas with a Mediterranean climate, and plays an important role in the economy of the south of Portugal, where it occupies an area of 83 000 ha, distributed by orchards where they are intercropped with other species (60 000 ha), pure formations (10 000 ha) and semi-wild conditions (13 000 ha) (*source*: Associação Interpresarial para o Desenvolvimento da Alfarrobeira 'AIDA' 2004).

1.1.4. *Cultivars*

Centuries of cultivation have resulted in a great intraspecific variability, and in a high number of local cultivars (Mitrakos 1988, Batlle and Tous 1997). Most cultivars are of unknown origin and represent the germplasm of each region. The information on genetic resources shows that carob cultivars have a high variation in morphologic and agronomic characteristics (Batlle and Tous 1997). However, isoenzyme and DNA analysis have

revealed low polymorphism between cultivars from different (Tous et al. 1992, Batlle et al. 1996) and the same origin (Barracosa et al. 1996).

Female trees have always been selected in preference to the hermaphrodites, since generally they are better pod bearers (Batlle and Tous 1997). Thus, the most common cultivars in commercial orchards are female and only few hermaphrodites have sufficient agronomic desirable attributes (Batlle and Tous 1997). Due to the spatial proximity, cultivars are pollinated, at least in part, by neighbouring wild-growing males and hermaphrodites. Consequently, many of the seeds produced by domestic female cultivars carry *cultivated* × *wild* hybrid combinations (Zohary 2002). However, gene exchange between the wild and cultivated gene pools in such contact places is not symmetrical. The cultivars, because they are handled by vegetative propagation, are largely kept apart maintaining their genetic identity (Zohary 2002). In contrast, the adjacent wild populations are constantly 'bombarded' by the seeds and pollen of the cultigens (Zohary 2002).

Most Portuguese cultivars have a medium to high seed yield (10-12%) and have pods with round kernels appropriated for gum extraction (Batlle and Tous 1997). The main cultivars grown in Portugal, mainly in the Algarve are 'Alfarroba de burro', 'Brava', 'Canela', 'Costela de vaca', 'Da Lapa', 'Galhosa', 'Mulata' and 'Preta de Lagos' (Graça 1994), and the recently selected 'AIDA' (Batlle and Tous 1997). The more valuable Portuguese cultivars are 'Mulata', due to its productivity, and 'Galhosa', for its high seed yield and gum content (Martins-Loução and Brito de Carvalho 1989). Nowadays the current trend is to establish mechanically harvestable carob orchards with dual purpose carob cultivars, with both acceptable seed and pod yield, and high gum quality. In Spain, preliminary breeding programmes were conducted aiming the improvement of the agronomic characters, including yield, regularity of yield, growth habit, harvesting ease and disease resistance and the processing qualities, such as kernel/endosperm yield, gum quality, pod size and sugar content (Batlle and Tous 1997).

1.1.5. *Economical importance*

Carob is one of the most useful trees of the Mediterranean basin. Portugal and Spain process approximately half of the world's commercial carob supply (FAO 2001). The

world carob pod production is approximately 315 000 tonnes per year and the main carob bean producers and exporters are Spain (42%), Italy (16%), Portugal (10%), Morocco (8%), Greece (6.5%), Cyprus (5.5%) and Turkey (4.8%) (FAO 2001).

The potential multi-use value that existed for carob in the past is still valid and new uses proven to be economically viable. Carob pods provide two main products (by weight): seed (10%) and pulp (90%), remaining after the removal of the seeds. The main products derived from the carob pod (pulp and seed) and their major uses are summarized on Table 1.1. Nowadays, the main application for carob pulp is the animal feed. For humans, the carob pods have been used mainly as a cocoa substitute in a few countries, due to its absence of caffeine and low price (Petit and Pinilla 1995, Batlle and Tous 1997, Yousif and Alghzawi 2000). However, most carob pods are discarded and not effectively used at present.

Table 1.1. Main products derived from the carob pod (pulp and seed) and their major uses (Batlle and Tous 1997).

Product	Processing	Uses
Pulp		
Kibbles	Any	Animal feed (horses and ruminants).
	Milled	Human food and animal feed (ruminants and non ruminants).
	Extraction and purification	Sugar and molasses.
	Fermentation and distillation	Alcohol and microbial protein production.
Powder	Extraction	Tannins as antidiarrhoea.
	Washing, drying, roasting and milling	Food ingredient; cacao substitute; preparation of dietary and pharmaceutical products.
Seed		
Endosperm	Grinding	LBG (E-410); food additive (stabilizer and thickener); dietary fibre; pet food; pharmaceuticals; cosmetics.
Embryo	Grinding	Germ meal; human and animal nutrition.
Coat	Extraction	Tannins for leather tanning.

Carob pulp contains besides high soluble sugar content (Batlle and Tous 1997), a high number of polyphenols (Avallone et al. 1997), and its chemical composition depends on the cultivar, origin and harvesting time (Batlle and Tous 1997). Polyphenols are known to have antioxidative, anticarcinogenic and antiproliferative activity (Owen et al. 2003). Furthermore, epidemiological studies evidenced that the consumption of foods rich in polyphenols could reduce the risk of certain types of cancer and the incidence of coronary heart disease (Duffy et al. 2001). Extracts of carob pulp and leaves showed high antioxidative activity and free-radical scavenging activity *in vitro* (Kumazawa et al. 2002), an antimicrobial effect against microorganisms with impact in public health (Kivçak et al. 2002) and in food-related bacteria (Tassou et al. 1997) and antiproliferative activity in liver cells (Corsi et al. 2002).

The economical importance of carob tree is due to the galactomannans present in the endosperm of the seeds that constitute locust bean gum (LBG) used in the food industry as viscosifier, stabiliser and gelling agent, in products such as dietetic beverages, baby foods and pet foods (Batlle and Tous 1997) (Table 1.2). Besides its industrial interest, LBG has proven efficient in adjunct dietary treatment of diabetes (Tsai and Peng 1981), and has found increased applications as a component of water-soluble dietary fibre, in programs of cholesterol management (Jensen et al. 1997).

The embryo of the seeds accounts for 20-25% of the total seed dry weight and is removed and often discarded during preparation of LBG (Martins-Loução and Brito de Carvalho 1989). Besides vitamin B_2 (Martins-Loução and Brito de Carvalho 1989), embryos are rich in proteins, namely caroubin, which represents 50% of the weight of the embryo (Wang et al. 2001). Natural carob fibre (NCF) is a new product obtained from the pulp, and contains high levels of dietary fibre and polyphenols (Pérez-Olleros et al. 1999, Haber 2002). Recent studies revealed that NCF might be effective in dietary treatment of hypercholesterolemia (Zunft et al. 2003), which is one of the major risk factors of cardiovascular diseases. Carob fibre has a high content of phenolic antioxidants, comparable to other Mediterranean products, such as olives (Owen et al. 2003), exhibiting a high antioxidative potential, superior to many other products rich in polyphenols, such as red wine (Kumazawa et al. 2002).

Table 1.2. Locust bean gum uses and technical applications (*source*: Martins-Loução and Brito de Carvalho 1989, Batlle and Tous 1997 and FAO 2001).

Uses	Applications
Food (75%)	Food additive (E410) in ice creams, baby food, sauces, soups, soft cheeses, bakery products, pie fillings, powdered desserts, salad creams.
Industrial (25%)	
Pharmaceuticals	Anticoelic products, pomades, pills, toothpaste, dietary treatment of diabetes, dietary treatment of hypercholesterolemia, anti-oxidant, anti-carcinogenic, antimicrobial.
Cosmetics	Emulsions and foams, shaving foam.
Textiles	Used either alone or in combination with starch and synthetics as a sizing agent; print-paste thickener.
Paper	Added during the paper-making process to improve its physical characteristics.
Chemicals	Glues, colouring, polishing, dyeing, matches, pesticides.
Petroleum	Flocculation additive to increase stability and thickness of welling.
Mining	Flotation product.
Well sinking	Wall reinforcement, moisture absorbent.
Concrete	To strengthen solidification.
Explosives	Water binding for explosives.

In Portugal, the most developed industries of raw carob pods and kernels are located in Algarve, processing 2 400 tonnes per year (*source*: AIDA 2004). Carob pods are kibbled to separate the pulp from the seed- first transformation industry, and in a second stage, in endosperm and germ- second transformation industry (Fig. 1.3).

In the Algarve there are eleven small factories that kibbles pods to separate the pulp from the seed. The pulp is then sold to companies to produce carob syrup, or to be used as animal feed. The seeds are canalised to processing factories, which extract and produce LBG. In the Algarve, there are two important processing factories- 'Danisco' Portugal and 'Industrial Farense'.

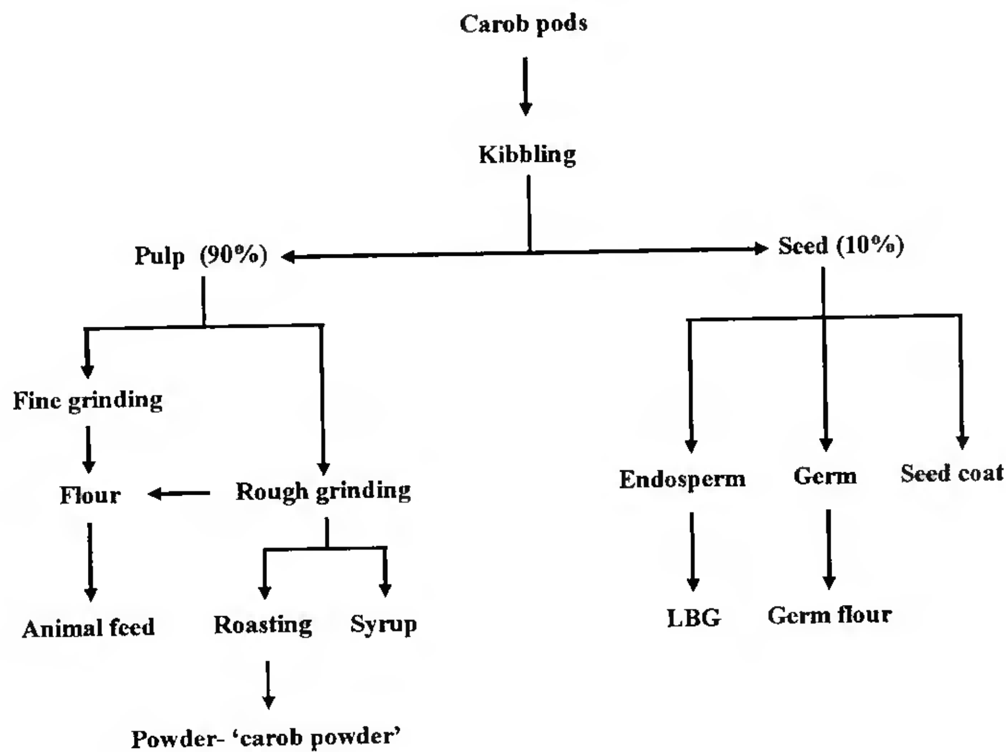

Figure 1.3. Carob industrialization in Portugal (Martins-Loução and Brito de Carvalho 1989).

Besides its economical importance, carob has also an important ecological value, and its adaptability to all types of soils at lower and middle altitudes, and its resistance to drought, makes it suitable for reforestation (Batlle and Tous 1997).

1.1.6. *Conventional methods of carob propagation*

Traditional carob propagation has been achieved by grafting 2-4 years old seedlings rootstocks with female buds of chosen productive trees (Martins-Loução and Brito de Carvalho 1989, Alorda and Medrano 1997, Batlle and Tous 1997). A 3-4 years period of growth of these buds is necessary to obtain a young tree, which only begins to bear fruit around 10-15 years of age (Alorda and Medrano 1997). Both canopy size and harvest increase during the next 10-15 years, and thus the fully shaped tree is only achieved 25-30 years after seed germination (Alorda and Medrano 1997).

Grafting of field-growing plants has been used since ancient times to perpetuate clones that cannot be readily maintained by other traditional asexual methods, such as cuttings or division (Hartmann et al. 1990). Carob grafting is not especially difficult if the rootstock stem diameter is between 2-4 cm (3-4 years old seedlings), and this method of asexual propagation dates back to its domestication (Alorda and Medrano 1997, Batlle and Tous 1997, Zohary 2002). The success of grafting or budding depends on the season and sap flow, with T-budding and patch budding being the most generally used techniques (Alorda and Medrano 1997). However, this method is extremely time consuming, and it is evident that current crop adaptations to market demands is severely hindered by this slow propagation system, with its long non-productive period. Furthermore, carob rootstocks are raised from open-pollinated seeds, and thus vary widely in vigour, habit and cold resistance, no rootstocks trials have been carried out and no rootstock selections are available (Batlle and Tous 1997).

Propagation by cuttings has been a horticultural practice for centuries and it is still the most extensive technique used to propagate ornamental, forest and fruit woody plants. Carob is usually referred to as a difficult-to-root species, and propagation by cuttings has been largely cited as inadvisable (Van den Heede and Lecourt 1981, Hartman and Kester 1985), altough some interesting results have been obtained (Medrano 1986, Alorda et al. 1987). Some aspects of the cuttings are important for rooting, namely collection time (seasonal variation), type of the shoot (age and position) and genotype (rooting potential) (Alorda and Medrano 1988). However, vegetative propagation by cuttings is not yet commercially available (Batlle and Tous 1997).

1.1.7. *New methods of propagation*

The high phenotypic variability within and between carob cultivars has important implications for selection, cultivation practices and establishment of new plantations and productivity optimisation of this crop (Batlle and Tous 1997). Furthermore, its improvement is hampered by its highly heterozygous nature and long reproductive cycle.

To date, improvement of carob cultivars has been carried out only empirically by growers, selecting promising chance seedlings and budding them onto less fruitful

genotypes as rootstocks (Batlle and Tous 1997). As carob is largely a dioecious species, genetic improvement for fruit characters is hampered by lack of information for the male parent regarding these characters (Batlle and Tous 1997). Mass selection after intercrossing the best individuals on the basis of their phenotypes would be the simplest method. This is based on the relatively high additive variance affecting the inheritance of most traits in fruit and nut trees (Bringhurst 1983).

The traditional methods of carob propagation have failed to meet the market demands for new, selected plant material. Recently, it has been shown that even mature carob trees from cultivars that are difficult to propagate asexually, can be successfully propagated by *in vitro* techniques (Sebastian and McComb 1986, Romano et al. 2002). Thus, the use of micropropagation seems to be appropriate in order to fulfil the increased demand for propagating this tree.

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques, and nowadays it is also associated with a mass production at a competitive price of many species and cultivars with economical value, offering several advantages over traditional methods (Bonga and Von Aderkas 1992). There are three basic methods of *in vitro* propagation: somatic embryogenesis, organogenesis and axillary shoot proliferation (Perik 1987, Bonga and Von Aderkas 1992). The first two methods have high initial multiplication rates, and embryo/shoot induction can be obtained either directly on the surface of the explants or indirectly through *callus* formation. In practice, both methods are applicable to a very few species, while production of plants by axillary shoot development has proved to be the most generally applicable and reliable method of *in vitro* plant propagation, and is commonly the most useful method for tree species (Bonga and Von Aderkas 1992).

1.2. AIMS OF THIS WORK

Since the early eighties carob has raised a considerable interest due to the generally sustained demand and increasing prices of the carob pods (pulp and seed). Carob is a crucial element of the traditional agro-forestry systems of the Algarve, where the most developed industry of raw carob pods and kernels is located. In optimum conditions carob

requires a minimum of inputs compared to most of the other fruit or vegetable crops. Furthermore, carob tree shows some outstanding features, like rusticity, drought resistance and reduced orchard management, being well suited to part-time farming. This crop is currently being re-emphasised as an alternative in dryland areas with subtropical Mediterranean climates for diversification of coastal agriculture.

Carob has been traditionally neglected by Research & Development programmes. Besides some recent research about the domestication of carob (Zohary 2002), its ecological status (Ramón-Laca and Mabberley 2004) and the conservation of carob populations (Talhouk et al. 2005), most studies on carob have focused on applied aspects, such as agricultural, industrial and commercial. There are some reports about reproductive and floral biology of carob (Haselberg 1986, Tucker 1992, Retana et al. 1994, Bosch et al. 1996, Ortiz et al. 1996, 1999, Arista et al. 1999), irrigation and fertilization practices (Cruz et al. 1988, Correia and Martins-Loução 1993, 1996, 1997, 2005, Correia et al. 2002), the use of micropropagation systems for mass cloning of elite mature carob trees (Sebastian and McComb 1986, Carimi et al. 1997, Romano et al. 2002, Custódio et al. 2004, Gonçalves et al. 2005, Osório et al. 2005), and other *in vitro* studies (Martins-Loução 1985). Nevertheless, it is clear that there are several areas of carob biology where more information is needed.

In this context, the general purpose of the work presented in this dissertation was to provide new approaches for the understanding of some aspects of the biology of carob, particularly those related with the reproductive process, having in mind that this is a field where more knowledge is still missing.

The specific aims of this work were:

1) To contribute to a better knowledge of the reproductive biology of carob tree, by making an overview of flowering, and a characterization of the main events occurring during micro and macrosporogenesis (Chapter II);

2) To study the composition of the volatile fraction of the scent emitted *in vivo* by the whole flower of males, females and hermaphrodites, and by isolated floral organs (Chapter III);

3) To study the macro and micronutritional composition of flowers and access the seasonal dynamics of mineral levels in leaves (Chapter IV);

4) To investigate the carbohydrate composition of flowers and fruits along their different stages of development, and the seasonal variations on the carbon levels in leaves (Chapter IV);

6) To investigate some factors affecting the cryopreservation of pollen (Chapter VI);

7) To study several factors influencing the induction of callogenesis and androgenesis by anther culture (Chapter VII).

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CHAPTER II

REPRODUCTIVE BIOLOGY

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2.1. INTRODUCTION

Early in their evolution, plants acquired a life cycle that alternates between a multicellular haploid organism, the gametophyte, and a multicellular diploid organism, the sporophyte (Yadegari and Drews 2004) (Fig. 2.1).

Gametophytes and sporophytes differ morphologically and functionally. The major function of the diploid sporophyte generation is to produce haploid spores, which are the products of meiosis. Spores undergo cell proliferation and differentiation to develop into gametophytes. The major function of gametophyte generation is to produce haploid gametes. The fusion of egg and sperm gives rise to the zygote, which is the beginning of the diploid sporophyte generation, thereby completing the life cycle (Yadegari and Drews 2004). During the angiosperm life cycle, the sporophyte produces two types of spores, microspores and megaspores that give rise to the male gametophytes and female gametophytes, respectively. The angiosperm gametophytes develop within sporophytic tissues that constitute the sexual organs of the flower.

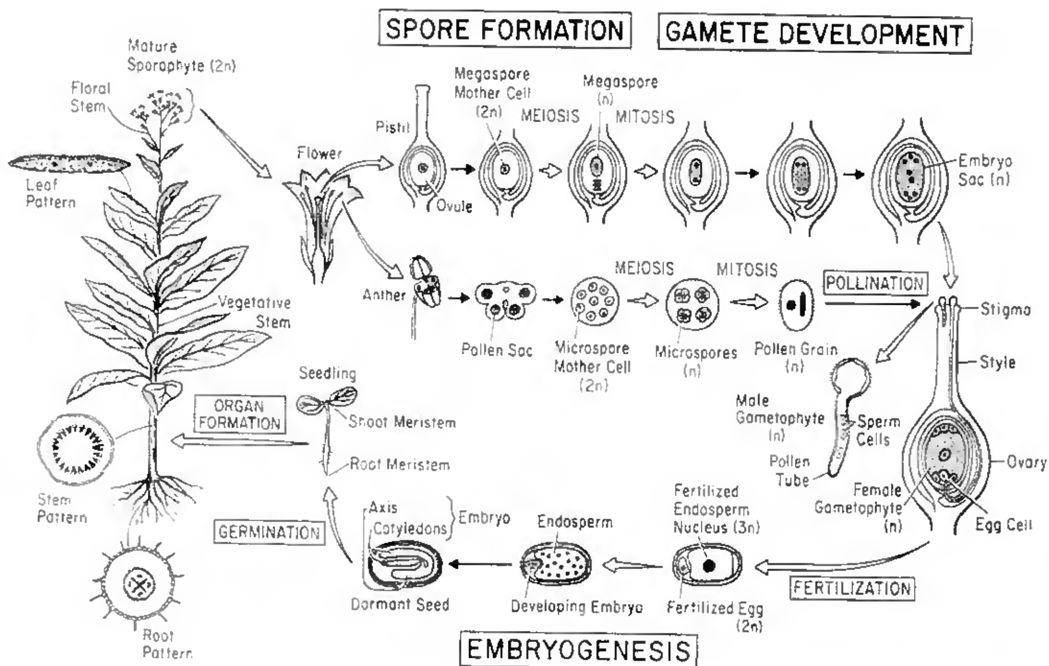

Figure 2.1. Life cycle of a flowering plant (Chasan and Walbot 1993).

In higher plants the reproductive processes take place within two specialized floral organs, the stamens and the carpels (Esau 1977, Raven et al. 1986, Goldberg 1988, Drews and Goldberg 1989). The stamens, collectively known as androecium, are the male reproductive organs of flowering plants, differentiated from primordia that are specified within the floral meristem following the transition from a vegetative to a flowering pathway (Esau 1977, Raven et al. 1986, Scott et al. 2004). They consist of two morphologically distinct parts: the anther and in most species a stalk-like filament, which transmits water and nutrients to the anther and positions it to aid pollen dispersal (Scott et al. 2004). The anther contains diploid cells that undergo meiosis to form haploid microspores that differentiate into the microgametophyte (pollen grains), which is a three-celled structure comprised of two sperm cells encased within a vegetative cell (McCormick 2004) (Fig. 2.2).

Microsporogenesis (Fig. 2.2) involves an array of extraordinary events, including cell division and differentiation, the transition from sporophytic to gametophytic generation, and modifications of cell division to produce structures that are unusual in plant development, including coenocytic tissues (the tapetum and the microsporocyte mass), and subsequently free cells (microspores) that give rise to self-contained units for genome dispersal (pollen grains) (Goldberg et al. 1993, Scott et al. 2004). This process begins when a diploid pollen mother cell, or primary microsporocyte, undergoes a meiotic division, followed by citokinesis to yield four haploid microspores which are still encased within a callose wall. The tapetal cells produces an enzyme (β-glucanase) which dissolutes the callose wall and releases the microspores. Later on, the exine is synthesized. As the young microspores grow, they fill with multiple small vacuoles which eventually coalesce into a single large vacuole. The nucleus is pushed to the site opposite the germ pore and organizes a separate cell attached to the microspore wall. Some days after meiosis, asymmetric division occurs (first microspore mitosis), producing two cells with very different fates. The bicellular product of the first microspore mitosis is, by definition, pollen (Nägeli 1998).

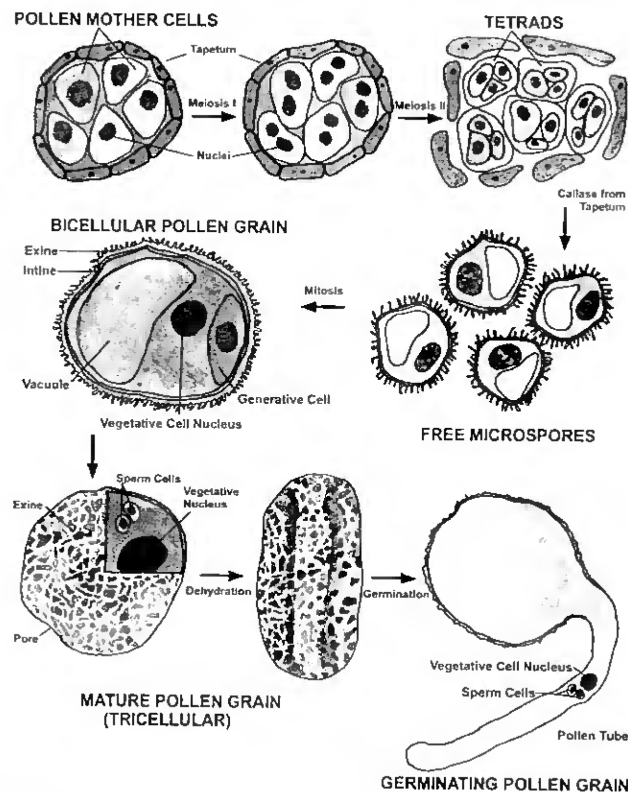

Figure 2.2. *In vivo* microspore development (microsporogenesis).

Usually, the second mitosis (where the generative cell within the young pollen divides again to form two sperm cells) takes place only after the germination of the pollen tube (Nägeli 1998). The pollen grain then secretes the intine, accumulates starch granules until the grain is entirely engorged, and dehydrates.

The female parts of an angiosperm flower are collectively referred to as the gynoecium, which consists of one or more ovule-bearing unit structures, the carpels. The term pistil is also commonly used to describe the female parts of a flower. It refers to a single carpel when individual carpels of the gynoecium are separated (simple pistils), or to a single structure formed by fusion of multiple carpels (compound pistil). However, there are authors that advocate that the term pistil is not the more adequate (Esau 1977, Fahn 1990).

At anthesis, the carpel consists of three parts: the ovary, at the base of the pistil containing the ovules and which differentiates into the fruit following fertilization; the style, an extension above the ovary, through which the pollen tubes grow toward the

ovules; and the stigma, at the top of the style, where pollen grains adhere and germinate (Esau 1977, Fahn 1990).

The female gametophyte, also referred to as the embryo sac or megagametophyte, develops within the ovule, which is found within the carpel's ovary. It is critical to many steps of the angiosperm reproductive process, including pollen tube guidance, fertilization, the induction of seed development upon fertilization, and maternal control of seed development after fertilization (Yadegari and Drews 2004)

Ovules are specialized structures, derived from the placenta of the ovary wall, that produce the megasporocyte (megaspore mother cell) and are the site of embryo sac formation, fertilization, and embryogenesis (Gasser and Robinson-Beers 1993, Reiser and Fischer 1993). The most common female gametophyte form consists of seven cells and four different cell types: three antipodal cells, two synergid cells, one egg cell, and one central cell (Maheshwari 1950) (Figs. 2.3D, 2.4 and 2.5).

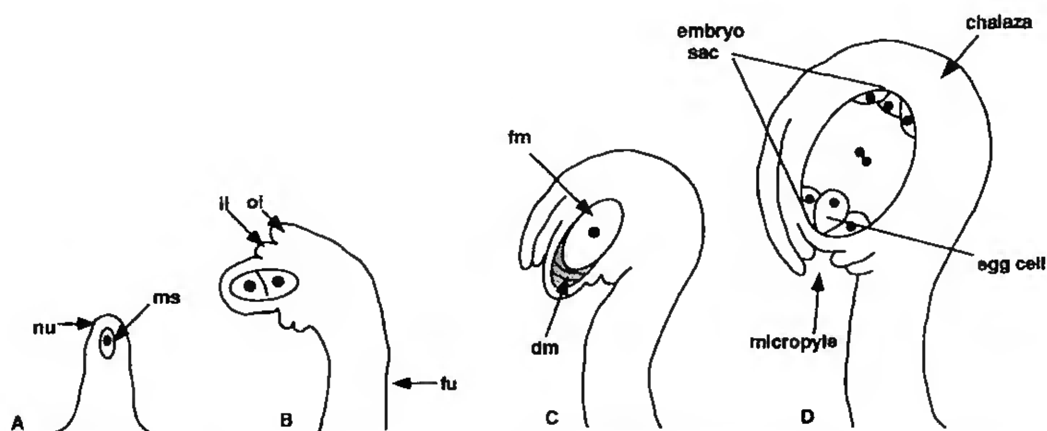

Figure 2.3. Ovule development. Stages are shown for an anatropous ovule with *Polygonum*-type embryo sac development. (A) Ovule shortly after initiation, with a single megasporocyte (ms), nu: nucellus; (B) Ovule after both integuments have been initiated. At this time, the megasporocyte has undergone the first meiotic division, fu: funiculus, ii: inner integument, oi: outer integument; (C) Ovule after meiosis. The functional megaspore (fm) at the chalazal end has expanded, and the non-functional megaspores are degenerated, dm: degenerate megaspores; (D) Ovule after megagametogenesis. The mature embryo sac contains seven cells and eight nuclei (Reiser and Fischer 1993).

The process of embryo sac development can be divided into two stages: megasporogenesis and megagametogenesis (Reiser and Fischer 1993). The nucellus

produces the megasporocyte or megaspore mother cell. During megasporogenesis, the megasporocyte undergoes meiosis and four megaspore nuclei are produced. After meiosis, usually three or four megaspores degenerates, and only one survives, becoming very large by absorbing the protoplasm of the other three (Mauseth 1991). Subsequent mitotic divisions, nuclear migration, and cytokinesis during megagametogenesis produce the mature embryo sac.

There is a considerable diversity in the patterns of embryo sac development among plant species, and in Fig. 2.4 is represented a small sample of some of them (Reiser and Fischer 1993, Russell 1993).

TYPE	MEGASPOROGENESIS				MEGAGAMETOGENESIS			
	mega-sporocyte	meiosis I	meiosis II	functional megaspore	mitosis	mitosis	mitosis	mature gametophyte
MONOSPORIC (Polygonum)								
MONOSPORIC (Oenothera)							—	
BISPORIC (Allium)					—			
TETRA-SPORIC (Adoxa)					—	—		

Figure 2.4. Patterns of embryo sac development (Reiser and Fischer 1993).

The most commonly observed form of embryo sac development is the *Polygonum*-type (Webb and Gunning 1990) (Fig. 2.4). In this type of development the nucleus of the megaspore undergoes three mitotic divisions, producing two, four and then eight haploid nuclei (Figs. 2.3, 2.4 and 2.5). The nuclei migrate through the cytoplasm, presumably

pulled by microtubules, until three nuclei lie at each end and two in the center. Walls form around the nuclei and the large eight-nucleate megaspore becomes a megagametophyte with seven cells, one of which is binucleate. The seven cells consist of one large central cell with two polar nuclei, three small antipodal cells, and an egg apparatus consisting of two synergids and an egg, the megagamete. These cells comprise four groups that function in fertilization, embryogenesis, and nutrition of the embryo sac and embryo. The egg cell is located at the micropylar end of the embryo sac and ultimately fuses with a sperm nucleus to produce a zygote.

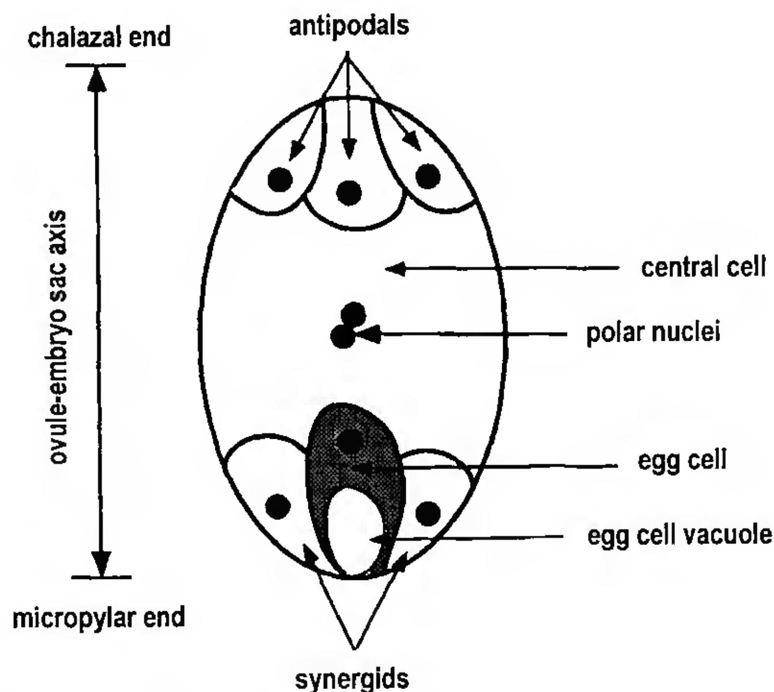

Figure 2.5. Organization of the embryo sac. The orientation of the embryo sac with respect to the chalazal-micropylar axis of the ovule is indicated by the vertical arrow on the left. The egg apparatus, including the egg cell and synergids, is located at the micropylar end, where the pollen tube enters the embryo sac. The central cell contains two nuclei. Three antipodal cells are located at the chalazal end of the embryo sac. The egg cell is actually adjacent to, rather than between, the two synergids (Reiser and Fischer 1993).

The synergids are located on either side of the egg cell and play an important role in fertilization (Reiser and Fischer 1993, Russell 1993). The pollen tube discharges its

contents into one of the synergids prior to incorporation of the sperm nuclei into the egg and central cells.

The central cell is positioned in the center of the embryo sac, and contains two nuclei, a large vacuole, and many cytoplasmic organelles. The polar nuclei originate at both the micropylar and chalazal ends of the coenocytic megagametophyte and migrate to the center after cellularization. The polar nuclei may partially fuse with each other before they are fertilized by a single sperm nucleus, generating the triploid primary endosperm nucleus (Russell 1993). The mature endosperm will provide nutrients for the developing embryo or seedling (Lopes and Larkins 1993). Three antipodal cells are located opposite to the egg at the chalazal end of the embryo sac. No specific function during reproduction has been attributed to the antipodals, but they are thought to be involved in the import of nutrients to the embryo sac (Reiser and Fischer 1993).

Breeding systems show great diversity in angiosperms, but the co-occurrence of males, hermaphrodites, and females within the same population, identified by the general term 'polygamy' (including trioecy and subdioecy), appears to be rare, representing 3.6% of species (Richards 1997). One advantage of gender dimorphism over hermaphroditism that has long been proposed is the advantage that unisexual plants may gain over hermaphrodites by specializing in one sexual role (Darwin 1877 *in* Davis 2002).

Carob has been variously described as a polygamo-dioecious or monoecious species (Tucker 1992, Batlle and Tous 1997), and some authors consider trioecy as one of the most peculiar biology features of this species (Retana et al. 1994). Individual carob trees may be male (with inflorescences carrying only staminate flowers), female (with inflorescences carrying only pistilate flowers) and hermaphrodite (with inflorescences carrying staminate flowers and others with pistilate flowers, or with inflorescences carrying hermaphrodite flowers). The flowers of carob tree are small and numerous, spirally arranged along the inflorescence axis in catlin-like racemes borne on spurs from old wood or in the trunk (cauliflory). The flowers are usually pentamerous with the calix placed on a short pedicel, and are unusual in lacking petals (Tucker 1992). The calix is disk-shaped, reddish green and has nectarines (Tucker 1992). The flowers are initially bissexual, but usually one sex is suppressed during late development of functional male or female flowers (Tucker 1992). Furthermore, inflorescences can be polygamous, and there is a high plasticity in

inflorescence and flower characteristics (Haselberg 1986, Tucker 1992, Batlle and Tous 1997).

The aim of this work was to make an overview of the main aspects of flowering: 1) inflorescence and floral morphology and 2) to study the major events occurring during micro and megasporogenesis and their relation with the male and female flower developmental stages.

2.2. Materials and methods

2.2.1. *Plant material*

This work was done during the flowering period of 2001 (June to December), on 14 years old male, female and hermaphrodite carob trees (three trees per sexual type), growing in a non-irrigated and non-fertilised limestone soil located in the south of Portugal (37°07'N; 7°39'W), with a typically Mediterranean climate (Mitrakos 1981). The males were seminal, while the females and the hermaphrodites were grafted on carob rootstocks raised from open-pollinated seeds and budded in the nursery, one year after germination, when the trunk reached a diameter of 1 cm.

2.2.2. *Phenological and morphological studies*

For phenological studies, three branches on each tree were selected on the outside of the canopy, and the inflorescence number was recorded during the period of full bloom. Additionally, the number of flowers per inflorescence was recorded from 30 inflorescences per sexual type. For morphological characterisation (eg. flower size, number of anthers, pistil and stigma characteristics), 25 flowers per developmental stage were observed from each of the three sexual types of carob. The average weight of 25 individual mature flowers of each sexual type and the weight of the stamens (including filaments and anthers), nectarial disks (including pedicel) and carpels, was assessed.

For the flowering characterization of the three sexual types, three trees were selected, 3 branches were marked per tree and 30 inflorescences were collected. For the study of the weight of whole flowers and isolated floral organs, 30 flowers per tree and sexual type

were used. The values obtained for each tree were considered independent replications, and the data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 11.0, SPSS INC). Significance between means was tested by the Duncan's New Multiple Range Test (P=0.05).

2.2.3. *Anatomical and histological studies*

For anatomical and histological studies, a minimum of ten unopened flower buds and isolated anthers and pistils removed from flowers at various stages of development were fixed in formalin-100% acetic acid-70% ethanol (FAA, 5:5:90, v/v/v) and later stored in 70% ethanol at 4 °C, in the dark.

For freeze-sectioning it is frequently desired to dissect pieces of tissue of living plants and freeze them immediately, usually in isopentane cooled in liquid nitrogen (Knox 1970). Freezing at very low temperature greatly reduces ice crystal size and growth rate (Knox 1970). However, to cut cryostat sections accurately, it is essential to support tissues in a suitable sectioning medium, such as gelatine, rather than be infiltrated with it (Knox 1970).

We started by supporting tissues in rectangular gelatine blocks and freezing them rapidly (Knox 1970). However, this technique revealed inappropriate, since after sectioning we observed the complete destruction of tissues. Thus, before sectioning, samples were taken from the 70% ethanol, placed in the supporting mechanism of the freezing microtome (LAICA CM 1850), together with some drops of arabic gum, where they froze immediately. Then, these materials were transversally and longitudinally sectioned. Twenty to thirty sections (40-60 μm) were mounted on slides, stained with cotton blue in lactophenol, and after coverslips were added, examined by microscope (Leitz DIALUX 20EP) and documented by a camera loaded with KODAK 100 or 200 ASA film for colour prints. Photographs were taken, scanned and subsequently prepared with Adobe Photoshop.

For scanning electron microscopy (SEM), material was dissected in 95% alcohol, removing bracts and larger organs that obscured the inner structures. Dissected structures were dehydrated further through an ethanol-acetone series, critical-point dried with liquid

CO_2 in a Polaron Critical Point Drier apparatus, and mounted on microscope supports. They were coated with gold-palladium in a Polaron sputter-coater (E 5300 Freeze Drier coupled to a Polaron E 5350). Twenty flowers were examined with a scanning electron microscope JSM-T100 (JEOL Technics Ltd.) at 15 kV, and about 50 micrographs were taken and registered in a Kodak TMX 120 film for black and white prints. The photos were scanned and prepared as described above.

2.2.4. *Cytological studies*

For cytological studies, unopened flower buds and isolated anthers were removed from male and hermaphrodite flowers and fixed in an acetic-ethanol solution (absolute ethanol:glacial acetic acid, 3:1, v/v) (Han et al. 2000) during 24 h at room temperature. They were then transferred to a similar solution, in proportions 1:1 (v/v), and kept at 4°C in the dark until observations were made.

To study the *in vivo* microsporogenesis a minimum of ten observations per developmental stage of the flower were made (unopened flowers buds or anthers), using conventional brightfield microscopy and with fluorescence. For conventional brightfield microscopy an acetocarmine 4% (w/v) stain was used. The acetocarmine solution was prepared by dissolving 4 g of carmine in 100 ml of acetic acid (50%, v/v), and heated at 60°C during 20 min. After cooling, the acetocarmine solution was filtered and stored in the dark at 4 °C. For observations, 2 anthers were squashed on a microscope slide in a drop of acetocarmine stain. After removing the anther debris, a cover slip was added and the microscope slide heated on a flame. Drops of stain where then placed in an extreme of the covers slide, and removed carefully with a filter paper in the other extreme (this procedure was repeated several times, not allowing the sample to boil). The samples were immediately observed using conventional brightfield microscopy. Photographs were made and prepared as described previously.

For fluorescence microscopy, a fluorescent DNA-specific dye [4,6-diamidino-2-fenilindole.HCl (DAPI) (0.01 mg ml^{-1})] (Detchepare et al. 1989) was used. DAPI is a nuclear counterstain for use in multicolor fluorescent techniques, and stains nuclei specifically, with little or no cytoplasmic labeling. DAPI absorb violet radiations (max 372

nm) and emits a blue fluorescence (max 456 nm), which stands out in vivid contrast to green, yellow or red fluorescent probes of other structures. The stock solution of DAPI was prepared by dissolving 1 mg of DAPI in 1 ml of dimethylsulfoxide (DMSO), and stored at 4 °C in the dark. For cytological studies, a fresh DAPI solution in a concentration 0.01 mg ml^{-1} was prepared, by diluting 10 µl of the stock solution in 1 ml of distilled water. For observations, 2 anthers were squashed on a microscope slide in a drop of DAPI. After removing the anther debris, a cover slip was added, and after 5-10 min the samples were observed in a microscope equipped with fluorescence. Photographs were made and prepared as described previously.

2.3. Results and discussion

2.3.1. *Overview of flowering phenology*

2.3.1.1. Flowering characteristics

Carob is usually referred as the only Mediterranean tree with the main flowering season in autumn (September-November), with a peak in October (Martins-Loução and Brito de Carvalho 1989), similarly to many truly tropical plants (Batlle and Tous 1997), and in contrast with other insect-pollinated trees from the Mediterranean region, where flowering occurs in spring. However, in this work it was observed that the first inflorescence primordia and inflorescences at early stages of development appeared in June, and that the flowering period lasts until December, peaking in September-October. The extended flowering season of carob compensates for the unstable weather at this time of the year, increasing the possibilities of a good pollination to occur (Batlle and Tous 1997). Both the time and length of the flowering period is conditioned by local climatic conditions. Retana et al. (1994) had already observed some receptive flowers out of the normal flowering period, in July and December.

It was also observed that the females bloom 3-4 weeks before the males and the hermaphrodites, and have a more extended flowering season, as already been reported by other authors (Retana et al. 1994). Since in females the synchronization of flower receptivity with pollen dehiscence periods is essential to ensure high levels of pollination, their extended flowering period increase the chances of overlapping with the flowering

period of males, which is shorter. However, this fact implies that the first female inflorescences have reduced possibilities of being pollinated. A similar situation has been reported for some wild dioecious species (Flanagan and Moser 1985). A possible explanation for that phenomenon has been suggested to be the competition for pollinators between sexual types (Barret and Helenurm 1981).

Carob exhibits some characteristics usually associated with wind pollination, such as dioecy, relatively small flowers in dense, often terminal inflorescences; a reduced, dull or green perianth with exposed fertile parts; anthers on long exserted filaments; and an expanded, exposed stigmatic surface (Lewis et al. 2003), but lack others, like the reduction of pollen apertures, from colporate to porate ones (Crane 1986).

The carob flowers are mainly visited by flies (*Diptera*), noctuid moths (*Lepidoptera*) and bees (*Hymenoptera*) (Retana et al. 1990, Ortiz et al. 1996, Arista et al. 1999), which is in agreement with the morphology of carob inflorescences, composed of conspicuous flowers with nectarines and stigmas easily accessible to unspecialized floral visitors, similar to the reported for other dioecious woody species (Freeman et al. 1980, Fox 1985). Moreover, the strong odour associated with the flowering period, the secretion of nectar and the high frequency of insect floral visitors suggest that carob is predominantly an entomophylous species (Ortiz et al. 1996). However, the flowering season of carob is longer than for other insect pollinated orchard species, such as almond, apple and peach whose flowering periods lasts only 15-25 days (Sedgley and Griffin 1989). The extended flowering season in carob compensates for the unstable weather at that time of the year, and ensures that at least some flowers will be pollinated in a spell of good weather and insect activity (Batlle and Tous 1997).

2.3.1.2. Phenological, morphological, and anatomical characterization of inflorescences and flowers

In the three sexual types the inflorescences primordia first appear as reddish spots with a diameter less than 1 mm, on the surface or 2 year old branches or on the trunk, in regions from which the leaves have shed (Plates 2.1A-C, 2.2A-C and 2.3A-C). When observed under SEM, we can see that inflorescence primordia are composed by four bracts, in opposite pairs, covered with hairs (Plate 2.6D). In some male trees we also observed

inflorescences developing on tumour-like zones (Plate 2.1H-J). Sometimes, inflorescences develop on the apical zone of young branches, which are located in older branches (Plate 2.1B, C and F) or in the trunk (cauliflourous inflorescences) (Plates 2.1E, 2.2G and 2.3B).

From the appearance of the inflorescence primordia until flower anthesis, the three types of inflorescences are morphologically identical, with conical shape and floral primordia covered by hairy bracts (Plates 2.5A,C,D and 2.6A-C,E). The organogenic stages are alike in the three sexual types of inflorescences (Tucker 1992), and all have perfect flowers, initiating both stamens and carpels (Plate 2.5A-E). The flowers of most angiosperms are "perfect," containing both androecium and gynoecium, but in others, a sex determination process causes the abortion of the primordia of one or the other type of sexual organ (Chasan and Walbot 1993). This is the case of carob. Flowers are initially bisexual, and differentiation of functional male or female flowers occurs when buds are less than 0.5 cm in size, when the stamen is about 0.35 mm height, and the carpel stops growing in the male flowers (Tucker 1992), corresponding to the developmental stage 0 described by Haselberg (1986). This was also observed in other leguminous with unisexual flowers, such as *Neptunia pubescens*, *Bauhinia malabarica* and *B. divaricata* (Tucker 1988a,b), where flowers are bisexual in early developmental stages and become functionally unisexual by subsequent organ suppression.

In the three sexual types a single floral primordium forms in each bract axil, in an acropetal sucession along the inflorescence, with paired bracteoles positioned laterally (Plate 2.5A, C and D). Flower development proceeds as described by Tucker (1992): sepal primordia, low ridges on the sides of the floral apex in antesepalous positions, forming an irregular encirclement around the base of the carpel primordium, and initiation of the carpel primordium. The flower development is successive along the axis, and the first flowers to become receptive are those located at the base of the raceme, and the last, those at the apex. Abundant trichomes cover the surface of the receptacle at the inner side of each sepal, encircling the carpels or carpels primordiums (Plate 2.5B and E).

Flowers are small (6-12 mm) and numerous (20-50), spirally arranged along the inflorescence, several cm long (Plates 2.1D,E,G, 2.2D,E, and 2.3A,D-F). The anatomical structure of the raquis of the inflorescence is similar to that of a typical stem, with a single layer of epidermis with short, trichomes, cortex, a split vascular cylinder, collenchyma cells and the pith (Plate 2.7A).

Inflorescences with only one type of flower were observed in all plants studied: functional male, with the presence of rudimentary carpels (Plate 2.4A,D), functional female, with the presence of small and rudimentary stamens (Plate 2.4B,E and G) and hermaphrodite flowers, with both fertile staminate and pistillate portion (Plate 2.4C,F).

Carob is regarded as one of the most archaic of the leguminous genera, together with *Gleditsia* and *Cercis*, due to its variable sexual arrangement of flowers and merosity, radial symmetry and unspecialised perianth (Tucker 1992). Hillcoat et al. (1980) summarized the great variation among carob trees reported in the literature as including: 1) male-flowered inflorescences with long stamen filaments and abortive pistils; 2) a similar type but with short filaments; 3) female-flowered inflorescences with abortive staminodia and fully developed pistils; 4) hermaphrodite inflorescences of flowers all having functional stamens and pistils; and 5) polygamous inflorescences of male, female and hermaphrodite flowers. Like in previous studies (Tucker 1992), in this work all types of inflorescences, except the last, were observed. Furthermore, we also observed in the same tree both racemose and cymose inflorescences and inflorescences with secondary branching. This variation in respect to the presence of terminal flowers and branching was already reported by Thompson et al. (1944, *in* Tucker 1992), who found an enormous variation within individual inflorescences of carob. Thompson hipothesised that carob represented a primitive state of flowering in which all floral organ primordia are uncommitted at initiation, allowing the number of parts and the proportion of male to female parts to vary unpredictably.

Carob flowers are incomplete (apetalous), turbinate, irregular (zygomorphic) and peryginous, with a calyx tube and five short sepal lobes, and a hypogynous nectarial disk that appears to be five-lobed (Plate 2.4A-F). The median sagital sepal is adaxial, rather than abaxial, which makes the position of the sepals in carob unique among Caesalpinioids (Tucker 1992). The number of organs varies around the typical pentamerous form (Tucker 1992), and tetramery, hexamery and septamery were also observed. Furthermore, when a terminal flower is present, it has usually several stamens or carpels (Plate 2.4H). The presence of an abnormal number of floral organs has already been reported in the same species (Tucker 1992).

The androecium of functional male flowers consist of a group of 4-7 stamens, inserted peripherically around the receptacle, each one standing opposite to the sepal lobe

(Plate 2.4A,D). Each filament is partly surrounded by a sepal and has dorsifixed introrse versatile anthers (Plate 2.4A,D). In the centre of the nectarial disk a rudimentary carpel is visible (Plate 2.4A). The stamens' filaments are caducous, and are short in some plants and long in others. Male flowers are reddish or yellow, depending on the type of pigmentation of the male type.

The gynoecium of functional female flowers is apocarpous having a short stipe, a peltate stigma, and abbreviated style and a superior ovary inserted in a central depression in the nectarial disk (Plate 2.4B,E). The carpel cleft is usually on the adaxial side, but sometimes it can be obliquely oriented or inverted (Fig. 2.5D). The ovule is unilocular containing many ovules arranged in two submarginal rows, and placentation may be described as marginal. Rudimentary stamens surrounded by sepals can be distinguished (Plate 2.4G,H).

Hermaphrodite flowers are a combination of male and female types having both the androecium and the gynoecium (Plate 2.4C,F). Fruits of both female and hermaphrodite trees are indehiscent pods (Plates 2.2H, I, 2.3G and H).

Anthers, of both male and hermaphrodite flowers, are tetrasporangiate and dithecal with microsporangia positioned laterally that open longitudinally when mature (Plates 2.4I, 2.7D and 2.8B). The connective, a zone of sterile tissue through which a vascular bundle passes, separates the two lobes (Plates 2.7D and 2.8B).

For a more complete flowering characterization of the three sexual types the number of inflorescences per branch and the number of flowers per inflorescence were compared. There were no significant differences ($P \geq 0.05$) between the different sexual types in either of the parameters studied (Table 2.1). Retana et al. (1994) working with Spanish cultivars, observed that the number of flowers per inflorescence was higher in males and hermaphrodites compared to female inflorescences. These differences can be due to genotype and growing conditions (Batlle and Tous 1997).

Table 2.1. Number of inflorescences per branch and number of flowers per inflorescence in male, female and hermaphrodite carobs. Number of inflorescences and flowers are means ± SE of 3 branches and 30 inflorescences, respectively. Absence of letters indicates no significant differences at $P \geq 0.05$.

	Number of inflorescences	Number of flowers
Male	36 ± 6	29 ± 2
Female	23 ± 3	36 ± 3
Hermaphrodite	29 ± 10	33 ± 3

Furthermore, we compared the weights of whole flowers and isolated floral organs of the three sexual types. Except for carpels, the sexual type significantly affected the weight of whole flowers and isolated flower parts ($P<0.05$). Both male and female flowers had similar mean weights, while the hermaphrodites were heavier (Table 2.2).

In the hermaphrodites the stamens were heavier than the nectarial disks, while in males and females the heavier flower parts were the stamens and the carpels, respectively (Table 2.2).

Table 2.2. Weight of whole flowers and isolated flower parts of the three sexual types of carob.

Flower weight (mg)	Type of flower		
	Hermaphrodite	**Male**	**Female**
Whole flower	100 ± 10Aa	30 ± 1Ba	30 ± 2Ba
Stamens	40 ± 1Ab	20 ± 1Bb	-
Nectarial disks	30 ± 1Abc	10 ± 0Bd	10 ± 2Bb
Carpels	20 ± 0c	-	20 ± 2c

In each column, statistical comparisons were made between different floral parts of the same sexual type, and are shown in lower case letters. In each row, statistical comparisons were made between sexual types, for the same flower part, and are shown in capital letters. Values represent means ± SE of 3 replicates with 30 flowers each. Values followed by different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

In the male flowers the stamens and nectarial disk comprised 64.6% and 33.7% of the total flower weight, while in the females, 58% of the total floral weight corresponded to the carpel, and 33.1% to the nectarial disk (Fig. 2.6). In the hermaphrodite flowers the contribution of stamens, nectarial disk and carpel to the total flower weight were 38.4%, 32.6% and 20.4%, respectively (Fig. 2.6).

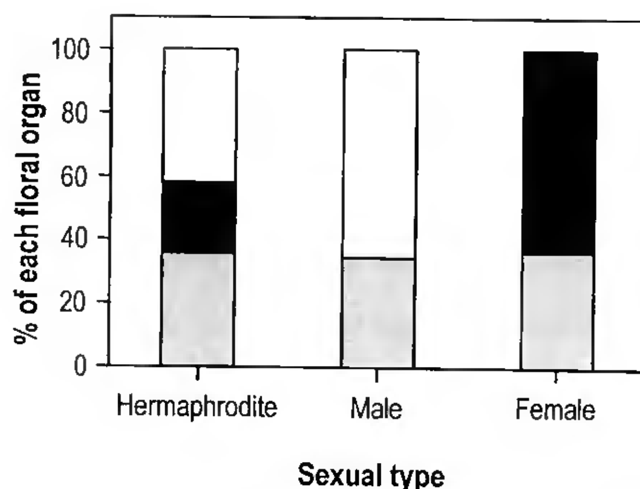

Figure 2.6. Contribution of stamens (□), carpels (■) and nectarial disks (□) to the mass of whole hermaphrodite, male and female flowers of carob.

2.3.1.3. Stages of flower development

The process of cell differentiation and dehiscence occurs in a determinate chronological order, and can be correlated with floral bud size (Koltunow et al. 1990, Scott et al. 1991). However, in carob it is more adequate to describe flower development in terms of developmental stages, rather than flower size (Haselberg 1986). This description has also been used to characterize the flower development in other species (Stauffer et al. 2002).

Based on the external morphological characters of the flowers, and on the description made by Haselberg (1986), six stages of development were assigned to male, hermaphrodite and female flowers of carob (Plate 2.4D-F). Thus, the degree of sepals aperture, the expansion of the filament, the size of the receptacle and carpel, the secretion of nectar and pollen release were selected as some characters that could be correlated with

the growth of the flowers. The developmental stages of male flowers are the following (Plates 2.1C,D,G,H, J and 2.4D):

Stage 0- from the formation of the floral primordium in the bract axil, until the flower is ± 1 mm size, and the androecium is covered by the calyx, formed by five sepals;

Stage I- the sepals open and the anthers (5-7) stretch and elongate, reaching their full size (2.0-2.5 mm); the flower has a star-shaped look and in the centre a rudimentary pistil can be seen;

Stage II- the anthers are pushed upward in the flower by filament extension, which grows up to 6.0-8.0 mm. The nectarial disc reaches its final size (2.5-3.0 mm). At the earliest stages of development the colour of the anthers appears to be of a reddish-brown. Depending of the pigmentation of the male or hermaphrodite type, later the colour turns to yellowish-green, orange or red;

Stage III- the mature flower shows secretion of a sticky fluid at its nectarial disc and pollen has not been shed;

Stage IV- tissue degeneration, dehiscence and pollen grain release occurs;

Stage V- the stamens dry and fall from the raceme.

The developmental stages of carob female flowers observed during this work can be described as follows (Plates 2.2D-G and 2.4E):

Stage 0- from the formation of the floral primordium in the bract axil, until the flower is ± 1 mm size, and the gynoecium is covered by the calyx, formed by five sepals;

Stage I- the pistil becomes visible and grows up to 2 mm;

Stage II- the carpel length increases and the nectarial disk becomes visible; the stigma is not swelling;

Stage III- carpel reaches its final length (6.0-8.0 mm), and the stigma tissue is growing;

Stage IV- nectar and stigmatic fluids are secreted at the nectarial disk; the colour of the stigma turns from greenish-yellow to yellow and brownish-yellow; brownish spots appear until the whole stigma turns into brown and black;

Stage V- after fertilization the carpel increases volume becoming flatly shaped.

As referred earlier the developmental stages of hermaphrodite flowers are six, presenting the characteristics of both male and female flowers (Plates 2.3A,D-F and 2.4F).

2.3.2. *Microsporogenesis, male gametophyte development and its relation to flower developmental stages*

Most plant organs derive from meristems groups of undifferentiated cells that divide to replenish themselves and to provide founder cells for organ primordia. Anther

development is unusual in that the microsporangia arise from single archesporial cells rather than from meristems (Goldberg et al. 1993, Scott et al. 2004). The key stages in this process are the establishment of adaxial-abaxial polarity, the specification of cell types, and the formation of the radially symmetrical microsporangia (Goldberg et al. 1993, Scott et al. 2004).

Microsporogenesis involves an array of extraordinary events, including cell division and differentiation, the transition from sporophytic to gametophytic generation, and modifications of cell division to produce structures that are unusual in plant development, such as coenocytic tissues (the tapetum and the microsporocyte mass), and subsequently free cells (microspores) that give rise to self-contained units for genome dispersal (pollen grains) (Goldberg et al. 1993, Scott et al. 2004).

Anther development can be divided in two general phases (Koltunow et al. 1990, Goldberg et al. 1993): the histospecification phase (phase 1) and the degeneration and dehiscence phase (phase 2). During the first phase, the stamen is partitioned into the anther and filament, specialized anther cell types differentiate from three primordia cell layers, the anther acquires its characteristic bilateral shape, four microsporangia and accessory cell types form, and microspore mother cells within each microsporangium undergo meiosis to produce haploid microspores (Goldberg et al. 1993). At the end of phase 1, a differentiated anther has several highly specialized cells and tissues that are responsible for carrying out nonreproductive functions (e.g., support and dehiscence) and reproductive functions (e.g., spore and pollen formation).

The second phase is characterized by enlargement of the anther, elongation of the filament, pollen grain differentiation, and a cell degeneration and dehiscence sequence that ends with the release of the mature pollen grains after breakage of a specific anther region, the stomium (Goldberg et al. 1993).

Since no differences were observed in microsporogenesis and anatomical characteristics of anthers between males and hermaphrodites, all the results presented refer to observations made in anthers taken from male flowers. The major events occurring during microsporogenesis in carob are shown in Table 2.3.

We observed that the histospecification phase takes place during stage 0 and early stage I of flower development, while the dehiscence phase begins in late stage I and proceeds until flower development is complete (Table 2.3).

Table 2.3. Description of anther and microsporogenesis in relation to flower developmental stages, observed from histological and cytological analysis of anthers from male flowers. PMC: pollen mother cell; PST: primary sporogeneous tissue; PPC: primary parietal cells.

Stages of flower development	Anther differentiation and microsporogenesis
0	Stamen primordia development; Formation of epidermis, endothecium, PST and PPC formation; PMC formation
I	PMC formation; Formation of tapetum and middle layers; First meiotic division; Second meiotic division; Formation of microspore tetrads; Microspore release; Immature pollen grains
II	Endothecial thickening; Maturation of pollen grains; Tapetum begins to break down
III	Degradation of the tapetum is completed
IV	Pollen is shed
V	Stamens fall from the flower

The male gametophytic cycle begins when flowers are ± 1 mm size (developmental stage 0), with the formation of the microsporangium in the anther, which consists of archesporial cells. By this time, in spite of the fact that developing anthers are visible in the staminal primordium, they consist only of protoderm and a mass of ground meristem (Plate 2.7B). The sporogeneous tissue develops from cell regions that are located in the angles of the developing anther, and can be seen in flowers in a late stage 0 (Plate 2.7C). Sporogeneous tissue fills the locular space, and is composed of individual cells with a polygonal shape, tightly abut (Plate 2.9A). In each of these regions there is a row of hypodermal initials that divide periclinally to form two layers. The inner layer of these initials constitutes the primary sporogeneous cells (PSC) and the outer layer the primary parietal cells (PPC), from which the wall of the pollen sacs and a large portion of the tapetum develop (Plate 2.7C).

In the same locule, both sporogeneous tissue and pollen mother cells (PMC) were observed (Plate 2.9A-C). PMC, or microsporocytes (Plate 2.9C), are the result of mitotic

divisions of the sporogenous tissue and dissociation of the combined cell walls. The tapetum is undifferentiated at this time.

On stage I all the tissues are well differentiated, and we can observe the epidermis, endothecium, connective, vascular bundle and the polinic sacs (Plate 2.7D). The endothecium and the connective have the same major functions of structure and support as the epidermis. Furthermore, the connective is responsible for joining teca together and connecting the anther to the filament (Goldberg et al. 1993). Besides providing nutrient and water supply, the vascular bundle makes the connection between the anther, the filament and the flower (Goldberg et al. 1993). The polinic sacs contain the sporogenous tissue which forms a primary parietal and sporogenous layer of cells as well as a secondary parietal layer, with specific middle wall layers: the tapetum and the endothecium (Plate 2.8A). Both the tapetum and the endothecium are non-reproductive tissues (Goldberg et al. 1993). The endothecium is the outermost layer of the parietal cells, located immediately below the epidermis of the anther, where the opening of the pollen sacs takes place, and is a structure and support tissue, involved in dehiscence (Goldberg et al. 1993). The tapetum apparently serves for the nourishment of the developing PMC, tetrads and microspores (Fahn 1990), playing a role in pollen wall formation (Goldberg et al. 1993). Furthermore, it produces enzymes involved in the microspore releasing from tetrads (Goldberg et al. 1993).

During stage I, microsporogenesis takes place in two successive meiotic divisions forming tetrahedral tetrads (Plates 2.9D and 2.10A). The majority of the tetrads occur in a tetrahedral configuration, and the callose of PMC is persistent through the tetrad stage (Plate 2.9D). Immediately before being released from the tetrads, the microspores are characterized by a small size, weak autofluorescence of the wall in UV and a large, centrally located nucleus (Plate 2.10A). Breakdown of the callose wall marks the transition from the tetrad stage to the free microspore stage (Plate 2.10B,C). Exine and intine becomes visible in the uninucleated microspores, with the nucleus slightly condensed and located mostly at or near the pole (Plates 2.9E and 2.10D). Microspore mitosis is asymmetric and produces nuclei that can be easily distinguished after staining with DAPI and acetocarmine (Plates 2.9F and 2.10E). The vegetative nucleus is large, diffuse and appears weakly stained, whereas the generative nucleus is small, condensed and appears intensely stained (Plate 2.10E). The tapetum, firstly distinguishable in flower

developmental stage I, is of the secretory type (Kreunen and Osborn 1999) (Plate 2.8C). The tapetum layer maintains a similar phenology throughout flower developmental stage I, and consequently during the PMC, tetrad and immature pollen grains stage.

When the flower reaches the developmental stage II, the anther wall is mature, and is composed of several cell layers: the epidermis, the endothecium and the middle layers (Plate 2.8C). Both epidermis and the middle layers are structure and support tissues, being involved in anther dehiscence, the epidermis also prevents water loss and allows gas exchange (Goldberg et al. 1993).

Tapetal degradation occurs simultaneously with maturation of pollen grains and endothecial thickening (Plate 2.8C). These thickenings were only observed in locules containing pollen grains, and were not detectable in earlier stages of ontogeny. Consequently, it is presumed that the cell walls thicken rapidly during the latest free microspore or earliest pollen grain stages (Kreunen and Osborn 1999). At this developmental stage the polinic sacs are completely open, and the tapetum begins to break down (Plate 2.8C). By this time, besides uni and binucleate microspores, we can observe completely formed pollen grains of spheroidal shape and tetra-pentacolpate (Plate 2.10F, G). Depositions of substances possibly secreted by tapetum are also observed (Plate 2.8C). The regions of dehiscence are in the form of longitudinal slits between the two pollen sacs of each lobe, the stomiums. The stomium is a specialized cell layer that runs along the lateral side of each anther half, or theca (Plate 2.8B). In this region, the epidermal cells are smaller than the neighbouring epidermal cells, and they are easily ruptured when the anther ripens (Fahn 1990).

By the time the degradation of tapetum is completed, we can only observe mature pollen grains ready to be released from the anther (Plates 2.8D and 2.9G), which corresponds to developmental stage III. The anthers split longitudinally, releasing the pollen, in developmental stage IV, and in stage V the stamens fall from the flower. *Leguminosae* pollen grains are commonly colporate with 2-6 apertures (http://www.biodibersity.uno.edu). *Ceratonia* exhibits some plasticity in pollen morphology (Fergusson 1980; Graham and Barker 1981): tetracolpate in *C. siliqua* (atypical from primarily tricolpate *Cassiae*) and tricolpate in *C. oreuthama* (Hillcoat et al. 1980). In carob, Fergusson (1980) reported a 36% level of pollen abnormality, including 12 apertures and spiraperturate grains. In this study pollen grains are of spheroidal shape

and are tetracolpate (Plates 2.6F and 2.10F). Ocasionally, pentacolpate pollen grains were observed (2.10G).

2.3.3. *Macrosporogenesis, megagametogenesis and its relation with flower developmental stages*

The ovule is the source of the megagametophyte known as megaspore mother cell (MMC), which is the site of embryo-sac formation, fertilization and embryogenesis (Reiser and Feisher 1993).

On the young female inflorescences we can observe that, like in male flowers, the floral primordia forms in each bract axil, with paired bracteoles positioned laterally (Plates 2.5D and 2.11A,B). The floral apex is convex and tangentially broad (Plate 2.11B).

Similar to what was observed in microsporogenesis, the transition between flower developmental stages is gradual, and within flowers in the same stage we can observe different stages of macrosporogenesis. In fact, in the same ovary, we observed ovules in distinct maturation stages.

The carpel share tissues with the vegetative plant body that are necessary for support, nutrition, and protection, such as ground tissue, vascular tissue, and epidermis, but has tissues that are unique to this structure, such as the stigmatic and transmitting tissues (Gasser and Robinson-Beers 1993). The stigmatic tissue is responsible for capture of pollen grains and is present in the two lobed peltate stigma covered by verrucate papillae (Plate 2.11H). The transmitting tissue facilitates the passage of the pollen tube to the ovules, and is present in a hollow canal in the style (Plate 2.11G). The suture is visible the full length of ovary, style and stigma.

The ovary is unilocular and stipitate, and in the loci, the ovules are arranged in two alternating rows along the placenta. Placentation can be described as marginal, along the ventral suture. The carpel is covered with tricomes (Plate 2.11F), and is circular until stage I (Plate 2.11C), but at stage II and onwards it flattens adaxially (Plates 2.11E and 2.12A).

Over 15 different patterns of female gametophyte development have been described (Haig 1990). The developmental pattern exhibited by most species is the *Polygonum*-type because it was first described in *Polygonum divaricatum* (Reiser and Fischer 1993), and is thought to be the ancestral type (Huang and Russell 1994). The development of the

Polygonum-type female gametophyte can be divided into two phases: megasporogenesis and megagametogenesis (Reiser and Fischer 1993). During megasporogenesis, a diploid megaspore mother cell undergoes meiosis to produce four haploid megaspores (Reiser and Fischer 1993). The chalazal-most megaspore survives, and the other three undergo cell death. During megagametogenesis, the functional megaspore undergoes three rounds of mitosis, producing an eight-nucleate cell (Reiser and Fischer 1993). Two nuclei (the polar nuclei), one from each pole, then migrate toward the centre of the cell. During this migration the embryo sac cellularizes to form the seven-celled structure.

The major events occurring during ovule and embryo sac formation are shown on Table 2.4. Ovule formation and macrosporogenesis occur during stages 0, I and II (Plate 2.11C-E), while megagametogenesis begins in stage III (Plate 2.12B-H).

Table 2.4. Description of ovary and embryo-sac differentiation stages, in relation to flower developmental stages, observed in histological and cytological analysis of carob female flowers. MMC: megaspore mother cell; PST: primary sporogeneous tissue; PPC: primary parietal cells.

Stages of flower development	Ovary and embryo-sac differentiation
0	Carpel primordium development Formation of PST and epidermis
I/II	Formation of integuments and placental cells Macrosporocyte formation Macrosporocyte maturation First meiotic division of megaspore Second meiotic division of megaspore
III	Macrogametogenesis Embryo-sac formed
IV	Embryo-sac formed Embryo initiation and development
V	Embryo formed

In the angiosperm female gametophyte, the embryo sac is completely embedded within the maternal sporophytic tissues of the ovule (Plate 2.12G). The female gametophyte plays a critical role in almost of the steps of the reproductive process. During

pollen tube growth, the female gametophyte participates in directing the pollen tube to the ovule (Higashiyama et al. 2003). During fertilization, cytoskeletal components within the female gametophyte directs the sperm cells to the egg cell and the central cell (Russell 1992, 1993, Lord and Russell 2002). Upon fertilization, female gametophyte–expressed genes control the initiation of seed development (Chaudhury et al. 2001).

The ovules from *Leguminosae* are usually anatropous, campylotropous to amphitropous, or hemianatropous (http://www.biodibersity.uno.edu). Ovules were first observed on stage I of flower development (Plate 2.11C,D), and are anatropous (Batista 1994), being attached to the placenta by a long funicullus (Plate 2.11D). At stage I fertilization has yet not occurred, and we can observe the nucellus and a tick tegument (Plates 2.11D and 2.12B). The nucellus has a well established polarity, determined by the opposition of the microphyle, relatively to the chalaza, and it has two distinct cell layers, the inner nucellus and the external nucellus (Batista 1994).

The flowers become receptive at late stage III, and with pollination the pollen grains containing the sperm cells are transferred to the stigma of the pistil, so that fertilization can occur in the embryo sac (Esau 1977, Raven et al. 1986).

Carob seeds have an oval shape and consist of three genetically distinct components: embryo, endosperm, and seed coat (Plate 2.12H). The embryo and endosperm are genetically identical except for ploidy level: the embryo is diploid and the endosperm is triploid (Lopes and Larkins 1993). Fertilization also initiates changes in maternal tissues (Reiser and Fischer 1993): the ovary develops into a fruit and the ovule integuments differentiate to form the protective seed coat. The embryo and endosperm develop from the zygote and megagametophyte central cell, both located in the nucellus of the ovule. Soon after fertilization, or even before, synergids and antipodals breakdown in most species. The nucellus expands somewhat but later is crushed by the expansion of the embryo and endosperm, and is usually not detectable in mature seeds. The integuments that surround the nucellus expand as the rest of the ovule grow and mature into the seed coat (also called the testa). As the ovule develops into a seed, the ovary matures into the fruit.

Embyogenesis is accompanied by the degeneration of the nucellus and by the formation of the endosperm (Plate 2.12H). Uni and bicelular embryos were observed on transversal sections of pistils excised from flowers on late stage III and early stage IV (Plate 2.12C-E), and embryos at the globular stage were observed starting on late stage IV

(Plate 2.12F). The globular embryos were surrounded by a dark stained tissue which resulted from the cells adjacent to the endosperm (Plate 2.12F). The endosperm persists after embryo development is completed and constitutes the major portion of the mature fruit. It stores starch, lipids, and storage proteins and acts as a vital source of nutrients during germination and early seedling development (Lopes and Larkins 1993, Martins-Loução et al. 1996).

2.4. Conclusions

In this work it was observed that the flowering period of carob lasted from June to December, peaking in September-October. Usually, the females bloomed 3-4 weeks before the males and the hermaphrodites, exhibiting a more extended flowering season.

The flower development of the three sexual types is successive along the axis, and the first flowers to become receptive are those located at the base of the raceme. The three sexual types of inflorescences are morphologically identical from the appearance of the inflorescence primordia until stage 0. Initially they are all bisexual, and the differentiation of functional male and female flowers occurs when buds are less than 0.5 cm in size, the stamen is about 0.35 mm height, and the carpel stops growing in the male flowers.

Carob flowers are apetalous, turbinate, irregular and peryginous, with a calyx tube and a hypogynous nectarial disk. They are small and numerous, spirally arranged along the inflorescence, several cm long, on the surface or 2 years-old branches or on the trunk (cauliflourous inflorescences), in regions from which the leaves have shed. Inflorescences with only one type of flower were observed in all plants studied: functional male, with the presence of rudimentary carpels, functional female, with the presence of small and rudimentary stamens or hermaphrodite flowers, with both fertile staminate and pistillate portion. An enormous variation within individual inflorescences of carob was observed, namely the presence of racemose and cymose inflorescences in the same individual, flowers with an abnormal number of stamens and carpels and branched inflorescences.

The androecium of functional male flowers consists of a group of 4-7 stamens, inserted peripherically around the receptacle. Each filament is partly surrounded by a sepal and has dorsifixed introrse versatile anthers. In the centre of the nectarial disk a

rudimentary carpel is visible. The gynoecium of functional female flowers is apocarpous with a short stipe, a peltate stigma, and abbreviated style and a superior ovary inserted in a central depression in the nectarial disk. The ovule is unilocular containing many ovules arranged in two submarginal rows, with and marginal placentation. Hermaphrodite flowers are a combination of male and female types having both the androecium and the gynoecium. During the development of male, hermaphrodite and female flowers six stages were observed.

Both the number of inflorescences per branch and the number of flowers per inflorescence where similar between males, females and hermaphrodites. The male and female flowers had similar mean weights, while the hermaphrodites were heavier. In hermaphrodite flowers the androecium had a mean weight two times superior than in males, and the mean weight of the nectarial disks was three times superior to both males and females.

Anthers of both male and hermaphrodite flowers, are tetrasporangiate and dithecal with microsporangia positioned laterally that open longitudinally when mature. The microsporogenesis is sucessive and results in binucleate pollen, which is released in flower developmental stage IV. Pollen grains have a spheroidal shape and are tetracolpate, sometimes pentacolpate.

The ovary is unilocular and stipitate, and in the loci, the ovules are arranged in two alternating rows along the placenta. Placentation can be described as marginal. It seems that macrogametogenesis is from the *Polygonum*-type, but further investigation is needed in order to study this process in detail. The reproductive organs of carob make this species a suitable object for further embryological investigations, due to the numerous and large ovules that are present in the developing pods.

2.5. References

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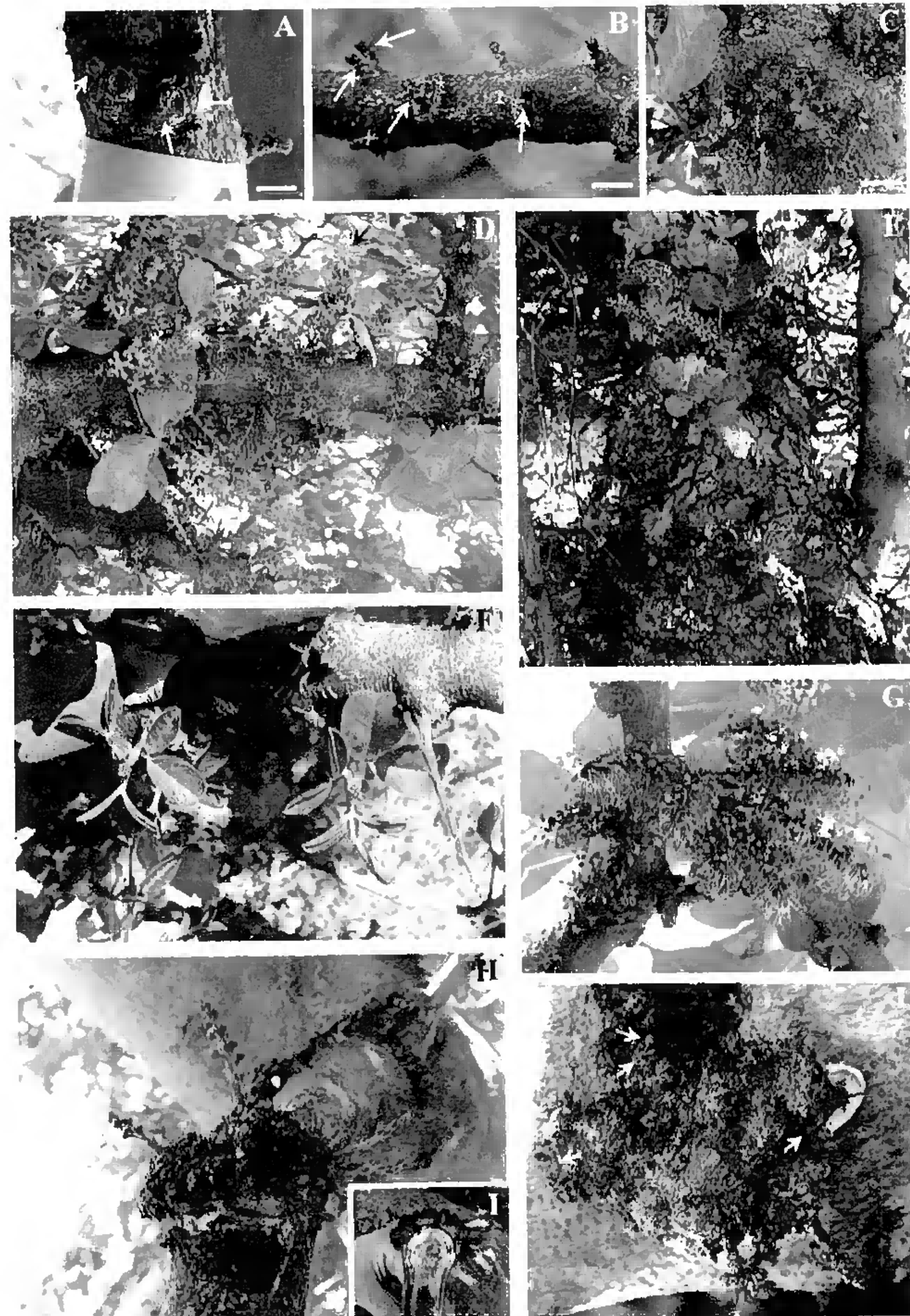

Plate 2.1. Male inflorescences of carob. (A) Inflorescences primordial on a branch (arrows), in a zone from which the leaves have shed, at the beginning of the flowering period; (B) inflorescences developing together with young branches, note the first leaflets (arrows); (C) inflorescences primordial and inflorescences with flowers at stage 0, one of them developing in the apical portion of a young branch (arrow); (D) inflorescences with flowers at stages 0 and I, in the beginning of the flowering period; (E) cauliflourous inflorescescences; (F) inflorescences developing on the axis of young branches; (G) inflorescences with flowers at stage IV at the end of the flowering period; (H-J) Inflorescences primordial (arrows) and inflorescences with flowers at stage 0, on tumour-like zones. Bars = 10 mm on Fig. A and 4.2 mm of Figs. B and C.

Plate 2.2. Female inflorescences and fruits of carob. (A-C) Inflorescences primordia on portions of branches from which the leaves have shed; (D) inflorescences with flowers at stages 0 and I in the beginning of the flowering period and pods developed during the previous season; (E) inflorescences with flowers at stages I and II in the middle of the flowering period; (F) inflorescences with flowers at stages IV and V at the end of the flowering period; (G) cauliflourous inflorescence with flowers at stage IV; (H) general view of pods and (I) pods at different stages of maturation. Bars = 5 mm on Fig. A, 42 mm on Figs. B and C.

Plate 2.3. Hermaphrodite inflorescences and fruits of carob. (A) Inflorescences at developmental stages I and II at the beginning of the flowering period; (B) cauliflourous inflorescences at different stages; (C) detail of inflorescence primordia on the trunk (arrows) (bar = 5 mm); (D) detail of inflorescescence at stage III being pollinated; (E) detail of flowers at stages III and IV with nectar and pollen; (F) inflorescence with flowers at stage V; (G) pods developing in the trunk; and (H) general view of pods.

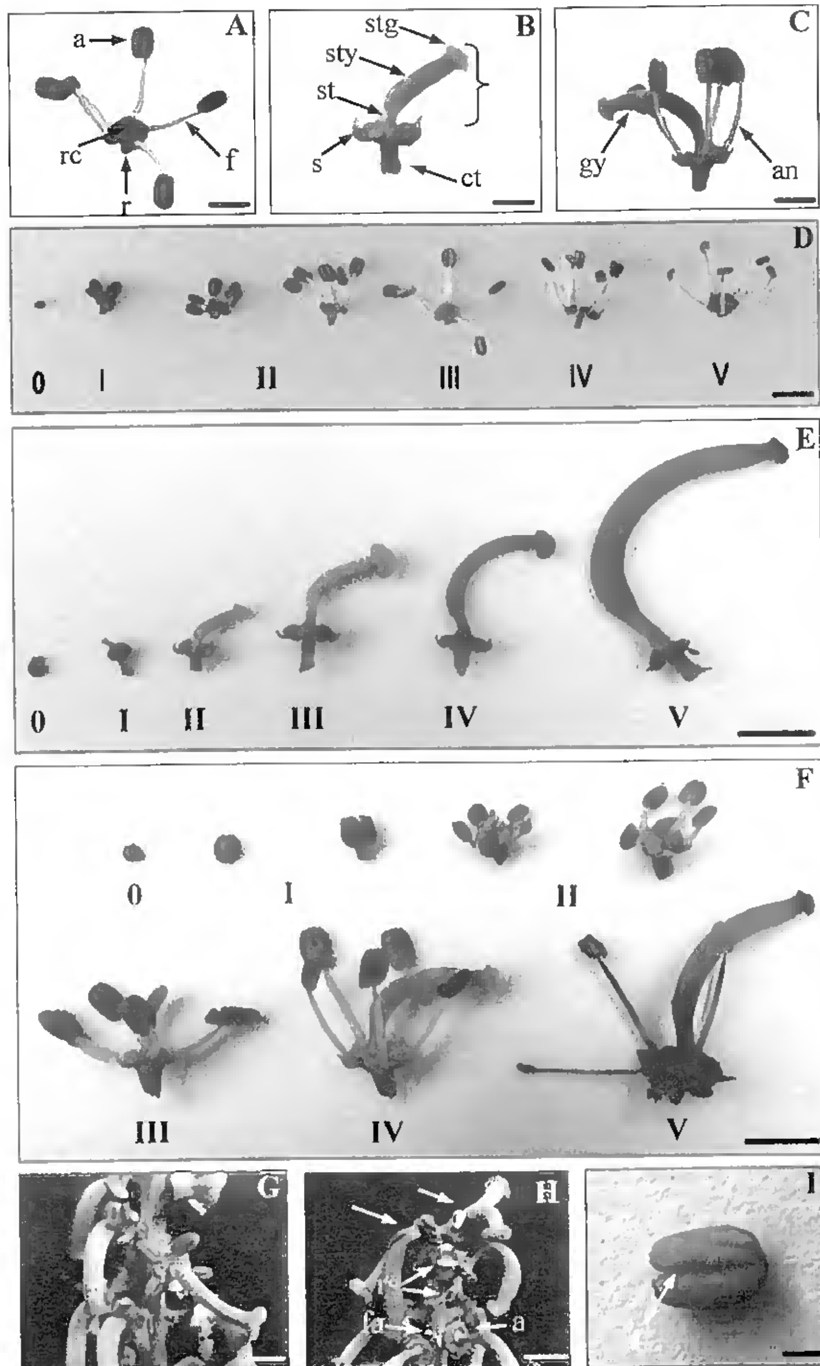

Plate 2.4. Aspects of carobs flowering. (A) Male flower with anthers (a), filament (f), rudimentar carpel (rc) and receptacle (rc) (bar = 8.8 mm); (B) female flower with a calyx tube (ct), sepals (s), and a pistil (pi) formed by a short stipe (st), an abbreviated style (sty) and a peltate stigma (stg) and (bar = 13.9 mm); (C) hermaphrodite flower with androecium (an) and gynoecium (gy) (bar = 7 mm); (D) male (E) female and (F) hermaphrodite flower developmental stages (bar = 4.4 mm); (G) detail of female inflorescence with rudimentar anthers (bar = 10 mm); (H) female inflorescence with abnormal number of pistils (arrows), (cf), rudimentar stamens (rs) and anthers (ra), and flower developing in the axil of other flower (fa) (bar = 4.7 mm); (I) anther, (c) connective (bar = 28.3 mm).

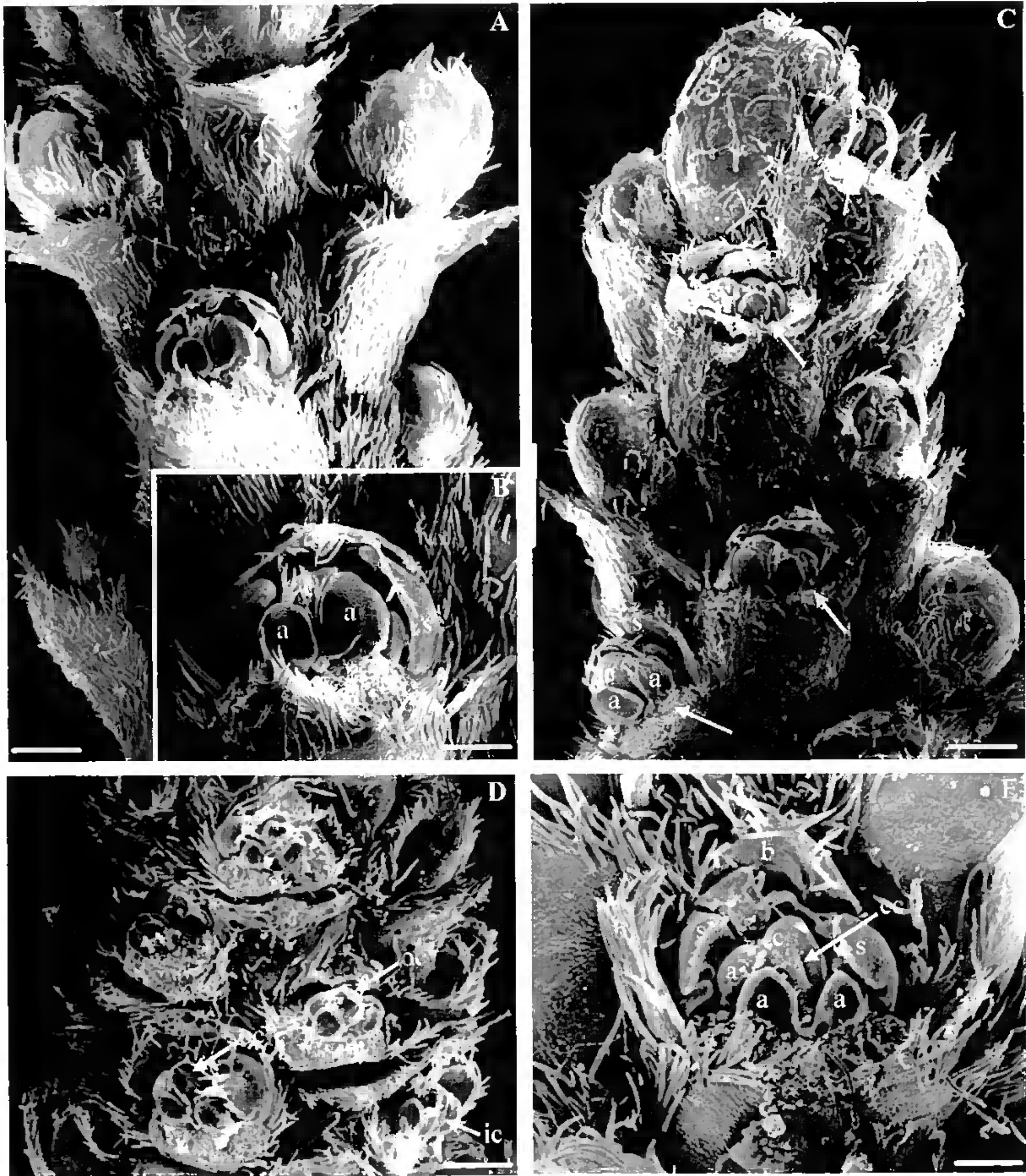

Plate 2.5. Inflorescences and flowers of carob (SEM micrographs). (A) Male inflorescence with flowers at stage 0 (bar = 312.5 μm); (B) male flower with one sepal removed to show the anthers (a) and the rudimentar carpel (c) (bar = 175 μm); (C) hermaphrodite inflorescence with bracts and sepals removed to show the flowers at stage 0 (at arrows) (bar = 354.2 μm); (D) female inflorescence with flowers at stage 0 with bracts removed to show two flowers with obliquous carpels (oc) and one with an inverted one (ic) (bar = 284.1 μm); (E) detail of female flower with one sepal removed to show the rudimentar anthers (bar = 111.1 μm).

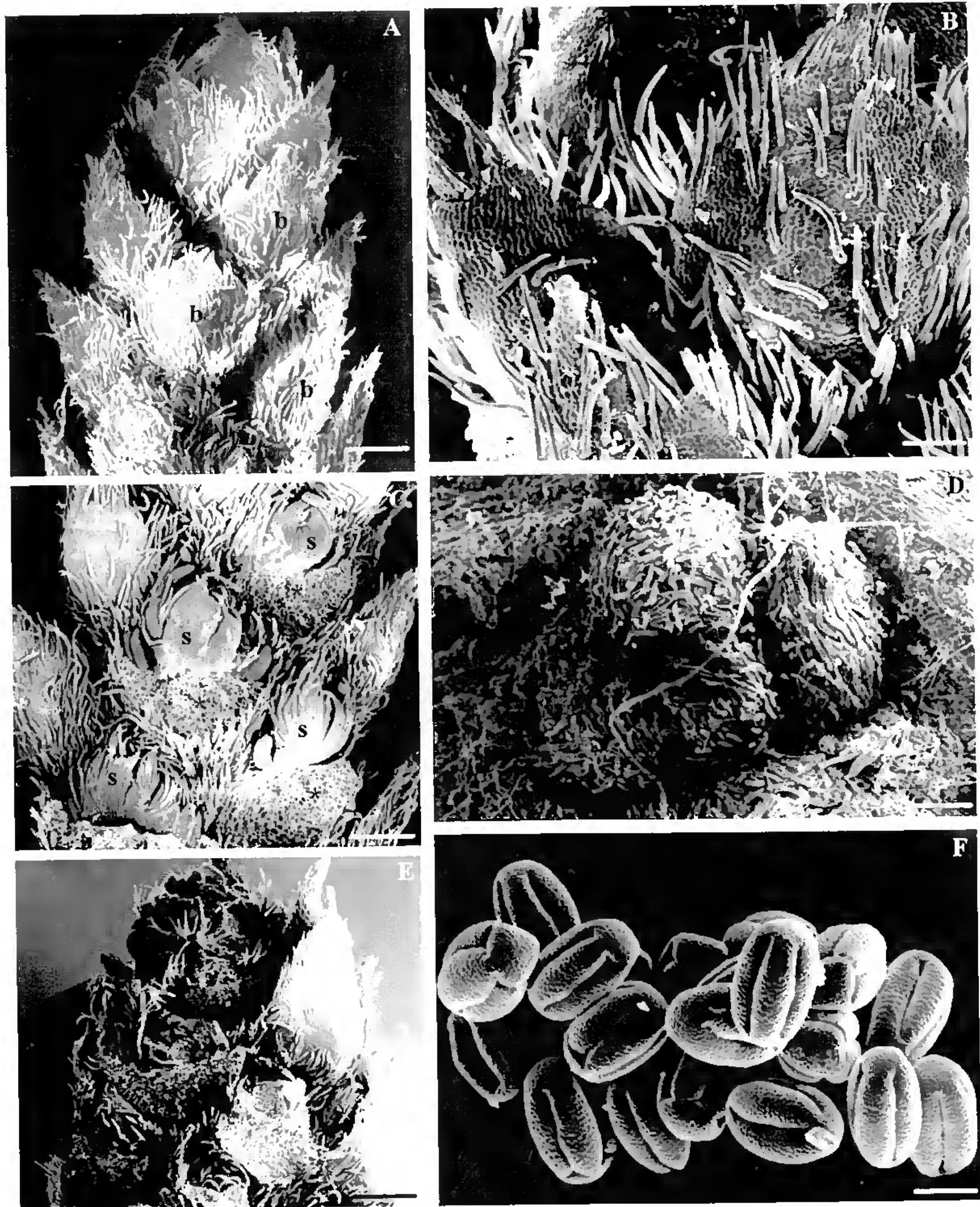

Plate 2.6. Inflorescences and pollen of carob (SEM micrographs). (A) Male inflorescence with floral primordia (bar = 312.5 μm); (B) detail of bracts with tricomes (bar = 50 μm); (C) male inflorescence with floral primordia. Bracts have been removed (asterisks) to show floral primordia covered with sepals in each bract axil (s) (bar = 208.3 μm); (D) inflorescence primordium in the surface of a branch (bar = 136.4 μm); (E) tip of young male inflorescence with helical phylottaxy and acropetal initiation (bar = 312.5 μm); (F) pollen grains (bar = 16.2 μm).

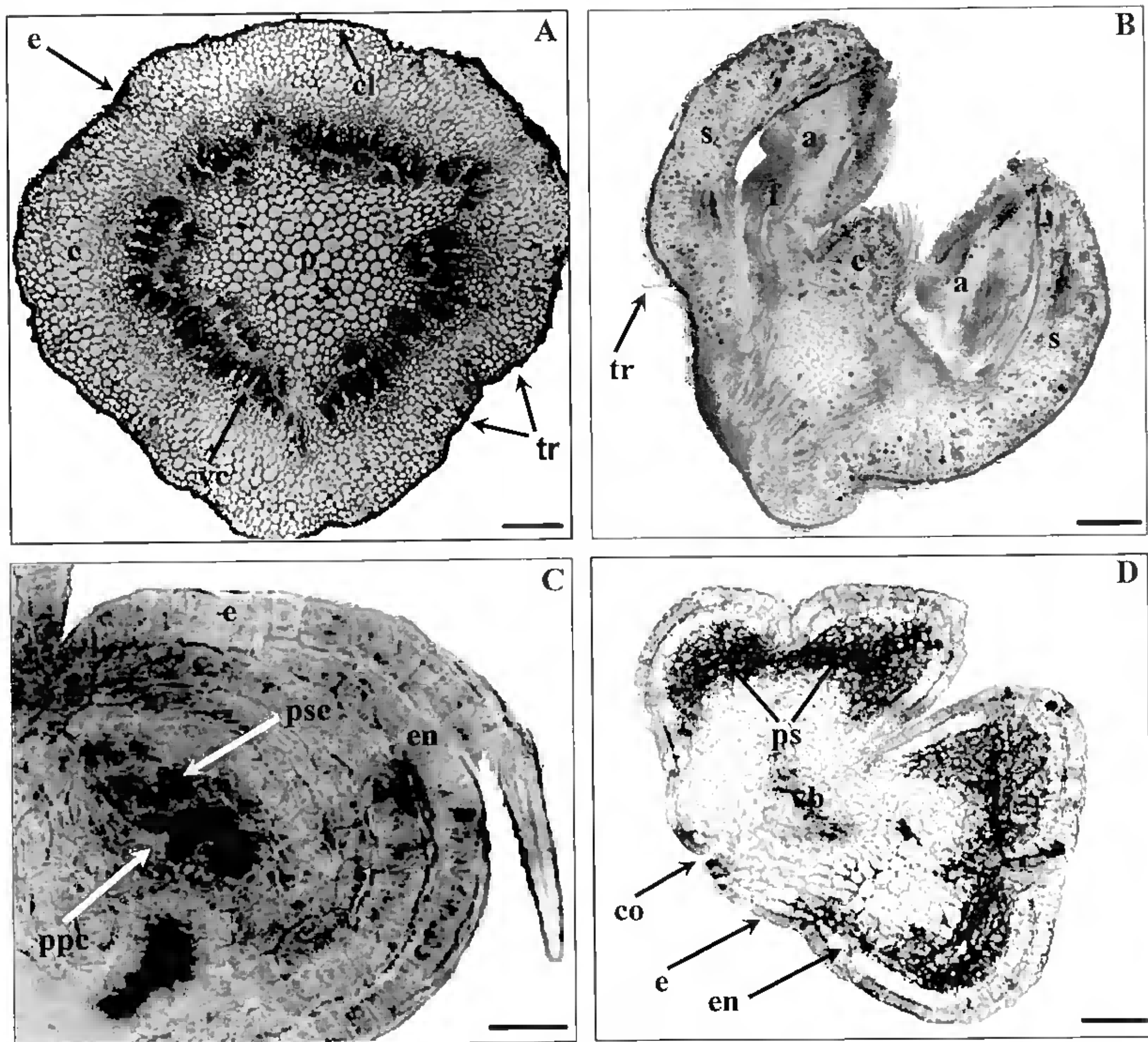

Plate 2.7. Sections of carob male reproductive structures at several stages of development. (A) Transversal section of the raquis, showing a single layer of epidermis (e), trichomes (tr), cortex (c), collenchyma cells (cl), the pith (p) and a split vascular cylinder (vc); (bar = 100 µm); (B) longitudinal section of flower on late phase 0, with anthers (a), a rudimentar carpel (c), filament (f), trichomes (tr) and sepals (s) (bar = 100 µm); (C) transversal section of anther on late phase 0 showing epidermis (e), endothecium (en), primary parietal cells (ppc) and primary sporogeneous cells (psc) (bar = 17.5 µm); (D) anther on early phase 1 with epidermis (e), endothecium (en), connective (co), polinic sacs (ps), and vascular bundle (vb)(bar = 100 µm).

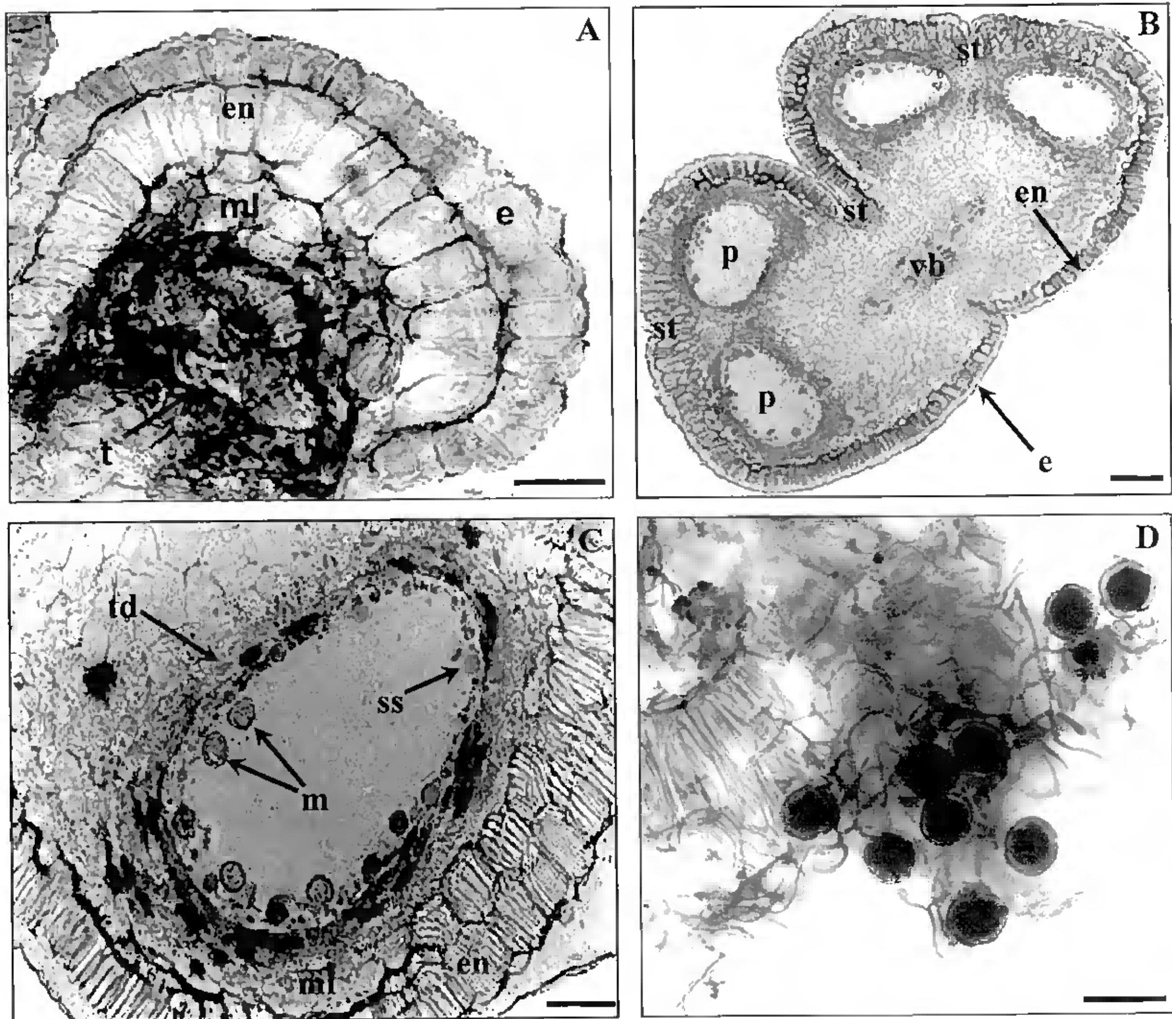

Plate 2.8. Sections of carob male reproductive structures at several stages of development. (A) Anther on late stage I with epidermis (e), endothecium (en), middle layers (ml) and tapetum (t) (bar = 43.5 μm); (B) anther on stage II with epidermis (e), endothecium (en) open policic sacs (p), stomium (st) and vascular bundle (vb) (bar = 100 μm); (C) anther on stage II showing endothecium with secondary thickenings (en), microspores at different developmental stages (m), middle layers (ml) secreted substances (ss) and destruction of tapetum (td) (bar = 43.5 μm); (D) anther on late stage III with mature pollen grains (bar = 10.2 μm).

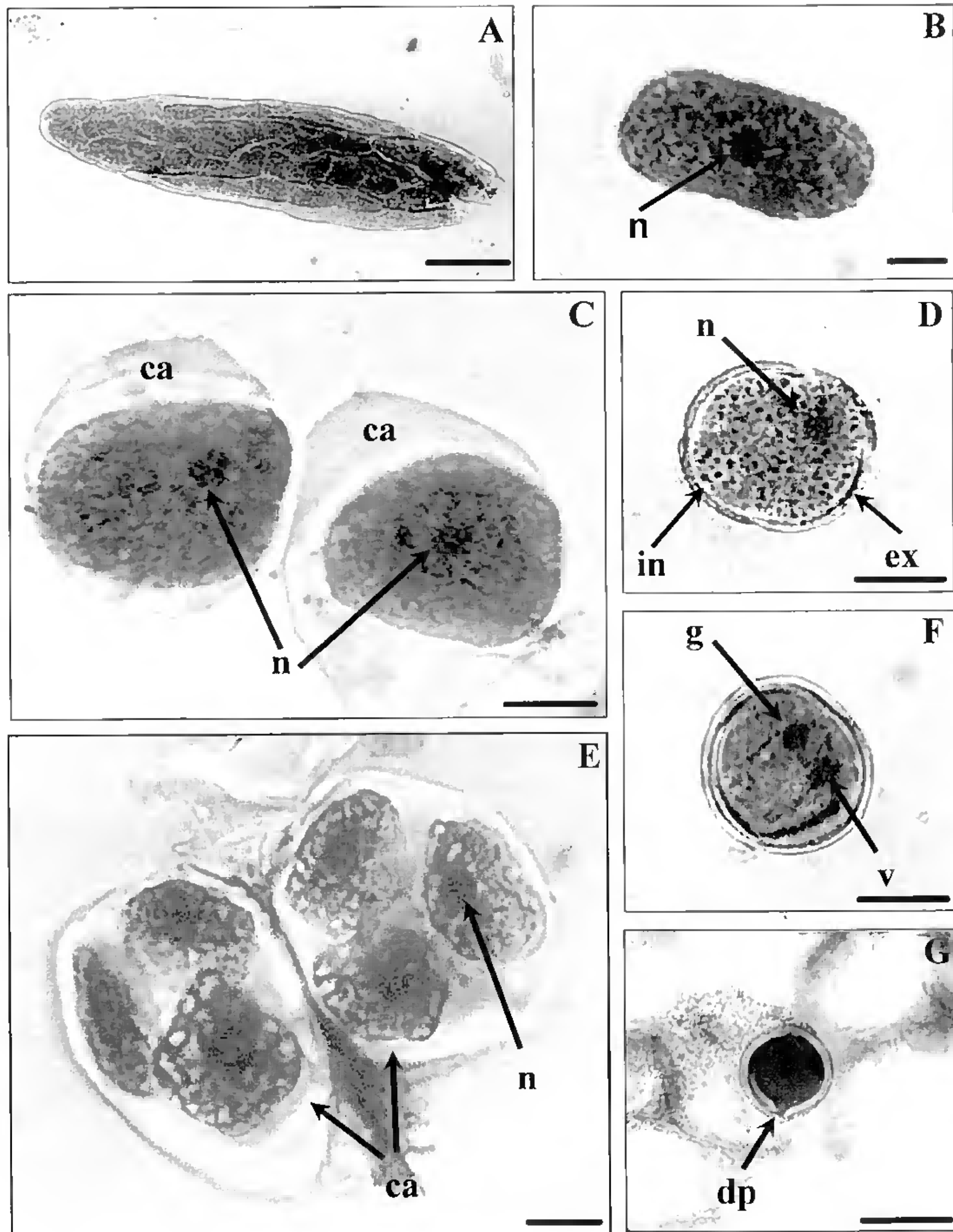

Plate 2.9. Microsporogenesis in carob. (A) Sporogeneous tissue constituted by elongated cells, tightly abut (bar = 17.5 μm); (B) pollen mother-cell with a poligonal shape, showing a nucleus (n) (bar = 12.2 μm); (C) pollen mother cells showing callose (ca) and nucleus (n) (bar = 12.2 μm); (D) uninucleated microspore showing exine (ex), intine (in) and nucleus (n) (bar = 12.2 μm); (E) tetrads showing callose (ca) and nucleus (n) (bar = 12.2 μm); (F) binucleated microspore showing a smaller generative nucleus (g) and a large vegetative nucleus (v) (bar = 12.2 μm); (G) pollen grain observed in anthers on phase III, showing a distal pore (dp) (early phase of pollen tube formation); (bar = 17.5 μm). All the preparations were stained with acetocarmine, except for (G) that was stained with cotton blue in lactophenol.

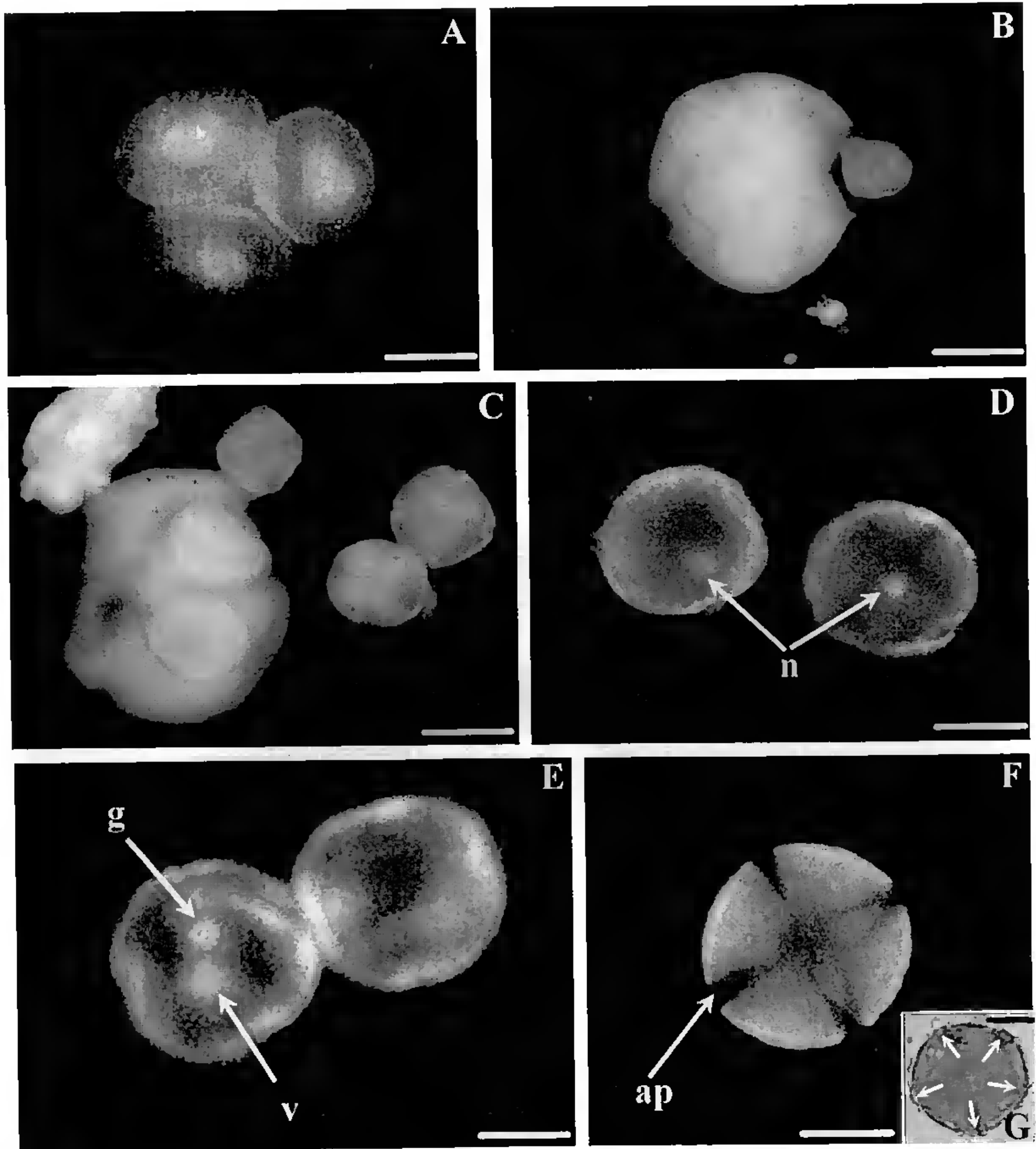

Plate 2.10. Microsporogenesis in carob. (A) Tetrads of microspores (bar = 12.2 µm); (B, C) tetrads releasing microspores (bar = 11.1 µm); (D) Uninucleated microspores (n) (bar = 17.5 µm); (E) binucleated microspores showing the the generative nucleus (g) and the vegetative one (v) (bar = 17.5 µm); (F) pollen grain showing four apertures (ap) (bar = 17.5 µm); (G) pollen grain showing five apertures (arrows) (bar = 8.7 µm). All the preparations were stained with DAPI, except (G) which was stained with acetocarmine.

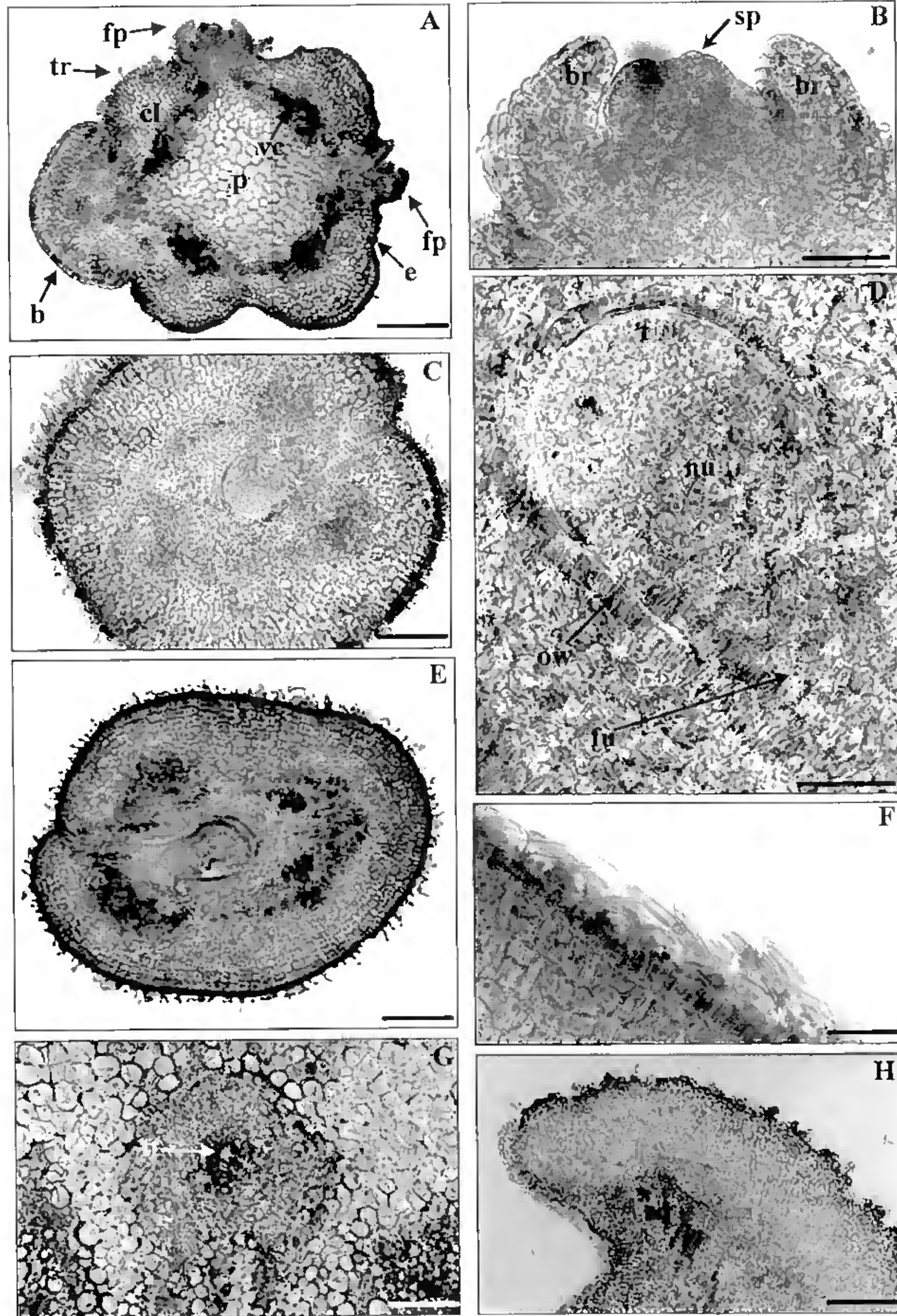

Plate 2.11. Transversal and longitudinal cuts of female reproductive organs of carob. (A) Female inflorescence with cortex (c), collenchyma cells (cl) a single layer of epidermis (e), a floral primordiums (fp), the pith (p), trichomes (tr) and the vascular cylinder (vc) (bar = 0.16 mm); (B) detail of female floral primordium with bracteoles (br) and sepal primordium (sp) (bar = 12.3 μm); (C) pistil 3 mm long from flower at developmental stage I (bar = 0.23 mm); (D) ovule in pistil 2 mm long from flower at stage I with funicullus (fu), nucellus (nu), ovary wall (ow) and tegument (t). The microphyle is not visible (bar = 0.02 mm); (E) ovule 5 mm long, from a flower at stage II (bar = 0.15 mm); (F) detail of tricomes on the carpel (bar = 0.04 mm); (G) style on a flower at stage III, with transmitting tissue (tt) (arrow) (bar = 20.07 μm); (H) detail of one lobe of papillate stigma (bar = 0.3 mm).

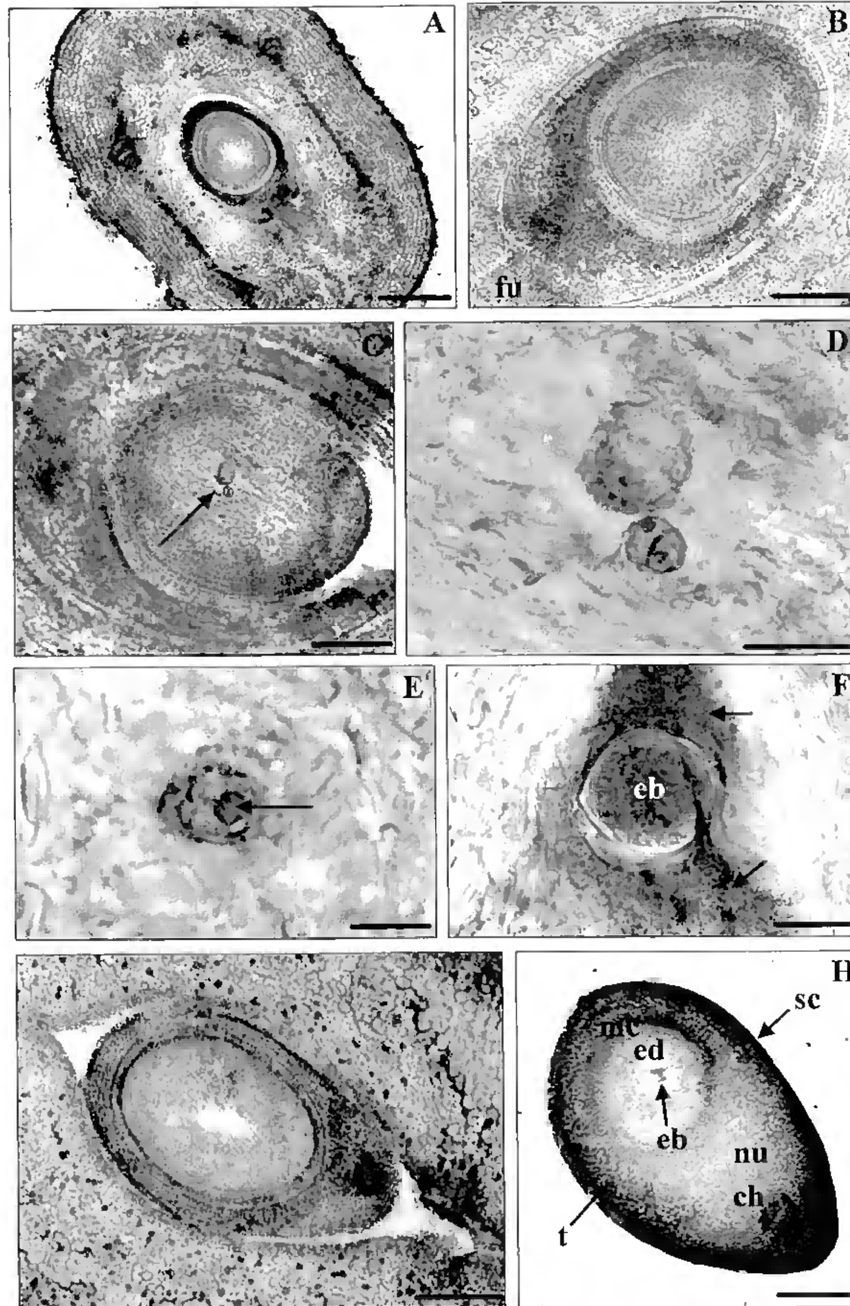

Plate 2.12. Sections of the gynoecia, ovules and seeds. (A) Mature ovule in pistil of flower at stage III (bar = 44 μm); (B) detail of ovule from flower at stage III with funiculus (fu) (bar = 0.09 mm); (C) ovule in ovary from flower at stage III, with embryos (arrow) (bar = 0.08 mm); (D) detail of a bicellular embryo of the previous photo (bar = 0.02 mm); (E) unicellular embryo in a ovule from flower at early stage IV with a proeminent nucleus (arrow) (bar = 35.08 μm); (F) gobular embryo (eb) in a ovule from flower at late stage IV surrounded by a darker stained tissue which resulted from the cells adjacent to the endosperm (arrows) (bar = 10.67 μm); (G) seed in newly developed fruit 26 mm long from flower at stage V (bar = 0.17 mm); (H) longitudinal section of seed in a fruit 41 mm long with a embryo (eb), chalaza (ch), a well developed endosperm (ed), microphylar zone(mc), a yet abundant nucellus (nu), seed coat (sc) and tegument (t) (bar = 0.17 mm).

CHAPTER III

ANALYSIS OF THE VOLATILES OF CAROB TREE FLOWERS

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3.1. Introduction

The ability of flowering plants to prosper throughout their long evolution has been strongly dependent on the constant development of strategies to lure pollinators (Vainstein et al. 2001). The colour of a flower is the first and foremost cue for pollinators attraction, but the scent of a flower is known to strong influence the flowers attractiveness not only to insect pollinators (Dobson 1994, Wright et al. 2002), but also to key insect pests (Gohole et al. 2003, Tasin et al. 2005) and may function as chemical cues for food, rest, mating, and/or larval development sites (Dobson 1994).

Flower scent is a composite character that is determined by a complex mixture of low-molecular volatile compounds that varies with the genotype, stage of plant development, and local environmental conditions (Dobson 1994, Dudareva and Pichersky 2000). Most of the volatiles emitted by flowers are also emitted by the leaves of artificially damaged crop plants or plants attacked by herbivores (Pellmyr et al. 1990). The fact that volatile compounds such as monoterpenes and fatty acid derivatives commonly occur in both (damaged) leaf and floral volatiles (Knudsen et al. 1993), suggests that floral volatiles may have originated from general leaf volatiles in the meshing of the life cycles of insects and plants (Pellmyr et al. 1990).

There is a high number of different flower volatiles (Knudsen et al. 1993), but they are biosynthesized by a relatively small number of often overlapping metabolic pathways (Croteau et al. 2000). Typically, fragrances consist of terpenoids (terpenes), phenylpropanoids (including benzenoids) and fatty acids derivates, which are often highly modified (oxidized, esterified, methylated, etc.) (Knudsen and Tollsten 1993, Croteau et al. 2000, Vainstein et al. 2001).

Terpenes are secondary metabolites comprising more than 20 000 natural products (McGarvey and Croteau 1995, Croteau et al. 2000). They are synthesized from isopentenyl diphosphate (IPP) by different mono- and sesquiterpene synthases (McGarvey and Croteau 1995, Theis and Lerdau 2003), and each of them is composed of 5-carbon (prenyl diphosphae) building blocks added together to make 10-carbon, 15-carbon, and even 2000-500 000-carbon chains, as in the case of rubber (Theis and Lerdau 2003).

In higher plants there are two biochemical pathways for generating terpenes: the classic melavonic acid pathway produces the precursors of sesquiterpenes (C_{15}) and

multiples thereof, for example, the tripenes (C_{30}), via the cytosol (Fig. 3.1A); and the plastid DOXP/MEP (deoxyxylulose phosphate/methylerythritol) produces units of C_{10}, such as the precursors of monoterpenes (C_{10}) and diterpenes (C_{20}) (Theis and Lerdau 2003).

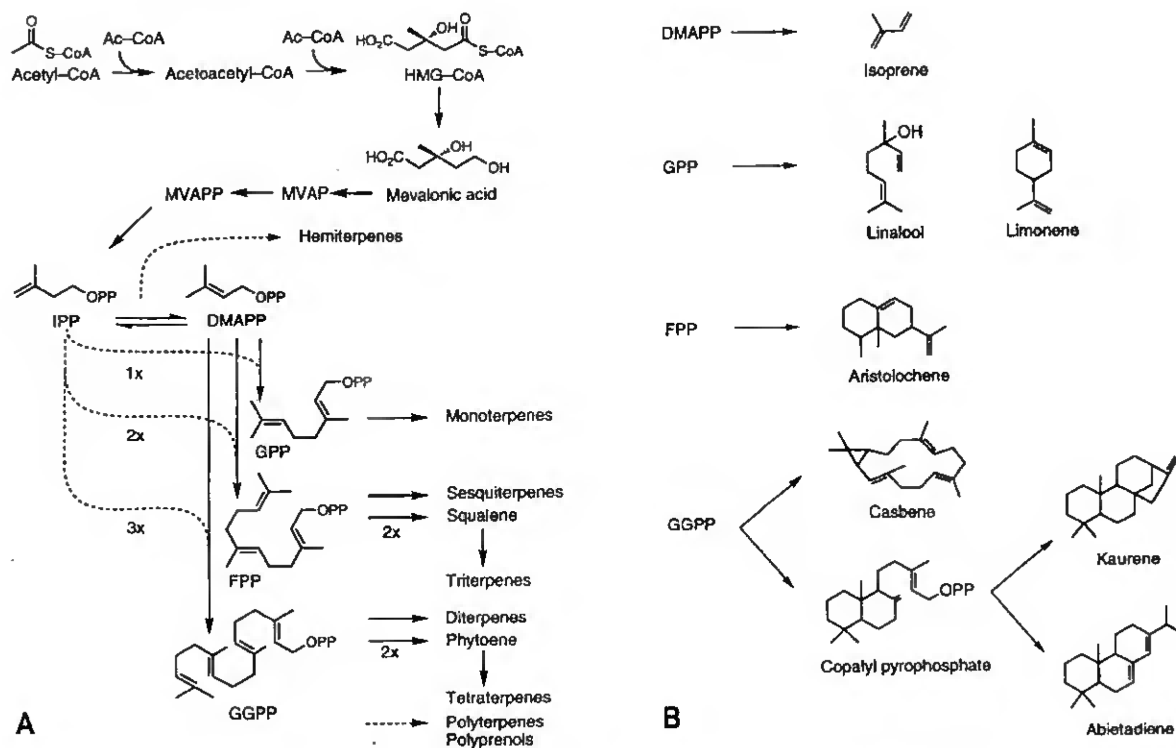

Figure 3.1. The melavonic acid pathway and terpenoid skeleton biosynthesis (A). lx, 2x, and 3x indicate the number of IPP units added; and representative terpenoids (B). DMAPP- dimethylallyl pyrophosphate, GPP- geranyl pyrophosphate, FPP- farnesyl pyrophosphate, GGPP- geranylgeranyl pyrophosphate (McGarvey and Croteau 1995).

The melavonic acid pathway (Fig. 3.1A), can be divided into four major processes (McGarvey and Croteau 1995), the first involving the conversion of acetyl-coenzyme A (CoA) to IPP, the 'active isoprene unit'. By the action of various prenyltransferases on this precursor, the higher order terpenoid building blocks are produced, namely geranyl pyrophosphate (GPP; C_{10}), farnesyl pyrophosphate (FPP; C_{15}), and geranylgeranyl pyrophosphate (GGPP; C_{20}) (Fig. 3.1B) (McGarvey and Croteau 1995). These branch point intermediates may then self-condense (to the C_{30} and C_{40} precursors of sterols and

carotenoids, respectively), be utilized in alkylation reactions to provide prenyl side chains of a range of nonterpenoids (including proteins), or undergo internal addition (that is, cyclization) to create the basic parent skeletons of the various terpenoid families (McGarvey and Croteau 1995). Finally, oxidation, reduction, isomerization, conjugation, or other secondary transformations elaborate the unique and manifold character of the terpenoids.

Isoprene, the simplest of the terpenes (Fig. 3.1B), is synthesized directly from the DMAPP by diphosphate elimination (McGarvey and Croteau 1995). Isoprene is produced and emitted by the leaves of many plants and accounts for a significant proportion of atmospheric hydrocarbons (McGarvey and Croteau 1995). Higher terpenoids, namely linalool and limonene (Fig. 3.1B) are generated via the action of prenyl transferases, which perform multistep reactions beginning with DMAPP (or a longer allylic pyrophosphate) and IPP to form higher isoprenologs (McGarvey and Croteau 1995).

The unique combination of volatile molecules making the small and not-so-small differences in fragrance spectra among flowers of different species can be distinguished by the olfactory receptors of insect antennae, enabling them to find and visit their flower(s) of choice (Raguso et al. 1996). Characteristic floral odours are often correlated with the type of pollinators. Species pollinated by bees and flies tend to have scents that are defined as sweet, whereas those pollinated by beetles have musty, spicy, or fruity odours (Dobson 1994). However, little is known about the innate ability of insects to detect specific volatiles or them innate and learned responses (attraction, repulsion, or indifference) to such compounds (Wright et al. 2002, Tasin et al. 2005).

Pollination is a critical step for the successful production of fruits and seeds, and thus, lack of an efficient pollination can lead to low crop yields. Since floral scent can be crucial in ensuring pollination, and therefore in determining fruit or seed set, the presence or absence of a scent attractive to the locally available insect pollinators may have a substantial impact on the yield of agronomically important crops (Dudareva et al. 2000, Vainstein et al. 2001). The intensity of an odour stimulus produced by flowers may play an important role in initially attracting pollinators and in causing them to seek out perceptually similar flowers (Dobson 1994). Many plant species produce floral arrays where individual flowers are arranged closely together, probably to influence pollinator

perception and recognition among species of plants by increasing a given plant's impact on an animal's sensory apparatus.

Although all floral organs can emit fragrance compounds, petals are the main source of scent in most plants (Pichersky et al. 1994). Some species have developed highly specialized anatomical structures, termed 'scent glands', for fragrance production, while in others the production and emission of scented volatiles occur in non-specialized floral epidermal cells (Dudareva and Pichersky 2000). Studies of floral scents and of their patterns within a single flower are important to better understand the chemical bases of plant-animal relationships and pollination ecology (Flamini et al. 2003), since qualitative and quantitative differences in the volatiles emitted from flower parts may be important in effecting pollinator attraction (Dobson et al. 1990).

For an objective evaluation of floral scent, proper instrumentation is needed. Mass spectroscopy (MS) detectors coupled to gas-chomatography techniques have enabled chemical analyses of floral scents components with high levels of sensitivity (Van Beek 1999). Sampling methods, however, have been somewhat more problematic. Since fragrance compounds are often emitted to the atmosphere, several methodologies have been designed to trap floral volatiles, namely headspace analysis and solid-phase microextraction (SPME) (Fig. 3.2) (Knudsen et al. 1993, Van Beek 1999, Pellati et al. 2005).

Headspace methods are considered the most helpful in studies of floral scents in relation to their role as cues for pollinators, since they give the most natural and true picture of the composition of the floral aroma as a pollinator meet it (Knudsen et al. 1993). The use of headspace techniques to sample volatiles in the air surrounding a plant organ has revealed that olfactory stimuli differ not only between species, but also among different organs within a single flower (McTavish et al. 2000, Fernando and Grün 2001, Flamini et al. 2003).

SPME is a unique sample preparation technique, which eliminates most drawbacks to extracting organics, including high cost and excessive preparation time and represents a valid alternative to hydrodistillation for gas chromatographic analysis of organic volatiles from different sources (Flamini et al. 2003, Pellati et al. 2005).

This method was developed by Arthur and Pawliszyn in 1990 (Pawlizyn 1997), and in a few years, its applications was extended to many fields, namely as a new clean-up

technology in environmental (Pawlizyn 1999) and food flavour analysis (Brunton et al. 2001), characterisation of the volatile fraction of aromatic and medicinal plants (Marriott et al. 2001, Smith 2003), and in the analysis of odours from different plants (Granero et al. 2004, Demirci et al. 2005, Tasin et al. 2005).

In SPME analytes are adsorbed from a solid sample by headspace extraction, using a polymer-coated fused silica fiber (Fig. 3.2). The compounds are then desorbed by exposing the fiber in the injection port of a gas chromatographic apparatus. This technique offers several advantages over dynamic headspace or hydrodistillation techniques, particularly the fact of not being time consuming, requiring smaller sample sizes, minimising the formation of artifacts since it does not damage the plant, and avoiding contamination or losses of compounds (Flamini et al. 2003, Pellati et al. 2005).

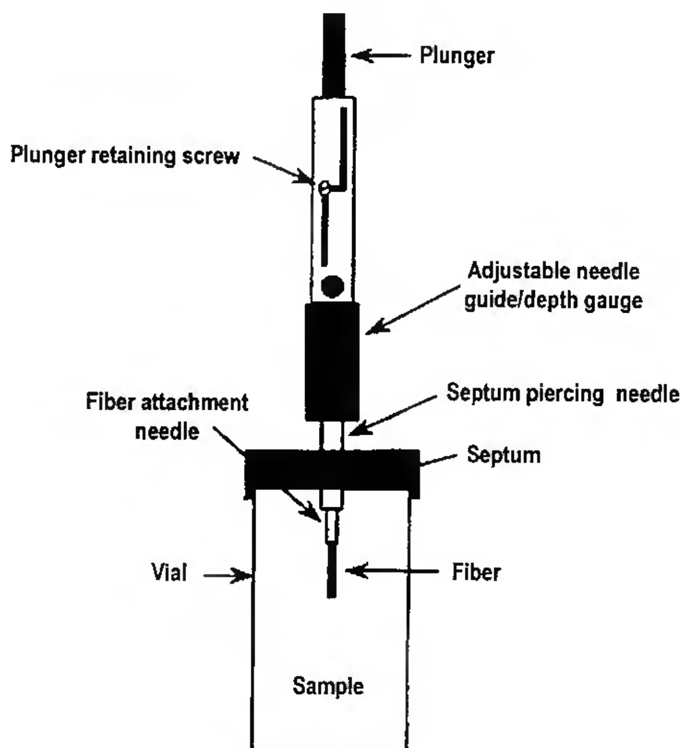

Figure 3.2. Solid-phase microextraction (SPME) diagram.

Carob exhibits some characteristics usually associated with wind pollination, such as dioecy, relatively small flowers in dense, often terminal inflorescences, a reduced, dull or

green perianth with exposed fertile parts, anthers on long exserted filaments, and an expanded, exposed stigmatic surface (Lewis et al. 2003), but lack others, like the reduction of pollen apertures, from colporate to porate ones (Crane 1986). Moreover, the strong odour associated with the flowering period, the secretion of nectar and the high frequency of insect floral visitors suggest that carob is predominantly an entomophylous species (Ortiz et al. 1996). The insect pollinators belong mainly to the orders *Hymenoptera* and *Diptera* (Retana et al. 1990, Ortiz et al. 1996, Arista et al. 1999).

The composition of the aroma volatile fraction of carob flowers is yet not known. Since this knowledge may be useful in understanding carob pollination, in this chapter headspace solid phase microextraction followed by capillary gas chromatography coupled to mass spectrometry detection (HS-SPME-CGC/MSD) was used to evaluate the main compositional volatiles in the scent emitted *in vivo* by whole flowers of male, female and hermaphrodite carob trees. Moreover, the composition of the scent of isolated flower parts was assessed, in order to evaluate the contribution of the volatiles emitted by those structures to the odour profile of whole flowers. The influence of cultivar on volatiles emission was also evaluated.

3.2. Materials and methods

3.2.1. *Plant material*

Inflorescences with flowers at different developmental stages (I, II, III and IV) were collected from the male, female and hermaphrodite carob trees (*vide* chapter II).

The study of the volatiles emitted *in vivo* by whole flowers was done in October of 2003, while the analysis of the volatiles emitted *in vivo* by isolated floral organs took place in July of 2004, and in this case, the two most representative female cultivars in the Algarve, Galhosa and Mulata, were also studied.

3.2.2. *HS-SPME-CGC/MSD analysis*

For the headspace-SPME analysis of volatiles emitted by whole flowers, 180 flowers at stage I, 150 at II and 100 at stages III and IV were picked from inflorescences from male, female and hermaphrodite carob trees. For the analysis of the volatiles emitted by isolated floral parts, samples (500 mg) of male, hermaphrodite and female flowers and of the different floral parts (stamens, nectarial disks and stigmas), were collected at the stages that were found to emit the higher number of volatiles.

After collection, the samples were immediately placed into 30 ml amber vials having screw caps (Supelco). The SPME device (Supelco Inc., Bellefonte, PA USA), coated with polydimethylsiloxane (100 μm) fiber, was first activated by inserting it into the GC injector port at 250°C for 1 h. For sampling, the fiber was inserted into the headspace for 15 min at room temperature (20°C). Following sampling, the SPME device was introduced in the injector port for chromatographic analyses and was allowed to remain in the inlet through the duration of the run, thus preparing it to the next analyses. Blank vials were conducted as controls.

Capillary GC/MSD analyses were performed in a Agilent 6890 gas chromatograph interfaced to an Agilent 5973 *N* mass selective detector (Agilent Technologies, Palo Alto, USA). A vaporization injector operating in the splitless mode (2 min) at 250°C was used into which a fused silica capillary column, 30 m × 0.32 mm ID × 0.25 μm d_f(HP-5MS; 5% diphenyl 95% dimethyl polydimethylsiloxane) was installed. The oven temperature program was 40°C for 2 min and then increased at 5°C min^{-1} to 180°C, followed by 15°C min^{-1} to 240°C and held isothermally for 15 min. Helium was used as carrier gas at 30 cm sec^{-1}.

Electron ionisation mass spectra in the range 40-400 Da were recorded at 70 eV. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280°C, respectively, and a turbo molecular pump (10^{-5} torr) was used. All data were recorded using a MS ChemStation (G1701CA; Rev C.00.01).

The identity of each compound was determined by comparison of its retention indices, relative to C_6-C_{24} n-alkanes (Adams 2001), as well as by comparison of the spectral data with the Wiley's library spectral data bank [G1035B; Rev D.02.00] or

homemade libraries (Flavour2.l). For semi-quantification purposes, the normalized peak area of each compound was used without any correction factor to establish abundance.

3.3. Results and discussion

3.3.1. *Volatiles of whole flowers*

In this section, the volatiles emitted *in vivo* by whole flowers of the three sexual types of carob were evaluated in the stages of flower development corresponding to the ones after anthesis and before drying of the stamens or fertilization (I, II, III and IV, *vide* chapter II).

The identified compounds in the scent of carob whole flowers, their percentages and retention indices listed in order of their elution are summarized in Table 3.1. The principal compounds (>5%) appear in bold face. The volatiles from carob flowers shown to be complex mixtures of several terpenes, mainly constituted by oxygenated monoterpenes (> 40%), as well as monoterpene hydrocarbons, esters and other minor compounds (Table 3.1). There were differences in the profiles of the volatile compounds between the sexual types and flower developmental stages.

Most of the compounds were detected in the three types of flowers, but the major volatiles were present in higher abundances in the male ones (Table 3.1, Fig. 3.3). In general, the stages with the most diversified composition were III and IV (Table 3.1), while a smaller number of compounds were detected on I and II. More than twenty-five compounds were positively identified on the headspace of carob flowers (Table 3.1), representing 74.1, 66.1 and 57.5% of the total constituents of stages III and IV from male, hermaphrodite and female flowers, respectively.

Terpenes are important allelochemicals and can act as antifeedant defense compounds as well as insect pheromones (Knudsen and Tollsten 1993, Dobson 1994, Pechous and Whitaker 2004). Furthermore, they play diverse functional roles in plants as hormones (gibberellins, abscisic acid), photosynthetic pigments (phytol, carotenoids), electron carriers (ubiquinone, plastoquinone), mediators of polysaccharide assembly (polyprenyl phosphates) and structural components (phytosterols) (McGarvey and Croteau 1995, Theis and Lerdau 2003).

Table 3.1. Constituents and compositional percentage of the volatiles of carob flowers (male, hermaphrodite and female) from field-grown plants by HS-SPME-CGC/MSD.

Compounds	*RI	**Male** (stage III)	**Hermaphrodite** (stage III)	**Female** (stage IV)
α-pinene	922	0.7		1.3
β-myrcene	1003			0.6
α-terpinene	1040			0.7
Limonene	1057	0.2		1.2
1,8-cineole	1061	1.1	0.8	
cis-ocimene	1069	1.4	0.4	0.3
γ-terpinene	1100			0.8
trans-linalool oxide	**1142**	**17.6**	**7.6**	**9.4**
δ-hexalactone	1148		0.9	1.1
Linalool	**1157**	**45.0**	**51.9**	**35.1**
Alloocimene	1197	1.0	0.3	0.3
Ethyl benzoate	1254	1.7	1.2	1.6
3-Methylbutyl *E*-2-methyl-2-butenoate	1284	0.8		
Methyl salicylate	1288			0.5
Decanal	1298		0.1	
Ethyl salicylate	1386	1.3	1.3	2.9
Ethyl nonanoate	1414	0.5		
Theaspirane A	1424	0.2	0.2	0.1
2-Methoxy-4-vinylphenol	1439	0.1	0.1	0.1
Theaspirane B	1445	0.1	0.1	0.1
Ethyl 2-methoxy-, methyl benzoate	1469	0.2		
Eugenol	1492		0.1	
α-copaene	1519	0.1	0.1	
Ethyl 2-methoxy-, ethyl benzoate	1547	0.8	0.3	0.7
1-Butanol, 3-methyl benzoate	1587	0.4		
Ethyl cinnamate	1619	0.2	0.2	0.3
α-fernesene	1665	0.6	0.4	0.1
Δ-cadinene	1687	0.1	0.1	0.3
Monoterpene hydrocarbons		3.3	0.7	5.2
Oxygenated monoterpenes		63.7	60.3	44.5
Esters		5.5	3.0	6.0
Others		7.1	5.1	7.8
Total identified		74.1	66.1	57.5

*Retention index relative to C_9-C_{17} n-alkanes on the HP-5MS capillary column; †Normalised peak areas without using the correction factors.

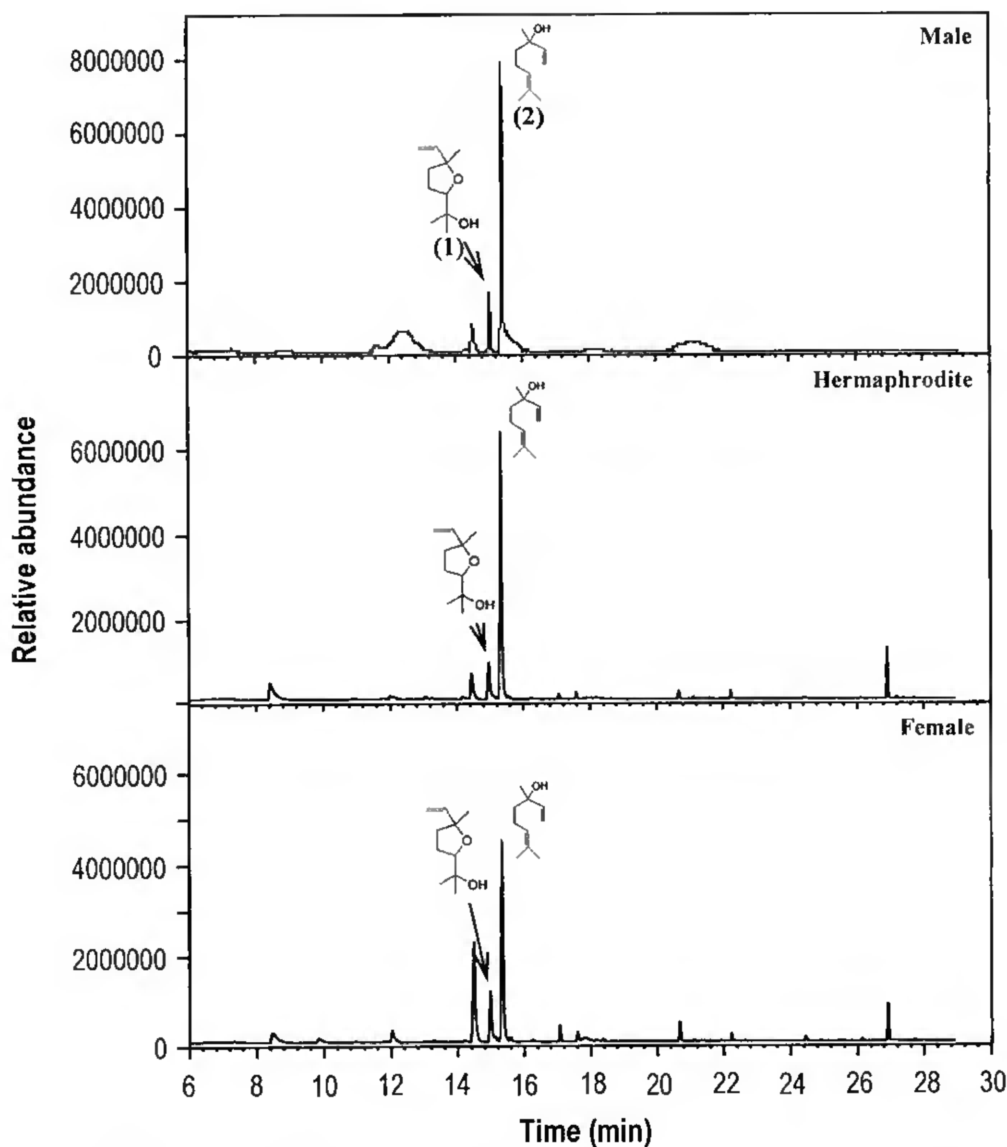

Figure 3.3. Total ion chromatograms of the headspace of male and hermaphrodite flowers at developmental stage III, and female flowers at stage IV. Peaks are 1) *trans*-linalool oxide and 2) linalool.

Many terpenes, including β-myrcene, (E)-β-ocimene, linalool and (E)-β caryophyllene, react readily with ozone and other reactive oxygen species, and protect the reproductive organs, with their valuable germ line cells, from oxidative damage (Loreto and Velikova 2001). A variety of monoterpenes and sesquiterpenes are also reported to have antimicrobial activity, acting in the defense of the floral organs from bacterial or fungal infestation, since the moist surface of the stigma may be an ideal environment for fungal growth (Deans and Waterman 1993).

Linalool (> 35%) and *trans*-linalool oxide (> 7 %) are the dominant volatiles in carob flowers and there are notorious differences in those compounds between different types of flowers as well as developmental stages (Table 3.1, Figs 3.3 and 3.4). Linalool is an acyclic monoterpene alcohol, and an important attractant pheromone for many insects (Raguso and Pichersky 1999, Borg-Karlson et al. 2003). It has been collected from the headspace of flowers of different species, namely in different species of *Citrus* (Altenburger and Matile 1990), *Malus domestica* and different species of *Nicotiana* (Loughrin et al. 1990), *Clarkia brewerii* (Dudareva and Pichersky 2000), *Magnolia kobus* (Azuma et al. 2001), and different species of *Gentiana* (Georgieva et al. 2005) and *Antirrhinum* (Wright et al. 2005). The antimicrobial and anesthetic activities of linalool-containing essential oils have also been reported (Mazzanti el al. 1998), being commonly found as a major component of the essential oils of several aromatic plant species, many of which are used in traditional medical systems as analgesic and anti-inflammatory remedies (Peana et al. 2002).

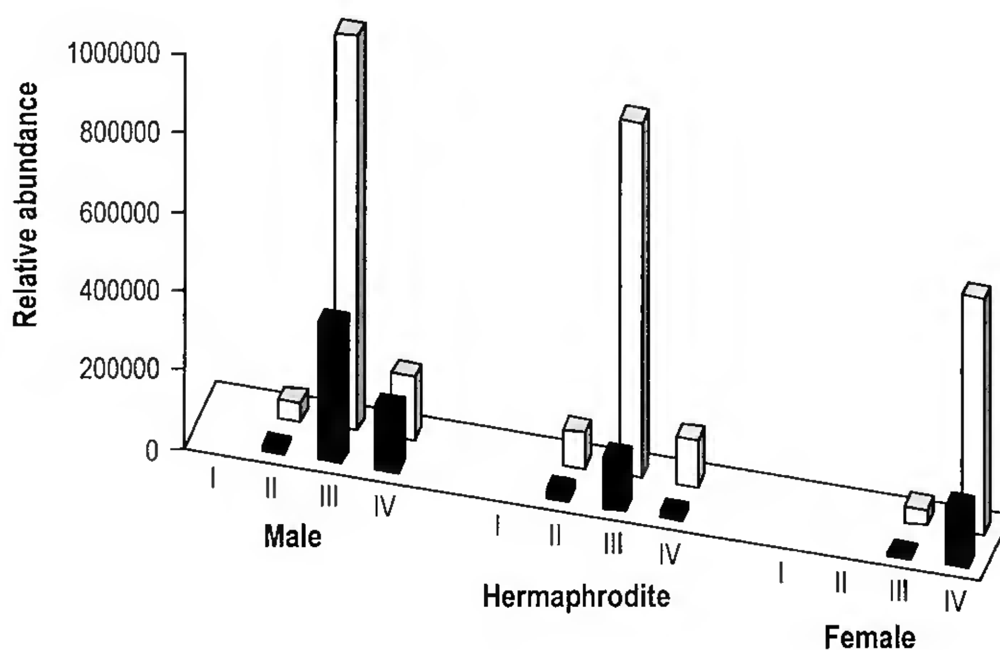

Figure 3.4. Abundance of linalool (□) and *trans*-linalool oxide (■) found in the volatiles of carob flowers in different developmental stages from field-grown plants by SPME/CGC-MSD.

Linalool and *trans*-linalool oxide are present in higher abundances in male flowers (Table 3.1, Figs 3.3 and 3.4). In earlier studies Ortiz et al. (1996) showed that in spite of producing significantly higher amounts of nectar, female flowers were much less fragrant than the male ones. Moreover, Ortiz et al. (1996) observed higher number of floral visitors in male trees, which could be correlated with the higher amount of volatile compounds present in the male flowers.

Linalool and *trans*-linalool oxide were not detected in stage I, in all types of flowers studied, and in stage II of female ones (Fig. 3.4). In male and hermaphrodite flowers the levels of those compounds peaked at stage III, and in the female ones at IV (Fig. 3.4). In fact, quantitative differences in flowers volatile compounds were reported within the same species (Dudareva and Pichersky 2000), which vary developmentally (Dudareva et al. 2000).

The higher amounts of volatiles identified in stage III in male and hermaphrodite flowers, and in the stage IV in the females, could be due to some characteristics associated with those stages. The stage III of male and hermaphrodite flowers is characterised by the secretion of nectar, just before pollen releasing in stage IV. The secretion of nectar is also observed in female flowers, but in stage IV. Nectar is a multi-component aqueous solution, and contains aromatic compounds, besides other substances (Tóth et al. 2003). Those aromatic compounds can contribute to the increase of the volatiles observed in the stages were this secretion is observed. Moreover, nectar is associated with the phenomenon of pollination, acting as a reward to the pollinator insect, and with floral scent, the flower is able to advertise truthfully when rewards, such as nectar or pollen, are available (Dudareva and Pichersky 2000).

Previous studies have shown that the production of a low percentage of fruits per raceme is a constant characteristic in carob (Arista et al. 1999, Bosch et al. 1996, Ortiz et al. 1999) and that fruit production can be limited by a deficient pollination (Arista et al. 1999). Various features of carob floral biology could be responsible for the deficient pollination, such as the racemes being caulogeneous and the female flowers much less fragrant than the male ones (Ortiz et al. 1996). The fact of the carob flowers being apetalous can also be a severe handicap for pollination, since those floral organs usually constitute the main source of scent emission (Dudareva and Pichersky 2000).

Besides male trees, hermaphrodites are sometimes used as pollinators in carob orchards, and since they are both pollinators and producers, they could be the preferred ones (Batlle and Tous 1997). However, only a few hermaphrodites have sufficiently desirable agronomic attributes (Batlle and Tous 1997). Since in this work it was found that the male flowers have a higher diversity and amount of volatiles, male trees could be more effective as pollinators by contributing to a more efficient dispersal of pollen by floral visitors. This should be taken into consideration when planning new carob orchards.

3.3.2. *Volatiles of isolated floral parts*

In this work it was initially made an evaluation of the volatiles composition in the scent emitted *in vivo* by whole flowers of the three sexual types of carob, along the different developmental stages (section 3.3.1). It was observed that both sexual type and developmental stage of the flowers affected the composition of the volatiles. On this basis, in this section the main compositional volatiles in the scent emitted *in vivo* by whole flowers and isolated flower parts of male, hermaphrodite and female (Mulata and Galhosa) carobs was evaluated, in order to assess the contribution of the volatiles emitted by the different floral parts to the odour profile of whole flowers. The stages of flower development used where those where the total emission of volatiles was found higher: stage III in male and hermaphrodites flowers and stage IV in the female ones.

The identified compounds in the scent of carob flowers and isolated floral parts, their percentages and retention indices listed in order of their elution are summarized on Table 3.2. The principal compounds (>5%) appear in bold face. The scent of carob's flowers is richer in monoterpenes than in sesquiterpenes (Table 3.2), and as reported in other species (Azuma et al. 2001), linalool and its oxides were the most abundant compounds.

Differences were observed on the composition of the volatiles emitted between the sexual types and the two female cultivars (Table 3.2 and Fig. 3.5). More than twenty-five compounds were identified, representing 84.5-98.6% of the total constituents of the volatiles (Table 3.2).

Table 3.2. Constituents and compositional percentage of the volatiles of whole flowers (WF) and isolated flower parts (ND: nectarial disk; Sta: stamens; Sti: stigma) from field-grown male, hermaphrodite and female carobs (cv. Mulata and Galhosa) by HS-SPME-CGC/MSD.

Compounds	*RI	†Composition (%)												
		Hermaphrodite				Male			Female (Mulata)			Female (Galhosa)		
		WF	ND	Sta	Sti	WF	ND	Sta	WF	ND	Sti	WF	ND	Sti
α-pinene	902	**17.9**	**8.5**	**14.1**	**41.9**	3.7	**14.4**	**21.4**	**60.0**	4.6	**22.6**	**10.2**	**12.3**	-
sabinene	944	0.5	0.3	-	0.3	-	0.8	-	0.9	-	-	0.3	0.7	-
β-pinene	947	2.2	1.0	-	3.2	-	2.0	-	5.2	-	-	1.0	1.3	-
β -myrcene	966	-	1.6	-	1.4	-	2.2	-	1.4	-	-	2.1	2.6	-
β -terpinene	991	-	-	-	0.6	-	0.1	-	0.5	-	-	-	-	-
limonene	1006	1.8	2.2	-	2.7	1.0	1.0	-	3.1	-	-	**13.1**	**12.9**	4.4
cis-ocimene	1014	-	0.9	-	0.5	-	0.8	-	0.5	-	-	2.5	2.3	-
trans- β -ocimene	1026	0.7	-	-	**9.6**	-	0.2	-	-	-	-	-	-	-
γ-terpinene	1037	-	-	-	1.5	-	0.2	-	0.8	-	-	-	0.3	-
cis-linalool oxide	1051	**6.1**	**8.6**	1.2	2.8	**9.0**	**8.9**	-	2.4	**9.5**	3.5	**7.8**	**6.1**	**6.4**
trans-linalool oxide	1068	**29.1**	**24.5**	**46.7**	**18.9**	**38.1**	**23.3**	**15.3**	**10.4**	27.9	**31.1**	**24.4**	**15.8**	**37.1**
linalool	1080	**7.4**	**17.2**	1.0	2.6	**11.4**	**24.1**	2.4	1.8	**11.5**	1.4	4.6	**11.5**	2.7
epoxy linalool	1158	**9.2**	**8.4**	**22.4**	3.4	**11.0**	**6.7**	**10.1**	2.5	**9.2**	**14.0**	**6.1**	**7.2**	**14.3**
4-terpineol	1164	-	-	-	0.9	-	-	-	0.4	-	3.5	-	-	-
α -copaene	1363	0.1	0.3	-	0.1	-	-	-	0.8	0.7	-	0.6	0.7	1.1
β -bourbonene	1375	-	-	-	-	0.5	-	-	0.5	0.7	-	-	-	-
β -guaiene	1453	-	0.3	-	-	-	0.2	-	0.5	-	-	-	0.2	-
valencene	1453	-	-	-	-	-	-	-	0.4	1.7	-	-	-	-
germacrene-D	1461	-	-	-	-	-	-	-	-	4.9	-	-	-	-
α -farnesene	1477	**6.5**	**9.6**	2.4	3.2	3.6	3.3	-	1.8	**6.9**	4.5	**12.7**	**9.5**	1.4
Δ -cadinene	1494	0.9	1.4	-	-	-	0.1	-	1.7	2.5	2.8	2.0	1.5	**8.4**
7-octen-2-one	1272	1.5	**4.4**	-	0.3	**1.6**	1.4	-	0.5	-	-	5.3	7.8	0.1
theaspirane A	1288	0.5	0.4	0.9	0.4	-	0.2	0.6	0.5	-	-	0.3	0.2	2.9
theaspirane B	1304	0.4	0.3	0.7	0.3	-	0.1	0.5	0.5	-	-	0.3	0.2	1.8
methyl eugenol	1384	-	0.9	-	-	3.2	1.8	-	1.5	-	-	3.3	5.7	-
phytol	1553	0.8	-	-	0.5	1.4	-	-	-	-	-	-	-	-
Monoterpene hydrocarbons		23.1	14.5	14.1	61.7	4.7	21.7	21.4	72.4	4.6	22.6	29.2	32.4	4.4
Oxygenated monoterpenes		51.8	58.7	71.3	28.6	69.5	63.0	27.8	17.5	58.1	53.5	42.9	40.6	60.5
Sesquiterpene hydrocarbons		7.5	11.6	2.4	3.3	4.1	3.6	-	5.7	17.4	7.3	15.3	11.9	10.9
Others		3.2	6.0	1.6	1.5	6.2	3.5	1.1	3.0	-	-	9.2	13.9	4.8
Total identified		85.6	90.8	89.4	95.1	84.5	91.8	50.3	98.6	80.1	83.4	96.6	98.8	80.6

*Retention index relative to C_9-C_{17} n-alkanes on the HP-5MS capillary column.

†Normalized peak areas abundances without using the correction factors.

The headspace of whole flowers of hermaphrodites, males and Galhosa were mainly constituted by oxygenated monoterpenes, accounting 51.8%, 69.5% and 42.9% to the total emission of the volatiles, respectively, while in Mulata, the monoterpene hydrocarbons were present in higher abundance (72.4%) (Table 3.2). The sesquiterpene compounds were present in higher abundances in the scent of hermaphrodites (7.5%) and females (Mulata: 5.7% and Galhosa: 15.3%) whole flowers (Table 3.2). The major components of the scent from the hermaphrodite and male flowers were *trans*-linalool oxide (29.1 and 38.1%, respectively), α-pinene (17.9 and 3.7%), epoxy linalool (9.2 and 11.0%), linalool (7.4 and 11.4%), α-farnesene (6.5 and 3.6%) and *cis*-linalool oxide (6.1 and 9.0%) (Table 3.2 and Fig. 3.5).

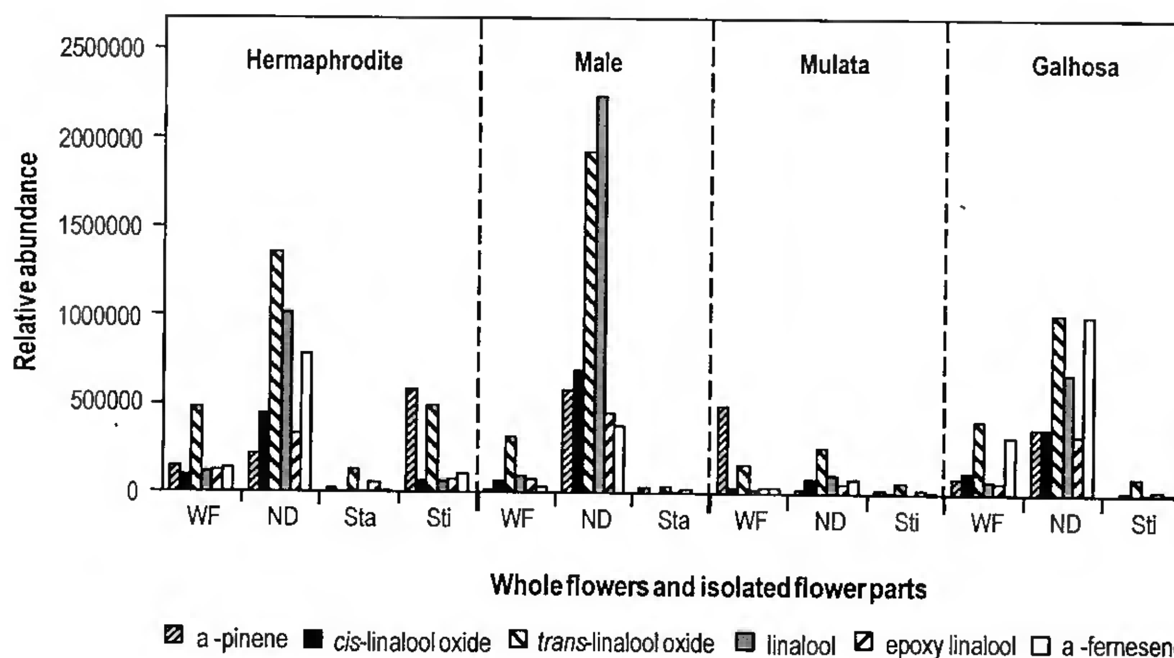

Figure 3.5. Abundance of α-pinene, *cis*-linalool oxide, *trans*-linalool oxide, linalool, epoxy linalool and α-fernesene found in the volatiles of whole flowers (WF) and isolated flower parts (ND: nectarial disk; Sta: stamens; Sti: stigma) from field grown male, hermaphrodite and female carob trees by HS-SPME-GC/MS.

Differences were observed in the number and abundance of the compounds emitted by whole flowers, between the two female cultivars (Table 3.2 and Fig. 3.5). In Mulata, 23 compounds were identified, and more than half of the fragrance of whole flowers consisted

of α-pinene (60.0%), a highly volatile compound; other important compounds were *trans*-linalool oxide (10.4%) and β-pinene (5.2%) (Table 3.2 and Figs. 3.5 and 3.6).

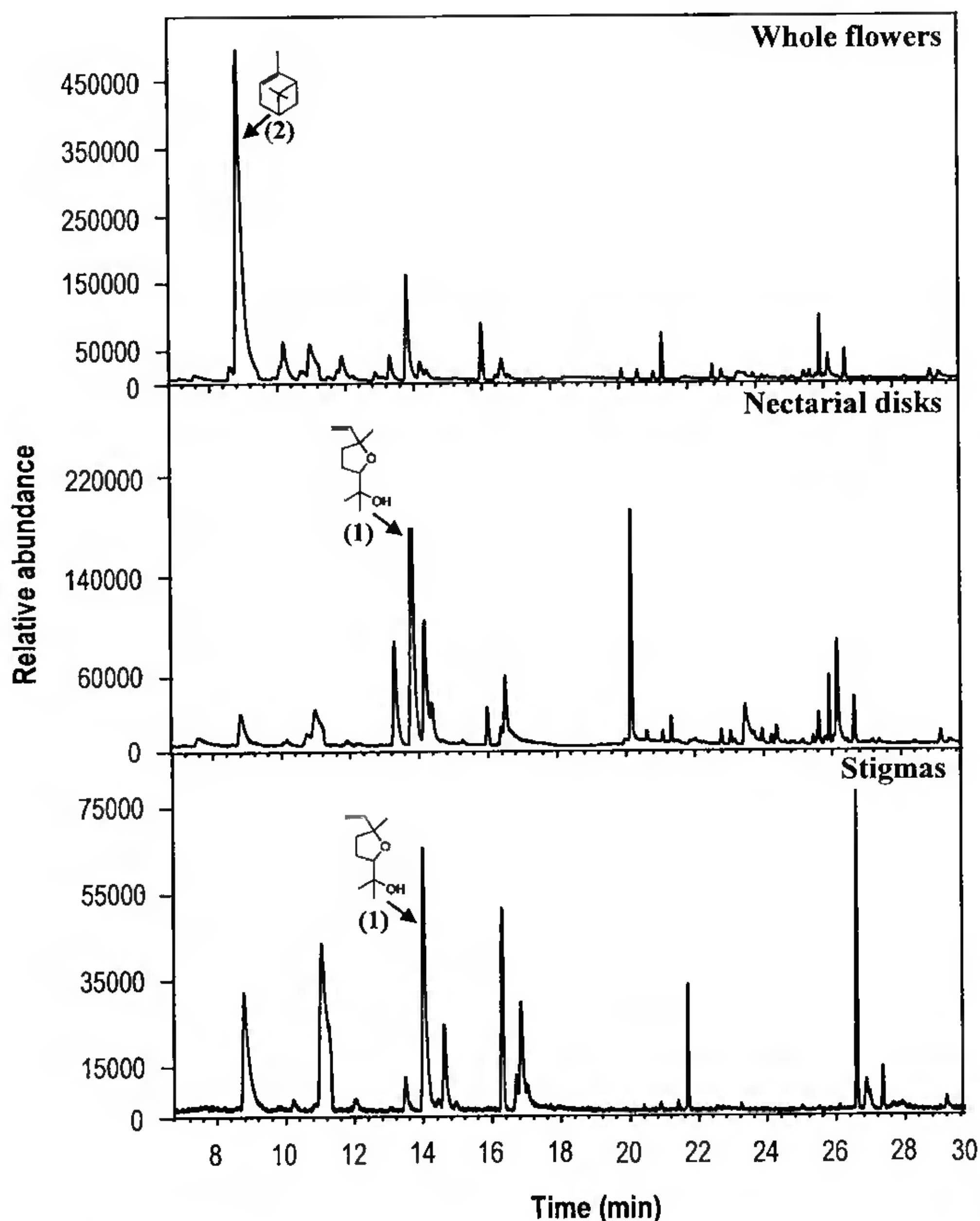

Figure 3.6. Total ion chromatograms of the headspace of whole flowers and isolated flower parts of Mulata at developmental stage IV. Peaks are1) *trans*-linalool oxide 2) α-pinene.

Galhosa had a lower content of volatile compounds and showed a different composition, dominated by *trans*-linalool oxide (24.4%), limonene (13.1%) α-farnesene (12.7%) and α-pinene (10.2%) (Table 3.2 and Figs. 3.5 and 3.6).

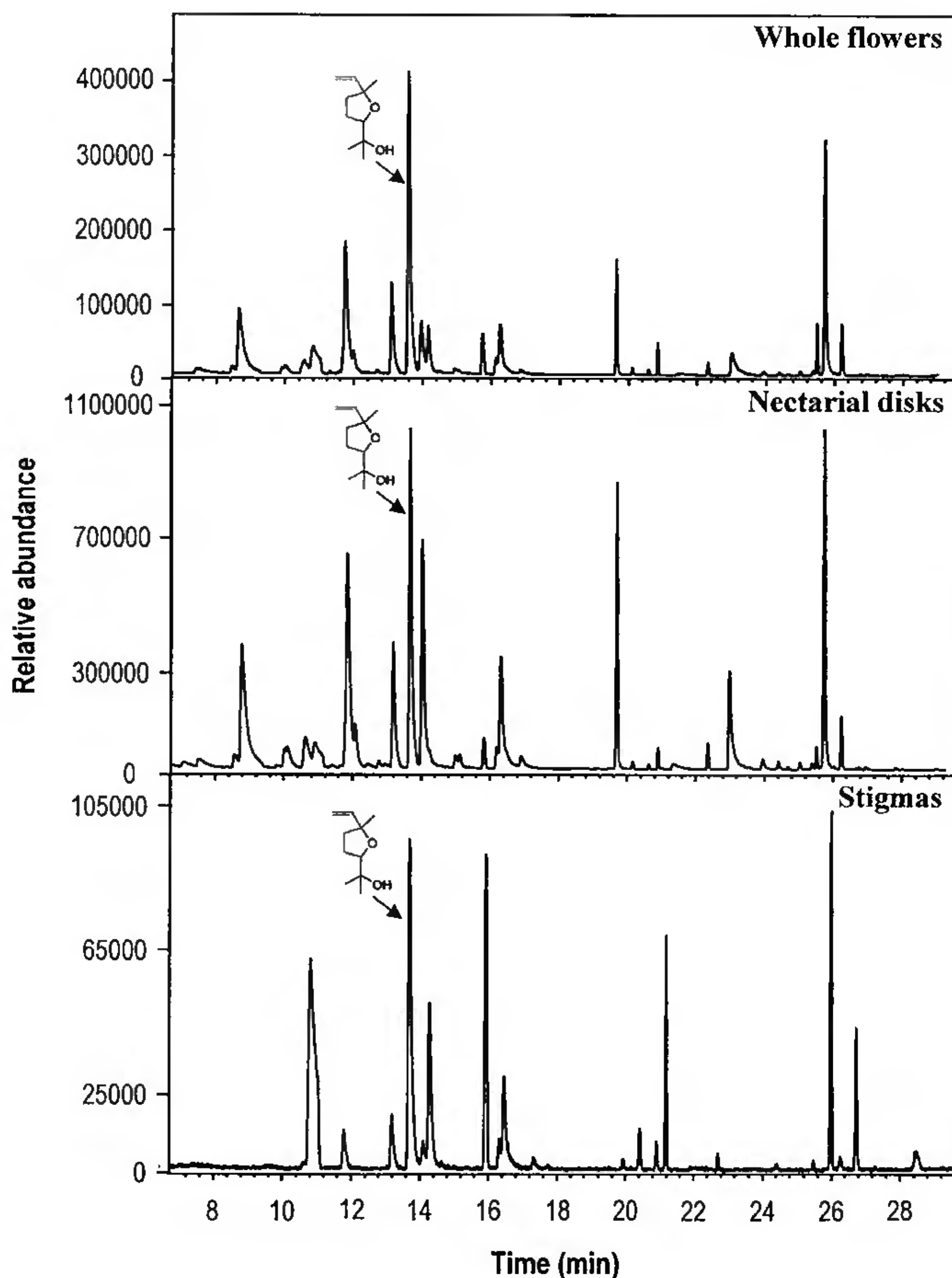

Figure 3.7. Total ion chromatograms of headspace of whole flowers and isolated flower parts of Galhosa at developmental stage IV. Peak is *trans*-linalool oxide.

The number, structure and concentration of volatile chemicals emitted by individual flowers vary substantially among species (Dobson 1994, Dudareva and Pichersky 2000). Furthermore, genetic variation within a species may also modify the amount and type of compounds in the odour profile (Wright et al. 2002), which may explain the differences in the volatile composition between the two female cultivars studied. Intraspecific variation in floral scent chemistry has been reported in other species namely *Platanthera bifolia* and *P. chlorantha* (Tollsten and Bergstrom 1993), *Clarkia brewerii* (Nam et al. 1999), *Antirhinnum majus* (Dudareva et al. 2000), and *Tanacetum vulgare* (Keskitalo et al. 2001). There may be many reasons for intraspecific chemical variation, namely the adaptation to different pollinator species, introgression effects involved in hybridization events, random genetic drift, adaptations to disrupt learning processes in the pollinators among deceptive (non-rewarding) flowers, etc. (Tollsten and Bergström 1993, Barkman et al. 1997).

The female Portuguese cultivars Galhosa and Mulata are economical and ecologically interesting (Martins-Loução and Brito de Carvalho 1989, Batlle and Tous 1997), and Mulata, in particular, is considered a very productive cultivar (Batlle and Tous 1997). Pollination is a critical step for the successful production of fruits and seeds, and thus, lack of an efficient pollination can lead to low crop yields. Different scent compounds or chemical compositions may attract different pollinator species, which may cause reproductive success to vary among scent-types, and the types will encounter different selective pressures from pollinators (Azuma et al. 2001). The higher amounts of volatiles identified in flowers from cultivar Mulata can contribute to a more efficient pollination and thus, to a higher production. However, this hypothesis needs further research.

Both quantitative and qualitative composition of flower volatiles observed in this study differed from the obtained in earlier findings (*vide* section 3.3.1). Contrary to the previous observations it was observed that the female flowers had a higher diversity of volatiles, than the males and hermaphrodites, and linalool was found in much lower abundance, while the amounts of *trans*-linalool oxide were comparatively higher (Tables 3.1 and 3.2).

Furthermore, besides linalool and *trans*-linalool oxide, other derivates of that acyclic monoterpene alcohol were identified, namely *cis*-linalool oxide and epoxy linalool (Table 3.2). Both studies were based on samplings taken in the field, therefore, environmental conditions, such as temperature, humidity, light, and time of day may, to some extent, have

affected the total amount and chemical composition of the floral scents (Azuma et al. 2001). Furthermore, those differences could be ascribed to the different germplasm tested, sampling period, or physiological status of the trees (Siani et al. 2002).

HS-SPME-CGC/MSD analysis allowed the detection, for the first time, the existence of spatial fragrance patterns within the flowers of carob (Table 3.2 and Figs. 3.5-3.9). The relative abundances of the major volatiles identified on whole flowers and isolated floral organs of carob are represented on Fig. 3.6.

There were differences in both the qualitative profile and the relative abundances in the volatiles emitted by the different floral parts (Table 3.3 and Fig. 3.6). In spite of the sexual organs accounted higher percentages to the total flower weight compared to the nectarial disks (*vide* chapter II), those structures seemed to be the major source of emission of the volatile compounds (Table 3.2 and Fig. 3.5). In fact, generally the fragrance from the whole flowers seemed to depend largely on the volatiles emitted from nectarial disks, which included among their major volatiles all the main compounds emitted by whole flowers (Table 3.2 and Fig. 3.5). Furthermore, the relative abundances of the major volatiles emitted by the nectarial disks were higher in the males, followed by the hermaphrodites, Galhosa and Mulata (Fig. 3.5).

In the hermaphrodite flowers the fragrance of the stamens consisted almost exclusively of monoterpenes and only one sesquiterpene (α–farnesene) was detected (Table 3.2). Almost half of the fragrance from the stamens consisted of *trans*-linalool oxide (46.7%) (Table 3.2, Figs. 3.6 and 3.8). Other important compounds were epoxy linalool (22.4%) and α-pinene (14.1%) (Table 3.2 and Fig. 3.5). In the nectarial disks the main compounds were *trans*-linalool oxide (24.5%), linalool (17.2%), *cis*-linalool oxide (8.6%), α-pinene (8.5%) and epoxy linalool (8.4%), while in the stigma, α-pinene dominated the headspace (41.9), together with *trans*-β-ocimene (9.6%) and *trans*-linalool oxide (18.9%) (Table 3.2 and Figs. 3.5 and 3.8). The nectarial disks and the stigma produced a higher number and abundance of volatiles, than the stamens (Table 3.2 and Fig. 3.5). Similar results were previously reported for other species (MacTavish and Menary 1997), however Flamini et al. (2003) observed the opposite.

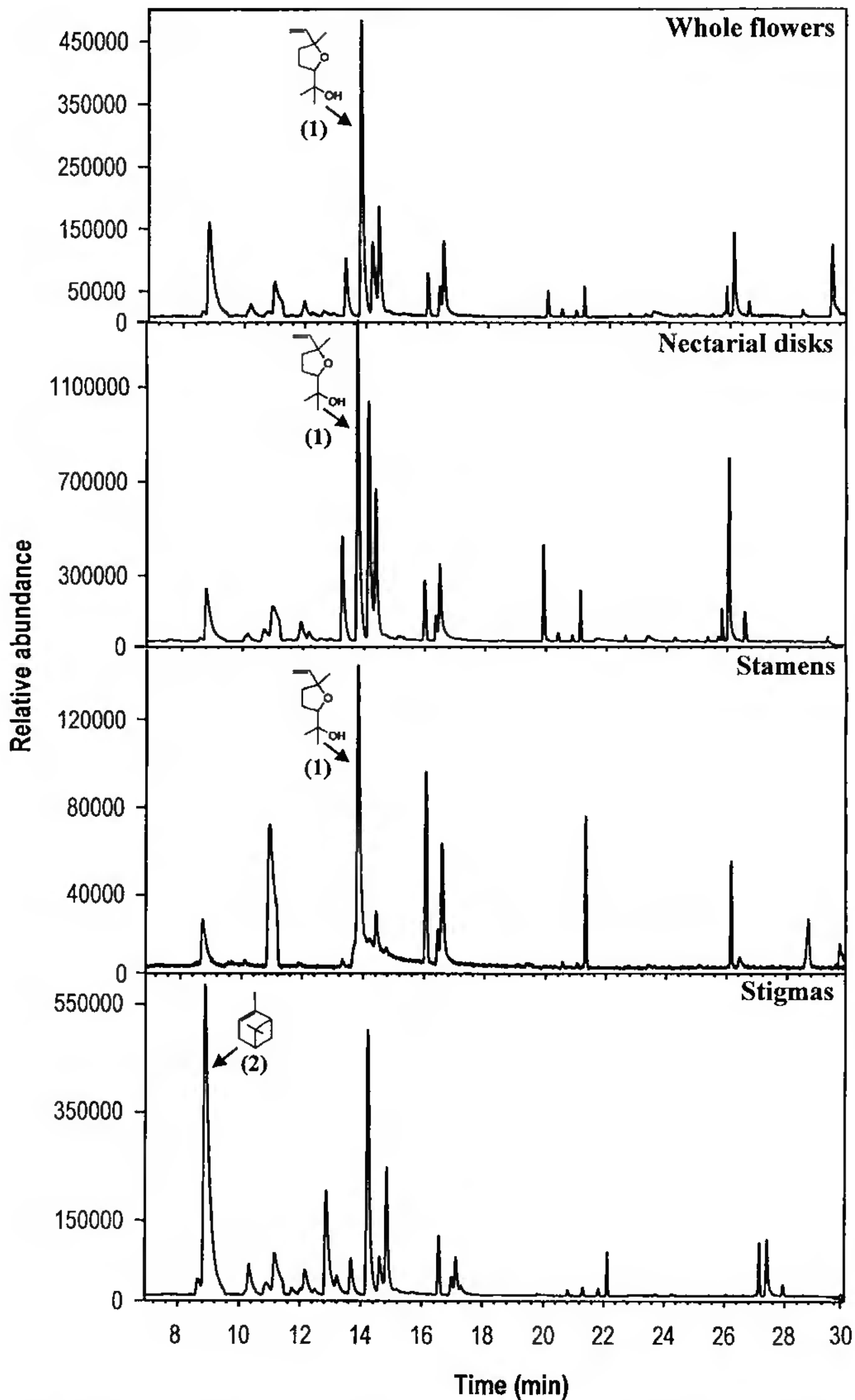

Figure 3.8. Total ion chromatograms of the headspace of whole flowers and isolated floral parts of hermaphrodites at developmental stage III. Peaks are 1) *trans*-linalool oxide and 2) α-pinene.

Similar to the observed in the hermaphrodites, in male and in both female cultivars the nectarial disks showed a richer blend of compounds compared to both stamens and stigma (Table 3.2). Noteworthy in the male flowers is the fact that only four monoterpene compounds were positively identified on the headspace of the stamens, representing 49.2% of the total constituents, dominated by α-pinene (21.4%), *trans*-linalool oxide (15.3%) and epoxy linalool (10.1%) (Table 3.2, Figs. 3.5 and 3.9).

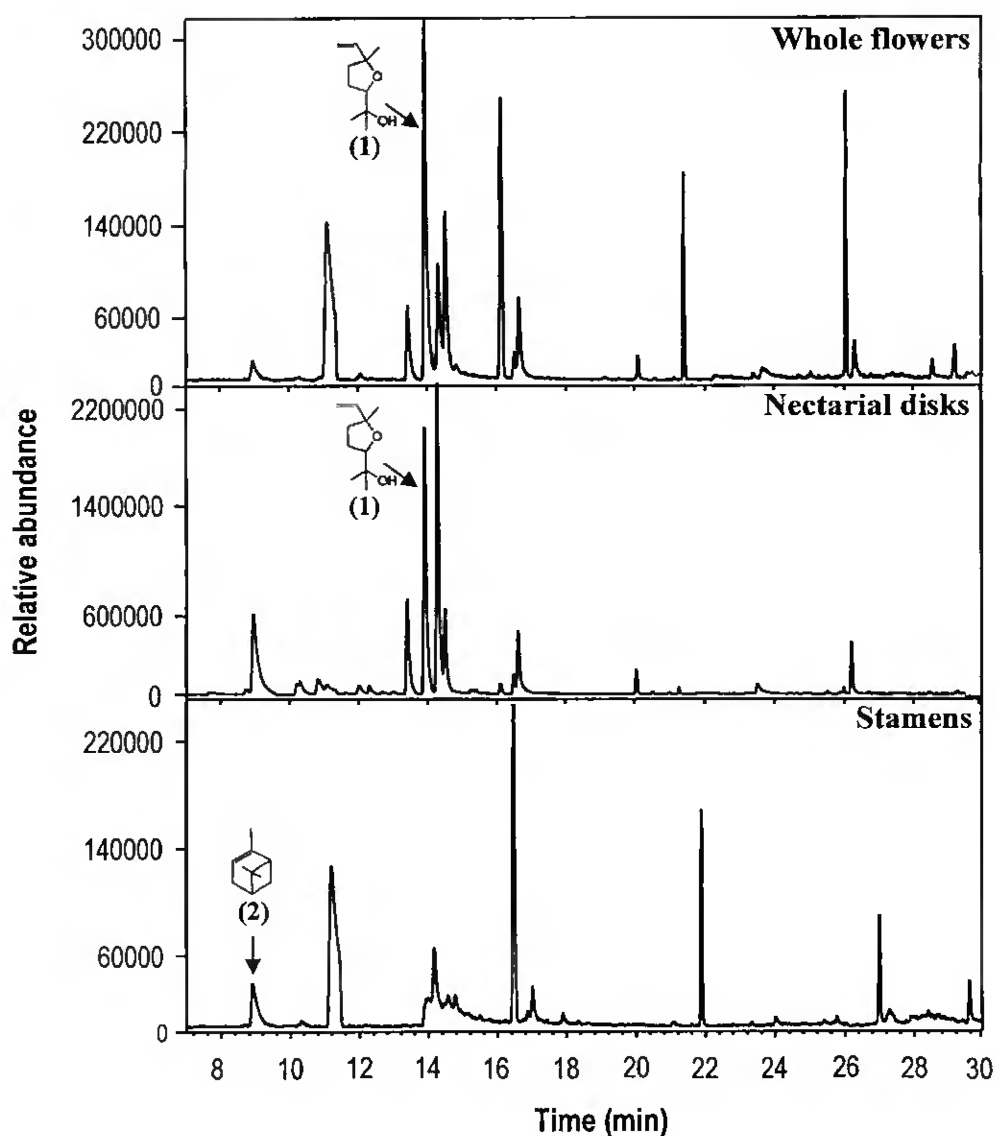

Figure 3.9. Total ion chromatograms of the headspace of whole flowers and isolated floral parts of males at developmental stage III. Peaks are 1) *trans*-linalool oxide and 2) α-pinene.

Usually all floral organs contain volatiles typical of that species (MacTavish and Menary 1997). In our work, the dominant volatiles (linalool, linalool derivates and α-pinene) where found in all floral organs, except in the stigma of Galhosa, that did not include α-pinene in its scent. However, quantitative differences between attractive, non-food structures (perianth) and attractive food sources such as the androecium and gynoecium are common (Dobson et al. 1990). Generally, the scent composition emitted by female organs is markedly different from emitted by the stamens (Pichersky et al. 1994).

There were remarkable differences, between the female cultivars, in the spatial fragrance patterns within the flowers (Table 3.2 and Fig. 3.5). In spite of Mulata emitted a higher number of volatiles compared to Galhosa, the abundance of the principal volatiles was lower (Table 3.2 and Fig. 3.5). In Mulata, the emission of its major volatile compound (α-pinene) depended largely on the emission by the stigma (22.6%). In Galhosa the volatiles profile of whole flowers and nectarial disks were very similar, except for γ-terpiene and β-guaiene, only present in nectarial disks, in very low amounts (0.3 and 0.2%, receptively) (Table 3.2). This may indicate that in Galhosa, the scent of the nectarial disks resembled more the fragrance of the whole flowers, than the gynoecium.

The higher amounts of volatiles identified on the nectarial disks support previous observations (*vide* section 3.3.1), where the high amounts of compounds present in the volatiles from developmental stage III in male and hermaphrodite flowers, and in the stage IV in the female ones, where attributed to the secretion of nectar by those floral organs. Spatial scent patterns within a flower may function like visual patterns. Thus, differences in the strength and/or quality of emitted volatiles between floral organs may serve as guides to insects, assisting them in finding food rewards or leading them to position themselves suitably on a flower to effective pollination (Dobson et al. 1990).

We observed the existence of organ specific synthesis of certain compounds: in hermaphrodite and male flowers only one component of the monoterpenes hydrocarbons fraction (α-pinene) is emitted by the stamens (Table 3.2). In the males the stamens do not produce sesquiterpenes (Table 3.2). Similarly, in Galhosa, a high number of the monoterpenes hydrocarbons are emitted by the nectarial disks, but only one is present in the headspace of the stigma (Table 3.2). This may be due to the fact that the pathway(s) for

synthesis and pattern of accumulation of terpenes differs considerably between the floral organs (Dudareva and Pichersky 2000).

3.4. Conclusions

HS-SPME coupled with CGC-MSD is a rapid and simple method that, for the first time, enabled the extraction and identification of the volatile compounds emitted from the whole fresh flowers and from different fresh flower parts of the three sexual types of carob. In the first part of this chapter, it was observed that both sexual type and stage of flower development affected the composition of volatiles of carob flowers, which shown to be complex mixtures of several components. Large amounts of monoterpenes and sesquiterpenes are responsible for the characteristic scent of carob flowers, and stages III and IV showed a higher number of volatiles. Linalool and *trans*-linalool oxide are the dominant volatiles, being present at higher abundance in male flowers. In male and hermaphrodite flowers the levels of those compounds peaked at stage III, and in the female at stage IV.

When the volatile compounds emitted by the different floral parts were studied, it was observed that both the qualitative profile and the relative abundances of the volatiles of whole flowers and isolated floral parts were different between the sexual types and cultivars. The female flowers had a higher diversity of volatiles, than the males and hermaphrodites, but a lower abundance of the major volatiles. Mulata had a higher number of volatile compounds than Galhosa, however, it had a lower abundance of the main compounds. In the hermaphrodite flowers, the nectarial disks and the stigma produced a higher number of volatiles, than the stamens. In male and in both female cultivars, the nectarial disks showed a richer blend of compounds compared to the stamens and stigma. The stamens emitted basically high volatile compounds (monoterpenes). The results of this work suggest that the fragrance from the whole flowers depend mainly on the volatiles emitted from nectarial disks than from the sexual organs.

The reason for the chemical variation observed in floral scent between the sexual types and female cultivars of carob is unknown. Studies on the effects of microhabitat and environmental conditions, and mechanism of floral scent emission, are required. In

addition, field observations of natural pollinators and bioassays using scent compounds are needed, since variation in floral scent chemistry may be a pre-requisite for adaptive evolution and specialization. It is also needed to access if the different scent compounds and chemical profiles found in the sexual types and female cultivars of carob are equally effective in attracting pollinators.

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CHAPTER IV

FLORAL ANALYSIS AND SEASONAL DYNAMICS OF MINERAL LEVELS IN LEAVES OF CAROB TREE

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4.1. Introduction

In carob, as in other evergreen species, growth in autumn and winter (from October until April in the Mediterranean basin) is minor or nil. From April to July, a steady flush of axillary and apical vegetative buds emerges (Martins-Loução and Brito de Carvalho 1989, Correia and Martins-Loução 1993, Correia 1996). Vegetative growth is normally uniform across most branches and may last until September, depending on the local climatic conditions.

During spring and summer, several phenological events occur simultaneously in carob: branch and leaf growth, enlargement of fruits set during the previous season (Martins-Loução and Brito de Carvalho 1989), and probably, floral induction (Correia 1996). Flower buds and first inflorescences appear in late June, and a clear flowering occurs in late August and September, when pollination occurs and fruit set is established just after pollination. Fruit enlargement begins in the following April and most pods are ready to be harvested in August. As a result, in mature trees there is a strong sink competition for resources from April to August, with biased impacts on vegetative and reproductive growth (Correia and Martins-Loução 2005). Therefore, this species, with long-lived leaves and an extended flowering period has to evolve mechanisms to optimise the use of nutrients for reproduction (Correia and Martins-Loução 1993).

It was mainly to the credit of Justus von Liebig (1803-1873) that the scattered information concerning the importance of mineral elements for plant growth was compiled and summarized. To be considered essential for plant growth, a mineral element has to fulfil three criteria (Marschner 1995): 1) a given plant must be enable to complete its life cycle in the absence of the mineral element; 2) the function of the element must not be replaceable by another mineral element; and 3) the element must be directly involved in plant metabolism, or must be required for a distinct metabolic step such as an enzyme reaction. According to this definition, the mineral elements that compensate for the toxic effects of other elements, or which simply replace mineral nutrients in some of their less specific functions, such as the maintenance of osmotic pressure, are not essential, but can be described as 'beneficial' elements (Marschner 1995).

In addition to carbon (C), hydrogen (H) and oxygen (O), the total number of mineral elements generally accepted as essential for the growth of plants now stands at 17, and

only 13 are required by most higher plants, although the known requirements for chlorine is yet restricted to a limited number of plant species (Marschner 1995). Depending on how great the growth requirement for a given nutrient, it is referred to as either a macro (principal or secondary) or a micronutrient. Nitrogen (N), phosphorous (P) and potassium (K) are considered the principal macronutrients, while calcium (Ca), magnesium (Mg) and sulphur (S) are the secondary ones. The micronutrients include the ones forming cationic forms: iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), and those present in the anionic form: boron (B), molybdenium (Mo) and chlorine (Cl).

The biological function, interactions (synergism and antagonism) and range values in leaves of some macro and micronutrients considered normal for most plant species are summarized on Table 4.1.

Most micronutrients are predominantly constituents of enzyme molecules and are essential only in small amounts (Marschner 1995). In contrast, the macronutrients are either constituents of organic compounds, such as proteins and nucleic acids, or act as osmotica (Marschner 1995). The main functions of mineral nutrients such as nitrogen, sulphur, and phosphorous that serve as constituents of proteins and nucleic acids are quite evident and readily described (Marschner 1995). Other mineral nutrients, such as magnesium and the micronutrients (except chlorine) may function as constituents of organic structures, predominantly of enzyme molecules, where they are either directly or indirectly involved in the catalytic function of the enzymes (Marschner 1995). Naturally, because of their low concentrations, micronutrients do not play a direct role in either osmoregulation in the maintenance of electrochemical equilibria (Marschner 1995).

The mineral nutrition of evergreen species in the Mediterranean basin has not been fully investigated, although some work has been done in different species of *Quercus* (Sabaté and Gracia 1994, Oliveira et al. 1996, Robert et al. 1996) and *Olea* (Jordão et al. 1994, Bouranis et al. 1999, Férnandez-Escobar et al. 1999).

Leaf-nutrient analysis is the established method to diagnose tree nutritional status and to determine fertiliser requirements (Correia and Martins-Loução 1996, Bouranis et al. 2001, Correia and Martins-Loução 2005). This technique integrates all the factors that might influence soil nutrient availability and plant uptake, and pinpoints the nutritional balance of the plant at the time of sampling (Pestana et al. 2004).

Table 4.1. Biological function, interactions (synergism and antagonism) and range values in leaves (RV) for the studied macro and micronutrients (Davis 1994, Marschner 1995).

	Biological function	Interactions	RV[1]
Macronutrients			
N	Structural element of aminoacids, proteins, apoenzymes, nucleic acids, cytochromes and chlorophyll. Important on the growing phase of plant development.	Synergetic relation with P, antagonic with K.	1.5 - 6.0
P	Structural element of enzymes, ATP, nucleic acids and phospholipids. Control function in photosynthesis and carbohydrate metabolism. Involved in the phytohormone balance.	Synergetic relation with N, antagonic with Zn.	0.15 - 1.0
K	Key role in osmoregulation. Involved in the processes of enzyme activation, protein synthesis, photosynthesis, cell extension, stomatal movement, phloem transport and in the cation-anion balance.	Synergetic relation with Fe, antagonic with Mg and Ca.	1.0 - 5.0
Ca	Structural element of cell wall and supporting tissues. Involved in cell division and extension. Essential for pollen tube growth. Low mobility from cell to cell and in the phloem.	Antagonic with K and Mg.	0.2 - 3.0
Mg	Central atom of the chlorophyll molecule. Involved in protein synthesis, cellular pH control, enzyme activation and energy transfer.	Antagonic with K. Its assimilation is inhibited by High levels of NH_4^+, K or Ca.	0.15 - 1.0
Micronutrients			
Fe	Important role in the biosynthesis of heme coenzymes and chlorophyll.	Fe solubility decreases with increasing concentrations of P. Antagonic with K.	10 - 1000
Mn	Activates and modulates some enzymatic systems.	-	15 - 50
Zn	Involved in the same enzymatic processes than Mn and Mg.	Antagonic with P.	15 - 50
Cu	Present in different forms of proteins, involved in enzymatic processes, and in carbohydrate and nitrogen metabolism.	-	

[1]Macronutrientes: g kg^{-1} of dry weight (DW), micronutrients: mg kg^{-1} of DW.

However, the leaf-nutrient analysis has limitations, such as dilution effects and the fact that the sampling date recommended for fruit trees is late in the growing season, generally very close to harvest. At this point is no longer possible to correct nutritional disorders in time to avoid decreases in yield (Sanz and Montañés 1995).

Due to the drawbacks associated with leaf-nutrient analysis new approaches, like flower analysis, have been developed for high profitable crops, namely orange (Pestana et al. 2004), pear (Sanz et al. 1994), peach (Sanz and Montañés 1995, Igartua et al. 2000) and almond (Bouranis et al. 2001).

The use of leaf analysis to guide carob fertilisation is not frequent, which may be caused, in part, by the lack of information on nutritional requirements of carob trees, and also by the limitations of leaf analysis when applied to particular nutritional imbalances (Sanz et al. 1993, Sanz and Montañés 1995, Pestana et al. 2004).

Since the mineral composition of flowers at full bloom can be related to the nutrient concentration in leaves taken later in the season, Sanz et al. (1993) proposed methods based on the mineral composition of flowers to determine the nutritional status of crops at an early stage. Since then, flower analysis has been developed for many deciduous fruit trees, such as pear (Sanz et al. 1994), peach (Sanz and Montañés 1995, Igartua et al. 2000), orange (Pestana et al. 2001) and almond (Bouranis et al. 2001).

In carob, the nutrient concentration of the inflorescences has been rarely studied (Cruz et al. 1988, Cabrita and Martins-Loução 1991) and therefore, nutrient demands of reproductive structures are not fully understood. Nevertheless, a positive correlation between leaf N concentration, the number of female inflorescences (Correia and Martins-Loução 1993) and leaf nutrient status and yield (Correia et al. 2002), was previously reported.

As referred earlier (*vide* chapter II) individual carob trees may be male (with inflorescences carrying only staminate flowers), female (with inflorescences carrying only pistilate flowers) and hermaphrodite (with some inflorescences carrying staminate flowers and others with pistilate flowers, or with inflorescences carrying hermaphroditic flowers), which led some authors to consider trioecy as one of the most outstanding biological features of this species (Retana et al. 1994). However, there are no data on seasonal nutritional dynamics and flower mineral concentrations between the three sexual types of trees. In this species flowering may reflect adjustments in response to past reproduction, or

may be controlled by resources other than light (Correia 1996, Batlle and Tous 1997). Furthermore, the total reproductive effort of a monoecious or dioecious plant is known to be different (Mauseth 1991).

In this chapter, the changes in nutrient concentrations of monoecious and dioecious trees during different phenological stages were compared. Furthermore, the nutrient content of flowers and the differences in nutrient concentrations between flowers and leaves at full bloom were also assessed. The knowledge of the seasonal variations in the mineral content of leaves, and the partitioning of nutrients between vegetative and reproductive organs can be helpful for understanding the needs of fertilisation to cover the costs of reproduction within the three sexual types of carob tree.

4.2. Materials and methods

4.2.1. *Study site*

This work was conducted along the year of 2003 on the male, female and hermaphrodite trees (three trees per sexual type), referred earlier on chapter II.

4.2.2. *Sampling and analyses*

Fully expanded leaves (Diamantoglou and Mitrakos 1981) were randomly collected from the middle third of the branches in all canopy orientations on four different phenological stages: vegetative rest (VR, early February), beginning of flowering (BF, late June), full bloom (FB, late August) and end of flowering (EF, early November). Flowering occurs during a relatively long time period, and it is not possible to specify full blooming with accuracy. To overcome that limitation, we set the harvest dates during the flowering period (BF, FB and EF) using the six developmental stages (0 to V) described earlier (*vide* chapter II). BF was when the first flowers reached stage I (the sepals open and anthers or pistils become visible); FB when at least 50% of the flowers had reached stage II (in male flowers this is when the anthers are pushed upwards in the flower by filament extension, in female flowers the carpel length increases and the nectarial disk becomes visible, and in the hermaphrodites a combination of both phenomenon is observed); and EF was when at least

50% of the flowers had reached stages III to V (the stages of nectar secretion to drying of stamens or swelling of carpels after fertilisation). Flower samples were collected at FB, simultaneously with leaves.

Leaves were washed with tap water, then with distilled water containing a non-ionic detergent, followed by immersion in a acid solution (10 mmol HCl l^{-1}) and finally rinsed three times with distilled water. Flowers were washed with distilled water only. Leaves and flowers were oven-dried at 60°C for 48h and ground for chemical analysis.

Standardized procedures (AOAC 1990) were used to quantify nutrient concentrations. Nitrogen concentration was determined by the Kjeldahl method, phosphorous was analysed spectrophotometrically, and K, Ca, Mg, Fe and Zn by atomic absorption spectroscopy (Pye Unicam, Cambridge, UK).

4.2.3. *Statistical analysis*

For each phenological stage and sexual type three trees were selected, and 30-40 mature leaves and 30 flowers were collected per tree. The values obtained for each tree were considered independent replications, and the data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 11.0, SPSS INC). Significance between means was tested by the Duncan's New Multiple Range Test (P=0.05).

4.3. Results and discussion

4.3.1. *Mineral composition of flowers and leaves*

Trioecy is one of the most outstanding biological features of carob, however, there is little information about differences in flowering phenology between its different sexual types (Retana et al. 1994). In this work we observed that there were no significant differences ($P \geq 0.05$) between the males, females and hermaphrodites in either the mean number of inflorescences per branch or the mean number of flowers per inflorescence (*vide* chapter II, Table 2.1) which led us to presume that the differences on the levels and allocation of nutrients could be mainly attributed to genotypic differences.

In this work it was studied the nutrients used in a model previously developed that estimates yield in carob (Correia et al. 2002). Although sulphur (S) is a secondary macronutrient, we did not include it in our studies since carob is apparently indifferent to its concentrations (Martins-Loução pers comm.).

In flowers at full bloom, the sexual type of the tree significantly affected the concentrations of N ($P<0.01$), P ($P<0.05$), Mn ($P<0.01$) and Zn ($P<0.001$) (Table 4.2).

Table 4.2. Effect of sexual type (male, female and hermaphrodite) and phenological stage on macro (g kg^{-1} dry weight) and micronutrient concentration (mg kg^{-1} dry weight) of carob tree leaves and flowers at full bloom (two-way ANOVA).

	Source of variation	Significance level	
		Leaves	Flowers
Macronutrients			
N	Sexual type	0.003	0.004
	Phenological stage	0.082	—
	Sexual type × phenological stage	0.823	—
P	Sexual type	0.133	0.018
	Phenological stage	0.000	—
	Sexual type × phenological stage	0.089	—
K	Sexual type	0.002	0.088
	Phenological stage	0.002	—
	Sexual type × phenological stage	0.004	—
Ca	Sexual type	0.000	0.270
	Phenological stage	0.000	—
	Sexual type × phenological stage	0.443	—
Mg	Sexual type	0.000	0.254
	Phenological stage	0.001	—
	Sexual type × phenological stage	0.110	—
Micronutrients			
Fe	Sexual type	0.532	0.973
	Phenological stage	0.344	—
	Sexual type × phenological stage	0.388	—
Mn	Sexual type	0.000	0.004
	Phenological stage	0.368	—
	Sexual type × phenological stage	0.580	—
Zn	Sexual type	0.239	0.000
	Phenological stage	0.008	—
	Sexual type × phenological stage	0.308	—
Cu	Sexual type	0.009	0.311
	Phenological stage	0.000	—
	Sexual type × phenological stage	0.000	—

The male flowers had a higher concentration of N, P and Zn and a lower concentration of Mn, than the female ones (Table 4.3).

Comparisons among flowers and leaves sampled at full bloom showed significant differences for most of the nutrients, and except for N in females, flowers had a higher concentration of the principal macronutrients (N, P and K) (Table 4.3). The high levels of N in flowers seems to be in agreement with its importance during flower development (Marschner 1995), and with the role of carob inflorescences as active sinks of N (Cruz et al. 1988, Correia and Martins-Loução 1993, Correia et al. 2002). Similar results were obtained in orange (Pestana et al. 2001), and in almond trees (Bouranis et al. 2001).

Table 4.3. Nutritional concentrations in flowers and leaves sampled at full bloom.

		Macronutrients (g kg^{-1})					Micronutrients (mg kg^{-1})			
		N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
Male	**Fl**	*20.7 a	*2.2 a	*14.9	4.5	1.3	30.9	6.2 ab	*24.3 a	*9.6
	L	13.3	0.7	7.1	*14.3	1.9	*63.5	*42.7	8.4	6.5
Female	**Fl**	18.5 b	*1.8 b	*14.1	6.1	1.2	29.0	8.0 a	12.2 b	10.4
	L	16.9	0.9	8.2	*11.8	*2.0	47.4	*20.2	5.5	7.4
Herma.	**Fl**	*22.1 a	*2.1ab	*17.1	3.8	1.3	30.2	1.5 b	*17.2 b	7.8
	L	13.1	0.8	8.4	*9.7	1.2	*74.7	*12.8	6.4	7.7

For each nutrient, statistical analysis was made between flowers (Fl) and leaves (L) for the same sexual type (*) and between flowers of different sexual types (lower case letters). Values followed by * or by different letters are significantly different at $P< 0.05$ (one-way ANOVA, Duncan's New Multiple Range Test).

The higher concentration of P in the flowers (Table 4.3) suggests a constant translocation from the sources- leaves, to the developing sinks- flowers, and in the case of the female and hermaphrodite trees, to fruits, which are also developing in the latest stages of flower development (*vide* chapter II), and confirms the negative relationship between yield and leaf P concentration observed by Correia et al. (2002).

Potassium was the second most abundant macronutrient present in flowers, and generally its concentration was higher in flowers than in leaves (Table 4.3). Similar results were observed in the same species (Cruz et al. 1988), and in orange trees (Pestana et al. 2001). Potassium is the most important inorganic solute in plants, and probably the only

mineral nutrient that is not a constituent of organic structures (Marschner 1995). Its function is mainly in osmoregulation (e.g. vacuoles), the maintenance of electrochemical equilibria in cells and their compartments and the regulation of enzyme activities (Marschner 1995). On the other hand, carob developing fruits show high concentrations of K (Martins-Loução and Brito de Carvalho 1989) suggesting a movement of that element towards these active sinks, which may be responsible for the correlation between leaf K concentration and yield variation (Correia et al. 2002).

Leaves were richer in Ca than flowers (Table 4.3), which could be related with the essential structural functions of this element, namely the regulation of membrane permeability and the strengthening of cell walls (Marschner 1995). The static behaviour of Ca and its importance in vegetative rather than in reproductive growth was already reported (Correia et al. 2002).

In females, the leaf content of Mg was statistically superior than in flowers (Table 4.3). Iron was the most abundant micronutrient present in carob flowers and leaves ($P < 0.05$), and in males and hermaphrodites its concentration in flowers was lower than in leaves (Table 4.3). These results agree with those reported in orange trees (Pestana et al. 2001), but contrast with the ones obtained by Sanz and Montañés (1995) in peach and pear, and may indicate that flowers of males and hermaphrodite trees do not behave as active sinks for Fe. Except for Cu, males and hermaphrodites exhibited a similar trend of micronutrients accumulation, with leaves being richer in Fe and Mn, and poorer in Zn (Table 4.3). In the females, we observed no differences in the micronutrient concentrations between leaves and flowers, except for Mn, which was highly concentrated on leaves (Table 4.3).

4.3.2. *Seasonal dynamics of mineral levels in leaves*

The concentrations of N, P, K, Zn and Fe in leaves of female trees were similar to those reported by other authors (Cruz et al. 1988, Correia et al. 2002), and to the ones needed for maximum fruit yield, except for P and Mn, which were near the values for the lowest production (Correia et al. 2002). Both seasonal and intraspecific variability were observed

in several nutrients in leaves, and a combined effect of the sexual type and phenological stage was also apparent, for K and Cu (Table 4.2).

Comparing the changes in nutrient concentrations between the three sexual types, we observe that, for the same phenological stage, the concentrations of nutrients in leaves were significantly different between the sexual type, with males and females accumulating more nutrients than the hermaphrodites, although in some cases hermaphrodites and females behaved similarly (Tables 4.4 and 4.5). Those differences were more evident during the period of VR and in the BF, when the hermaphrodites accumulated lower concentrations of most of the nutrients (Tables 4.4 and 4.5). During FB and EF, hermaphrodites accumulated more Fe than males and females (Table 4.5). In leaves, Mn presented the most consistent variation with the sexual type of tree, with males having a higher concentration of that nutrient in all the sampling dates (Table 4.5). Manganese is essential for the development of seeds (Marschner 1995), which may explain the lower amounts of that element in leaves of females and hermaphrodites, possibly due to the translocation towards the developing fruits.

Regardless of the phenological stage, leaf N concentration was significantly affected by the sexual type ($P<0.01$) (Table 4.2), with the females accumulating more N in leaves than males and hermaphrodites. However, N was the least variable nutrient among the different sexual types, with almost steady values throughout the year (Table 4.3). Since no significant decrease of leaf N concentration during phenological events was detected, the N allocated to flowers may derive from trunk and roots rather than from a retranslocation from leaves. These results support the importance of N supply for yield increment (Correia and Martins-Loução 1993) and the maintenance of tree nutritional status needed to guarantee both vegetative and reproductive growth (Correia 1996, Correia and Martins-Loução 1997, Correia and Martins-Loução 2005). In carob the number of inflorescences and thus, carob yield, is affected by the time and the level of N application (Correia and Martins-Loução 1996). Without any fertilization the investment of N towards inflorescence development is ensured by N remobilization, an efficient N uptake from the soil, or both. In the studied orchard root N uptake was able to supply shoot sink demands in order to buffer significant leaf N seasonal variations, which is observed under more unfavourable edaphic conditions (Correia and Martins-Loução 1997).

In the male trees, an accumulation of P in leaves was observed in VR and in the end of the flowering period, with minimum levels in the beginning of the flowering period and in full bloom (Table 4.4). An increase in the P contents was also observed from FB o the end of flowering in the females and the hermaphrodites (Table 4.4), thus suggesting a mobilisation of that element from leaves to support the inflorescences development. The levels of K in leaves from male trees reached a maximum level in the BF, decreasing thereafter, while in the opposite was true for the hermaphrodites (Table 4.4).

The leaf Ca concentration followed the same variation pattern in the males and females, dropping from VR to BF, and remaining constant thereafter, while in the hermaphrodites a peak was observed in the EF (Table 4.4). Calcium has low mobility within the plant, and its significant decrease in leaves from males and females from VR to BF (Table 4.3) may be due to a dilution effect related with tree growth, which is maximum during this phenological stage.

The concentration of Mg in leaves from females and hermaphrodites did not show any clear seasonal pattern, remaining constant throughout the year, while in the males an increase was observed from VR to BF (Table 4.4). Similar results were observed by Correia et al. (2002) in the same species and by Oliveira et al. (1996) in cork oak. In pistachio (Picchioni et al. 1997) and fig (Brown 1994) the leaf Mg values increased throughout the phenological stages, but the opposite was true in Japanese pear (Buwalda and Meekgins 1990).

The leaf Fe content remained constant along the year in the female trees (Table 4.5), while in the males its concentration increased significantly from the BF to FB, remaining constant thereafter (Table 4.5). In the hermaphrodites the levels of Fe dropped from VR to a minimum in BF, increasing significantly thereafter and peaking in EF (Table 4.5). In the females, no significant variations were observed in the leaf Zn concentrations, while in the males and hermaphrodites a maximum level was observed in VR (Table 4.5). An accumulation of Cu was observed in the three sexual types FB (Table 4.5).

In male trees, the leaf Mn remained constant thorough the year, while in females and hermaphrodites, reached maximum concentrations in FB (Table 4.4), when the fruit is mature (Warden et al. 1980, Batlle and Tous 1997). A similar pattern has been reported for olive (Férnandez-Escobar et al. 1999). In deciduous fruit trees the concentration of Mn in leaves shows a tendency to increase along the year (Buwalda and Meekings 1990).

However, the reverse has been reported in some evergreen species (Broschat 1997), which suggests that the pattern of Mn variation is species dependent.

One advantage of gender dimorphism over hermaphroditism that as long been proposed is that unisexual plants may gain over hermaphrodites by specializing in one sexual role (Darwin 1877 *in* Davis 2002). Unisexuals are able to allocate the resources previously spent on the opposite sexual role more efficiently and, consequently, increase fitness (Davis 2002). The complete separation of sexes, or dioecy, is expected to evolve when fitness gain curves accelerate as resources investment in one sexual function increases (Charnov et al. 1976 *in* Davis 2002). Similar resource use between the two sexual functions and intersexual competition for space and nutrients are among the factors that may contribute to this condition being met (Charnov et al. 1976 *in* Davis 2002, Givnish 1980). Even if phenotypic dimorphism is low when dioecy first arises, once dioecy has been established, further changes in resource allocation patterns are expected to occur because of sex specific selection (either sexual or natural selection), increasing differences between the sexes (Meagher 1994, Ashman 2000). These differences need not to be adaptive in nature and may arise as a consequence of physical reproductive differences between the sexes or correlated responses to selection on other traits within one sex (Ashman 1999).

Theoretically, the higher total reproductive effort of females and hermaphrodites would be a major factor contributing to a higher sink demand for nutrients by flowers and leaves, when compared to males. The total reproductive effort means the consumption of resources needed to complete the reproductive cycle of a crop (Mauseth 1991). In this chapter we observed that females were able to allocate more nutrients to leaves than male trees, even though male flowers were richer in particular elements such as N and Zn. However, flowers of females showed a lower nutrient concentration compared to males or hermaphrodites. Although the energetic cost of reproduction linked to fruit set and fruit enlargement was not determined in this work, hermaphrodites and females are expected to require an additional nutritional retranslocation from sources to sinks (Mauseth 1991).

Table 4.4. Concentration of macronutrients (g kg^{-1}) in leaves sampled at vegetative rest (VR), beginning of flowering (BF), full bloom (FB) and end of flowering (EF), of male, female and hermaphrodite carob trees.

	Male					Female					Hermaphrodite				
	N	P	K	Ca	Mg	N	P	K	Ca	Mg	N	P	K	Ca	Mg
VR	17.5	A 1.9 a	7.8 b	A 18.4 a	A 2.5 a	20.6	B 1.3 ab	9.8	B 13.4 a	A 1.9	14.7	C 1.1 b	8.6	B 9.7 b	B 1.2
BF	15.2	B 1.0 b	A 11.4 a	A 9.3 b	1.6 b	18.8	A 1.3 ab	A 11.8	A 8.8 b	1.6	14.7	B 0.9 b	B 7.2 b	B 4.8 bc	0.8
FB	13.3	0.7 b	7.1 b	14.3 ab	.1.9 ab	16.9	0.9 b	8.2	11.8 ab	2.0	13.1	0.8 b	8.4	9.7 b	1.2
EF	16.6	1.6 a	B 6.8 b	15.6 ab	1.9 ab	18.0	1.8 a	A 10.0	10.0 ab	1.6	16.5	1.7 a	B 8.0	10.5 a	1.2

In each column, statistical comparisons were made between phenological stages, for the same nutrient and sexual type, and are shown in lower case letters.

In each row, statistical comparisons were made between sexual types, for the same phenological stage, and are shown in capital letters.

Values followed by different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). In the absence of letters, there were no significant differences at $P\geq0.05$.

Table 4.5. Concentration of micronutrients (mg kg^{-1}) in leaves sampled at vegetative rest (VR), beginning of flowering (BF), full bloom (FB) and end of flowering (EF) of male, female and hermaphrodite carob trees.

	Male				Female				Hermaphrodite			
	Fe	**Mn**	**Zn**	**Cu**	**Fe**	**Mn**	**Zn**	**Cu**	**Fe**	**Mn**	**Zn**	**Cu**
VR	51.3 ab	A 43.5	15.2 a	A 4.2 c	59.7	B 15.5 ab	16.2	B 3.2 b	66.1 c	B 10.2 c	17.7 a	B 3.3 d
BF	A 39.0 b	A 34.6	A 8.0	5.1 b	A 36.1	B 16.1 ab	A 8.7	5.2 a	B 29.8 d	B 12.6 b	--	5.6 c
FB	B 63.5 a	A 42.7	8.4	6.5 a	B 47.4	B 20.2 a	5.5	7.4 a	A 74.7 b	B 12.8 a	6.4	7.7 a
EF	B 57.0 a	A 41.4	9.0	B 4.9 bc	B 53.1	B 12.7 b	8.3	C 3.7 b	A 100.5 a	B 8.7 d	8.7	A 6.2 b

In each column, statistical comparisons were made between phenological stages, for the same nutrient and sexual type, and are shown in lower case letters.

In each row, statistical comparisons were made between sexual types, for the same phenological stage, and are shown in capital letters.

Values followed by different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). In the absence of letters, there were no significant differences at $P\geq0.05$.

Resources allocated to reproduction are diverted from somatic growth and maintenance (Williams 1966 *in* Grison-Pigé et al. 2001). The direct cost of reproductive male function comprises resource expenditure in stamens and often in devices favouring pollen dispersal (Grison-Pigé et al. 2001). Wind-pollinated plants invest more in pollen production than insect-pollinated plants (Cruden and Lyon 1985). However, insect pollinated species also have to invest in pollinator attraction and reward (Dobson 1994).

4.4. Conclusions

The results observed show that the differences in nutrient concentrations between vegetative and reproductive organs are influenced not only by the phenological stages, but also by the sexual type of tree. The females showed a lower nutrient concentration in flowers, and a higher nutrient concentration in leaves compared to males. However, those differences had no influence on the number of inflorescences formed or in the number of flowers per inflorescence. In terms of total cost for reproduction this means that the nutritional investment of female leaves on flowering initiation and development was less effective than the male ones. It was also observed that females and hermaphrodites were able to allocate more nutrients to leaves than male trees. This allocation pattern, similarly to what was described for other dioecious species, is explained by the need to sustain the reproductive effort in females, which is longer than in male trees. Furthermore, the hermaphrodites support the development of both inflorescences and fruits with a lower seasonal variation and a lower leaf nutrient concentration, as compared to the other sexual types, which may indicate a more efficient use of resources.

The knowledge of the nutritional demand of the different sexual types of carob and the variation in leaf nutritional concentration along the season can have practical implications for fertilisation guidance, namely the optimization of the amounts and the timing of fertiliser's application.

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CHAPTER V

SEASONAL VARIATIONS ON THE PARTITIONING AND ALLOCATION OF CARBON IN LEAVES, FLOWERS AND FRUITS

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5.1. Introduction

Plants produce, store, invest and loose carbon compounds. The size of the mobile fraction of these compounds at a given time may 1) reflect passive accumulation for no other reason than a periodic disparity between net-uptake and need; 2) it may represent a required, in part transitory pool of solutes (transport, metabolic and osmotic requirements); 3) it may be tied to defence compounds; or 4) represent 'intentionally' stored reserves (Chapin et al. 1990). Except perhaps for defence compounds and osmotics, the size of the mobile C-pool is always likely to mirror a plant's overall carbon supply status, with the greatest fraction of this pool commonly present as non-structural carbohydrates (NSC, sucrose, glucose, fructose and starch) (Würth et al. 2005). Other carbohydrates, such as cell wall structural pollysaccharides (CWSP), as for instance galactomannans, are also related to the NSC, as they are synthesized from or converted to them. These carbohydrates are chemically inert and display different degrees of solubility in water. This feature prompts advantages that are similar to starch (packing and low reactivity) and makes possible the existence of a cell 'compartment' (the cell wall) which permits water to flow with considerable degree of freedom (Buckeridge et al. 2000a).

NSC have an essential function in plant metabolism, providing the building blocks for all compounds present in cells, namely amino acid and fatty acid biosynthesis (Smeekens 2000) and comprehending the main forms of food reserves for most woody plants (Loescher et al. 1990). These carbohydrates are the major substrates for respiration during winter as well as for respiration, growth and development early in the subsequent year (Loescher et al. 1990), allowing trees to grow roots, flush leaves or complete other phenological developments, without the benefits of current photosynthesis (Landhäusser and Lieffers 2003). Moreover, they act as a buffer for insufficient source activity (photosynthesis) due to external factors (Li et al. 2002, Würth et al. 2005). Sugar status further modulates internal regulators and environmental cues that rule growth and development (Koch 1996, Sheen et al. 1999, Smeekens 2000).

Biochemical, molecular, and genetic approaches have supported a central role for sugars in the control of plant metabolism, growth, and development and have revealed interactions that integrate light, stress and hormone signalling (Lalonde et al. 1999, Sheen et al. 1999, Smeekens 2000, Gazzarrini and McCourt 2001, Finkelstein and Gibson 2002,

Rolland et al. 2002) and coordinate carbon and nitrogen metabolism (Coruzzi and Bush 2001).

Photosynthesis is active primarily in mature mesophyll cells of source leaves, allowing for the assimilation of carbon, the resulting primary photosynthate being transported afterwards to meristems and developing organs such as growing young leaves, roots, flowers, fruit, and seed, that is, to sink tissues (Kozlowski 1992, Lalonde et al. 1999, Rolland et al. 2002).

Sucrose and its derivatives represent the major transport forms of photosynthetically assimilated carbon in plants (Kozlowski 1992, Pollock and Farrar 1996, Lalonde et al. 1999), although for a minority of plant species, main translocated sugars comprehend sugar alcohols (mannitol and sorbitol) and the raffinose series (raffinose, stachyose, and verbascose) (Rolland et al. 2002). However, in most cases in which such sugars predominate, sucrose is also present (Rolland et al. 2002). Its metabolizing pathway is highly regulated, mainly through the modulation of the respective synthesizing enzyme, sucrose-P synthase (SPS) (for a review see Huber and Huber 1996).

Instead, starch, is the most widespread storage polymer, followed by fructans and CWSP (Buckeridge et al. 2000a). Its synthesis is initiated by ADP glucose pirophosphorylase (ADPGPPase), a highly regulated enzyme involved along with SPS, in carbon partitioning (for reviews see Preiss 1991, 1997, Martin and Smith 1995), while it is mobilised either by hydrolysis or by a mechanism that involves direct phosphorylation of the terminal glucosyl residues (Buckeridge et al. 2000a). Starch accumulates whenever a high level of sugars builds in, and is degraded to sugars when they exhibit a low content (Kozlowski 1992). The transitory accumulation of starch in source leaves can therefore be altered according to sink demands. Apparently, sucrose is the sugar of transport that will take the products of storage mobilisation (carbon and energy) to the growing embryo (Buckeridge et al. 2000a). Starch is transiently produced in the cotyledons (Buckeridge and Dietrich 1996) and Dirk et al. (1999) proposed that CWSP degradation and starch synthesis might be biochemically related.

Galactomannans (GM) are one type of CWSP, which are usually classified into three groups: mannans, xyloglucans and galactans, the mannans being also divided into pure mannans, glucomannans and galactomannans (Buckeridge et al. 2000a). GM are the energy-reserve polysaccharides mainly found in the endosperm of seeds from the

Leguminosae family (Reid 1985, Buckeridge et al. 2000b, Ganter et al. 2001), but they are also components of the endosperm cell walls of the *Annonaceae*, *Convolvulaceae*, *Ebonaceae* and *Loganaceae* (Matheson 1990). GM are linear polysaccharides composed by a linear backbone of β-(1→4) linked D-mannose units, to which various amounts of single α-(1→6)-linked D-galactose residues are attached (Daas et al. 2000). The main field of GM application is their use as thickening or gelling agents (only when associated with other polysaccharides) in food industries. Its synthesis is initiated by the action of two membrane-bound glycosyltransferases, which catalyses the transfer of mannose (Man) and galactose (Gal) residues to the mannan backbone and galactosyl side chains, respectively (for a review see Edwards et al. 2002). During sprouting they are degraded enzymatically and used as nutrients. The main property of this natural polysaccharide is the high viscosity of the solution in water, over a wide range of temperature and pH (García-Ochoa and Casas 1992). In *Leguminosae*, the three subfamilies (*Caesalpinioideae*, *Mimosoideae* and *Faboideae*) can be distinguished by the mannose:galactose ratios of their seed galactomannans (Buckeridge et al. 2000a). In carob, the galactomannans are composed of mannose and galactose units in a ratio 4:1 (Fig. 5.1).

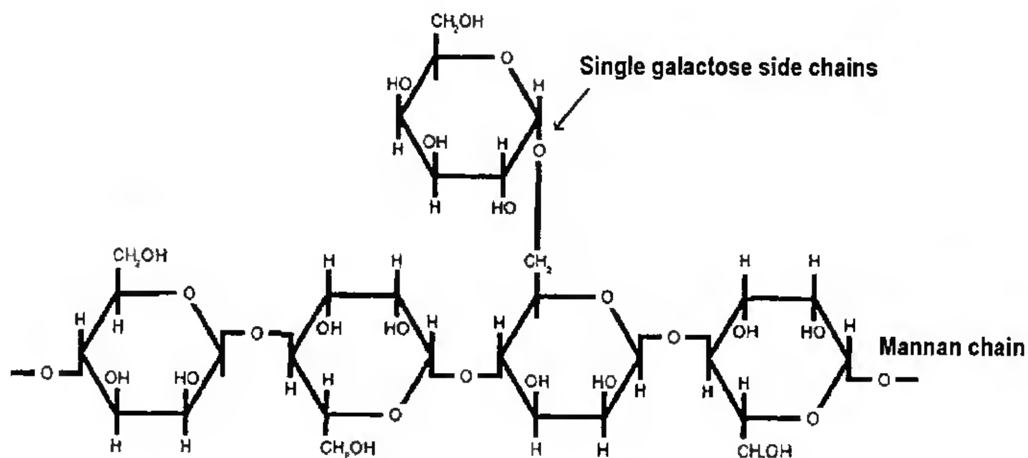

Figure 5.1. Molecular structure of the galactomannans of locust bean gum (Batlle and Tous 1997).

Both the structural and physicochemical characteristics of galactomannans depend on the plant source and on the extraction conditions (Reid and Edwards 1995, Izydorczyk and Biliaderis 1996, Petkowicza et al. 1998). The mobilisation of the galactomannan is

performed through hydrolysis, being disassembled to its monosaccharide constituents (free mannose and galactose) at the same time as sucrose is produced (Buckeridge et al. 2000a) (Fig. 5.2). The mobilisation of galactomannan starts after germination (i.e. radicle protrusion) (Buckeridge et al. 2000a). It has been demonstrated that in the endosperm of seeds of *Trigonella foenum-graecum* and *Ceratonia siliqua*, α-galactosidase and endo-β-mannanase, in the former, and α –galactosidase, in the latter, are synthesised *de novo* (Reid and Meier 1973 *in* Buckeridge et al. 2000a). It is reasonable to think that either mobilisation is induced after or held during germination by some factor.

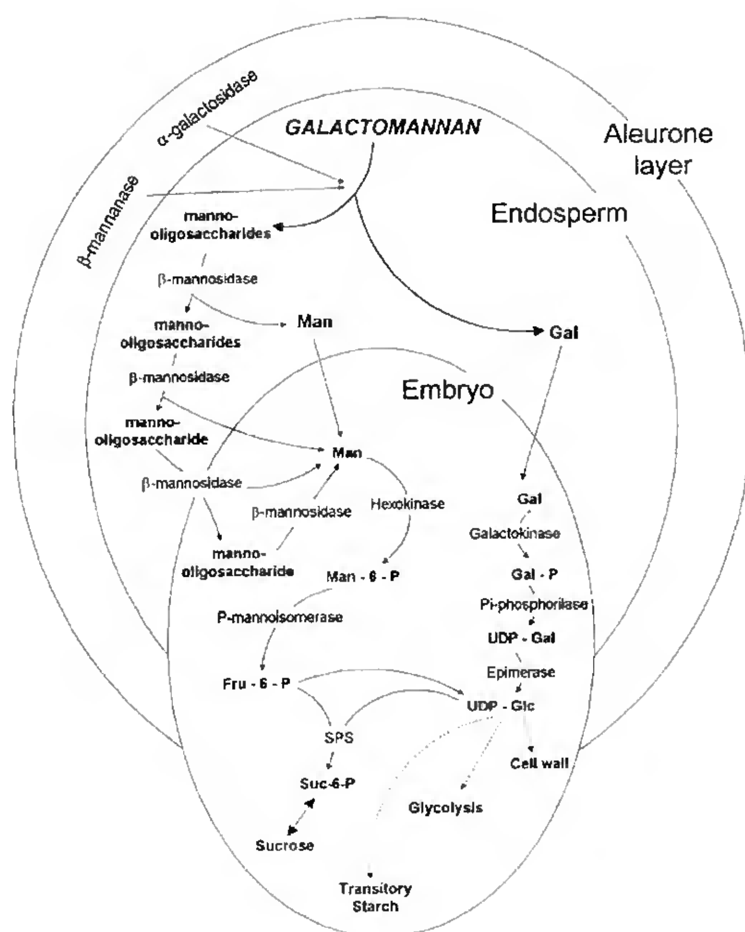

Figure 5.2. Biochemical pathways involved in the catabolism of galactomannan and further metabolism of its products by the embryo. When an aleurone layer is not present, it is likely that hydrolases are produced within each endosperm cell and secreted outwards to the cell wall where the same reaction occurs (Fru-6-P: fructose-6-phosphate; Gal: galactose; Man: mannose; Man- 6-P: mannose-6-phosphate; SPS: sucrose phosphate synthase; Suc-6-P: sucrose- 6-phosphate; UDP-gal: uridine diphosphate galactose) (Buckerige et al. 2000a).

Besides playing a role as a post-germinative reserve, galactomannans can also serve as imbibing substances in early stages of germination (Buckeridge et al. 2000a). It takes up proportionally high amounts of water and distributes it around the embryo. The imbibed endosperm protects the embryo against desiccation by buffering it against water-loss during post-imbibition drought (Reid and Bewley 1979 *in* Buckeridge et al. 2000a).

More than two-thirds of the dry weight of woody plants consists of transformed sugars and therefore their growth depends fundamentally on the control of carbohydrates translocation from sources to sinks and orderly assimilation into new tissues (Kozlowski 1992). Partitioning and allocation of carbon in plants can be influenced by a range of factors, exhibiting a changeable temporal and spatial dynamics, which depends on the plant physiological and ontological state, on the organ, and on the environmental conditions. For instance, despite the capacity to photosynthesize, flowers of woody plants are at certain stages of their development, for example during nectar and/or superfluous pollen production, very powerful carbohydrate sinks as shown by preferential movement of stored carbohydrates and current photosynthate into rapidly growing reproductive tissues (Kozlowski 1992). This is commonly associated with reduction in growth of vegetative tissues (Kozlowski 1992). Otherwise, the effect of carbon allocation on organ and whole plant architecture is illustrated most dramatically by carbohydrate storage and the concomitant cell expansion in reserve organs such as roots, fruit, seed, and tubers (White et al. 2000, Giovannoni 2001).

Light and sugars regulate growth activities by a coordinated modulation of gene expression and enzyme activities in both carbohydrate-exporting (source) and carbohydrate-importing (sink) tissues (Lalonde et al. 1999, Rolland et al. 2002). This ensures optimal synthesis and use of carbon and energy resources and allows for the adaptation of carbon metabolism to changing environmental conditions and to the availability of other nutrients (Coruzzi and Bush 2001). In general, low sugar status in sink tissues enhances photosynthesis, reserve mobilization, and export from source leaves, whereas the abundant presence of sugars in sink tissues promotes growth and carbohydrate storage in the source (Koch 1996). In addition, NSC pool becomes larger when active sinks are removed, such as when trees are debudded or girdled, or when sources become stronger, for instance through photosynthetic stimulation by atmospheric CO_2-enrichment or high compared to low light (Chapin and Wardlaw 1988, Wong 1990). Sink limitation

causes source activity to decline ('end product inhibition'), whereas active sinks stimulate source activity (Stitt and Knapp 1999). Each woody plant is a highly integrated system of competing carbohydrate sinks. The rate and direction of carbohydrate transport are controlled by the placement of vascular connections as well as the relative strengths of variously located sinks and their proximity to stored and/or currently produced carbohydrates (Kozlowski 1992).

Although seasonal NSC variations can be induced by seasonal temperature and water regimes or by phenological patterns that these regimes induce, leading to responses which result from these factors interaction (Kozlowski 1992, Mandre and Klõšeiko 1996, Iürth et al. 2005), it is known that in the majority of broad-leaved trees, soluble sugars content is high at the beginning of the vegetation period but it decreases along development. Otherwise, changes in starch content are somewhat smaller than these, its peak varying among species (Larcher 1995, Mandre et al. 1998). In respect to the direct effect of environmental conditions on NSC status, it has been agreed that insufficiency of light, low temperature and disturbed water supply reduce the content of carbohydrates in plants and bring about changes in the anatomic properties of the plants (Sauter 1988).

Carob tree is a typical woody evergreen species of areas with a Mediterranean climate, such as the Algarve (Mitrakos 1981), characterized by dry summers and cool winters. Low temperatures during winter are generally considered to be the primary constraints to the productivity and dynamics of vegetation in Mediterranean-climate regions (Werner et al. 1999), and in association with high light intensities may lead to photoinibition, which can limit net carbon assimilation, and thus, productivity and growth (Long et al. 1994).

Retention of leaves for longer than one growth period, evergreenness, has been proposed as an adaptation to resource limited systems that allows plants either to take advantage of suitable conditions during generally unfavourable periods (Moore 1984), or to store limiting resources such as nitrogen (Rundel and Parsons 1980). In temperate areas, the survival of evergreen foliage through the winter depends on the development of cold tolerance, which is associated with changes in the quality and quantity of foliar carbohydrates (Senser et al. 1971 *in* Amundson et al. 1995). Foliar carbohydrate reserves of evergreen trees change seasonally with changes in photosynthetic activity, growth and cold tolerance, reflecting changes in source-sink strengths (Kozlowszi 1992).

Sugar is known to accumulate in response to several environmental constrains, such as low temperature or drought. Under extreme low temperatures, high sugars content would lead to a lower freezing point and increase the intracellular osmotic potential, which would reduce the amount of dehydratation during extracelular freezing (Levitt 1980, Watanabe et al. 1999). Sugars also act as colligative cryoprotectants for cell membranes and proteins by diluting electrolytes and toxic compounds that accumulate during freezing (Santarius 1982, Popova and Busheva 2001). Under a moderate chilling, this accumulation could maintain growth processes going and forward photosynthetic metabolism, by counteract thermodynamic restrictions on major enzymes (Strand et al. 1999). During dehydration stress, the hydroxyl groups of sucrose may replace water by hydrogen binding to the phospholipids head groups of the membrane (Anchordoguy et al. 1987).

In this chapter, the seasonal variation of the NSC in vegetative structures of carob tree was studied by monitoring the sugar and starch content in leaves of male, hermaphrodite and in the two most representative female cultivars of carob tree in the Algarve, Mulata and Galhosa. The NSC content of male, hermaphrodite and female flowers along the stages of flower development, and in fruits during their different development stages was also assessed. Furthermore, the levels of galactomannans in seeds, were determined.

5.2. Materials and methods

5.2.1. *Plant material*

Sampling for the analysis of starch, sucrose, glucose and fructose, NSC, in leaves and flowers was done at midday along the year of 2003 on the male, female and hermaphrodite trees, referred earlier on chapter II. Fully expanded leaves were randomly collected from the middle third of the branches in all canopy orientations, every two months, starting in January. Flowers at all the developmental stages were collected along the flowering season (*vide* chapter II).

The samples for the analysis of the contents of NSC and galactomannans during fruit development were collected in March, May and July of 2004 (corresponding to stages I, II and III of fruit development, respectively) on the hermaphrodite and on the two female

cultivars (Mulata and Galhosa) (three trees per sexual type and cultivar). In order to study and compare the levels of carbohydrates in different parts of the fruit, the seeds were manually separated from the pulps.

5.2.2. *Extraction and analysis of NSC*

Soluble carbohydrates. For the analysis of the soluble carbohydrate (sucrose, glucose and fructose), leaves, flowers, pods and seeds were oven-dried at 60°C during 48 h and ground into fine powder with a coffee grinder. Samples (0.5 – 1 g) were extracted in 20 ml of boiling 80% ethanol. After extraction, the homogenates were centrifuged at 5000 rpm at 4°C during 15 min, and the pellets were saved for starch extraction. The supernatants were collected, evaporated to dryness at 30°C, resuspended in 2.5-5 ml of ultrapure water (Milli-Q PFPlus ultrapure water system, Millipore S.A.) and centrifuged at 1000 rpm at room temperature (RT) during 10 min. The solution was first filtered through a Sep Pack filter (Sep-Pack Cartridges, Waters Corporation), followed by a filter with 0.45 µm pores (white surfactant free, HATF 13 µm, Milipore). Twenty microliters of sample solution was used for sugar analyses by high-performance liquid chromatography (HPLC) with UV 166 detector (Gold Beckman). The mobile phase consisted of 0.0007% (v/v) sulphuric acid (H_2SO_4) in water. The column (Waters C18, 4.6 mm ×150 mm) was operated at RT. Peaks were identified and quantified by comparison with retention times, and peak areas relative to standards of sucrose, glucose and fructose.

Starch. The pellets resulting from the extraction of soluble sugars were used for starch extraction. Pellets were resuspended in 5 ml of ultrapure water and centrifuged three times at 4000 rpm during 30 min at RT. The solution was then resuspended in 5 ml of ultrapure water, and autoclaved at 121°C and 1.1 Kg cm^{-2} during 20 min, in order to induce the burst of starch grains. For starch cleavage, a 100 µl aliquot of the autoclaved suspension was added to 500 µl of Na-acetate buffer (pH 4.8) containing 1.4 U amyloglucosidade and 2 U a-amylase and the samples were incubated overnight at 37°C. The samples were then centrifuged at 10000 rpm during 10 min at RT, and the supernatant was used to quantify glucose. The solution was filtered and analysed by HPLC. Original starch concentration was estimated as glucose equivalents.

5.2.3. *Extraction and analysis of galactomannans*

The pellets resulting from the extraction of starch were used for galactomannans extraction. They were resuspended in 5 ml of ultrapure water and placed overnigth in a water bath at 80°C. The solutions were centrifuged three times at 5000 rpm during 20 min at 5°C. The supernatants were collected, resuspended in 10 ml of ethanol and kept overnight at 5 °C, for galactomannans precipitation. This solution was then centrifuged, the pellet was saved and evaporated to dryness at 30°C, resuspended with 5 ml of sulphuric acid at 72% (v/v), and autoclaved (121°C and 1.1 Kg cm^{-2} during 20 min) for galactomannans hydrolysis. After autoclaving, the solutions were brought to a final volume of 50 ml, by adding ultrapure water. The solution was filtered and analysed by HPLC. Peaks were identified and quantified by comparison with retention times, and peak areas relative to standards of mannose and galactose. Original galactomannans concentration was estimated as mannose and galactose equivalents.

5.2.4. *Statistical analysis*

For each sexual type and date of sampling, three trees were selected, and 30-40 mature leaves, a minimum of 30 flowers *per* stage of flower development and 10 fruits in each stage of development, were collected *per* tree. The values obtained for each tree were considered independent replications. The data were subjected to analysis of variance (ANOVA) using the SPSS statistical package for Windows (release 11.0, SPSS INC). Significance between means was tested by Duncan's New Multiple Range Test (P=0.05).

5.3. Results and discussion

5.3.1. *Concentration and seasonal dynamics of NSC in leaves*

Long-lived evergreen foliage may provide an important year-round storage of carbohydrates, which many authors agree as being necessary for growth, lignification and biomass formation of trees (Wardlaw 1990, Kozlowski 1992). The seasonal variation of NSC content should reveal the sink/source relationships, since the pool size of NSC in

trees reflects the balance between net photosynthetic carbon uptake (source) and the irreversible investments in structures or loss of carbon (sink) (Würth et al. 2005).

As referred earlier (*vide* chapters I and II), trioecy is one of the most outstanding biological features of carob (Retana et al. 1994). However, to our knowledge there is no information about differences in the carbon contents and partitioning between the different sexual types. Therefore, in this section the differences between the NSC contents in the leaves of male, hermaphrodite and in the two most representative female cultivars in the Algarve, Mulata and Galhosa, were assessed, as well as its seasonal variation.

In the males and Mulata the leaf starch content was significantly higher ($P<0.05$) than the total soluble sugars (calculated as the total concentration of sucrose, glucose and fructose) (Fig. 5.3), similar to the observed by other authors in the same species (Cavaco et al. 2002).

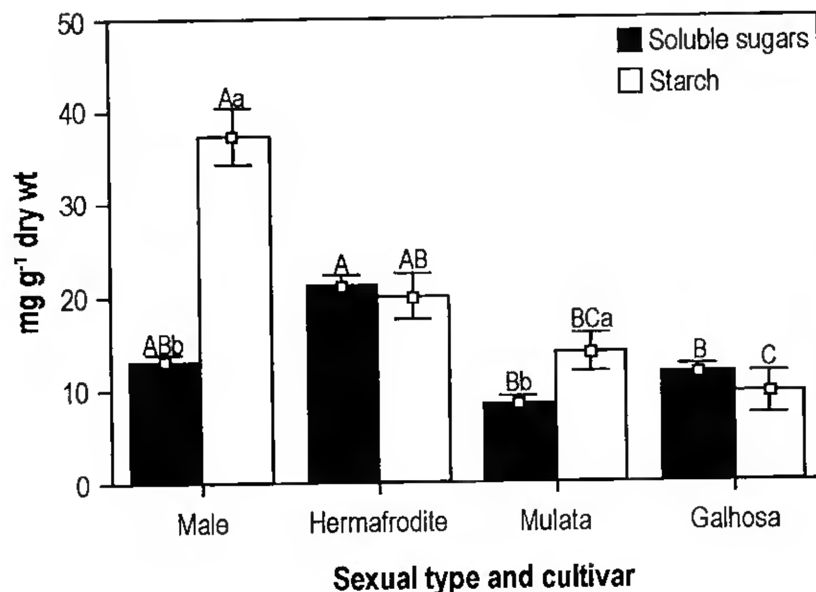

Figure 5.3. Annual mean leaf concentrations of total soluble sugars (sucrose + glucose + fructose) and starch on the three sexual types and two female cultivars of carob tree. For the same sexual type and female cultivar (lower case letters) and for the same sugar of different sexual types and cultivars (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

Starch is synthesized in leaves during the day from photosynthetically fixed carbon and is mobilized at night, being the dominant carbohydrate storage material in higher plants (Kozlowski 1992, Johansson 1993, Martin and Smith 1995). Its content has also been used to assess tree vigour and stress effects and its major site of accumulation is in storage organs, including seeds, fruits, tubers, and storage roots (Martin and Smith 1995, Wang et al. 1998). The accumulation of starch is only transient in photosynthetic organs such as leaves (Wang et al. 1998).

Regardless the time of the year, leaf content of total soluble sugars and starch were affected by the sexual type ($P<0.05$). The leaves of the hermaphrodites were richer in total soluble sugars than females, while starch was more abundant in the leaves of male trees, than in the females, and in the hermaphrodites, compared to Galhosa (Fig. 5.3

Regardless of the time of the year there were significant differences ($P<0.05$) between carbohydrates. Contrary to other reports (Cavaco et al. 2002), glucose was the main soluble carbohydrate present in leaves of all the sexual types and cultivars (Fig. 5.4). Starch was the main carbohydrates present in leaves of males and hermaphrodites, and in the females it was as important as glucose (Fig. 5.4). All the samples were oven-dried at 60°C during 48 h, before being analysed, which may have caused some reduction in the sugar content of carob (Yousif and Alghsawi 2000). Such reduction might be attributed to the Mailard reaction and caramelisation during drying (Calixto and Canellas 1982).

The leaf content of glucose was not affected by the sexual type or female cultivar ($P\geq0.05$) (Fig. 5.4). Leaves of hermaphrodite trees were significantly richer in sucrose than males or females ($P<0.05$) and the starch content was higher in males, than in the female cultivars (Fig. 5.4). Fructose was detected only in hermaphrodites, in very small amounts (Fig. 5.4).

Seasonal variations in the content of total soluble sugars were less pronounced than those taking place in starch (Fig. 5.5), which is opposite to the observed in other species by other authors (Mandre et al. 1998). In the hermaphrodites, the concentration of total soluble sugars was significantly affected by the time of sampling ($P<0.05$): the highest accumulation of soluble sugars was observed in the period of vegetative rest (January), significantly higher than in the other sampling dates, and higher than males and female cultivars in the same sampling date (Fig. 5.5).

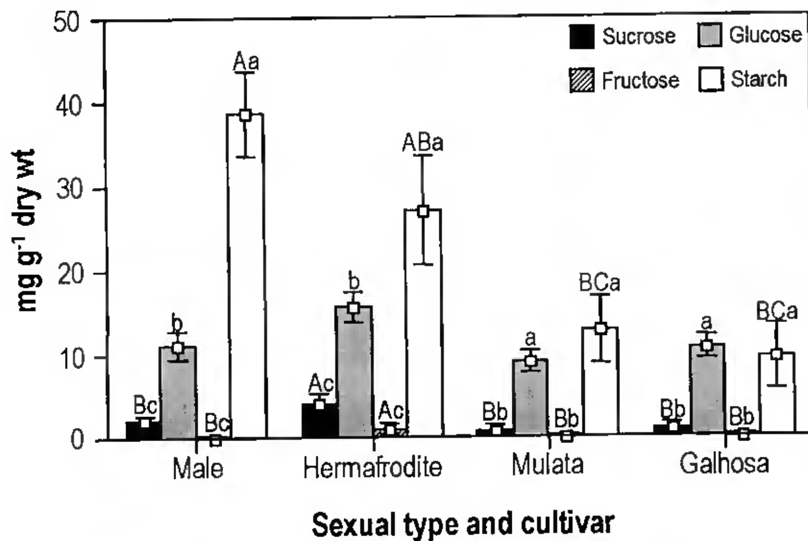

Figure 5.4. Annual mean leaf concentrations of sucrose, glucose, fructose and starch on the three sexual types and two female cultivars of carob tree. For different sugars of the same sexual type and female cultivar (lower case letters) and for the same sugar of different sexual types and cultivars (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

Although without statistical significance, some interesting variation tendencies were observed in the levels of total soluble sugars, along the year (Fig. 5.5).

In males, hermaphrodites and Mulata, levels of total soluble sugars showed a decreasing tendency from the period of vegetative rest (January) until early spring (March), when carob growth is mainly vegetative with the formation of new leaves, and the fruits are in development stage I, while in Galhosa an opposite tendency was observed (Fig. 5.5). Similarly, data reported for the majority of broad-leaved trees strongly suggests that soluble sugars content is high at the beginning of the vegetation period decreasing along the development of new shoots (Mandre et al. 1998). Foliar carbon stores could be of particular benefit in supporting early spring shoot growth when low soil temperatures limit the translocation of carbon reserves from roots (Chabot and Hicks 1982). Carbon gains during the cold season could benefit evergreen trees by providing additional resources for spring bud break and growth (Kozlowski 1992).

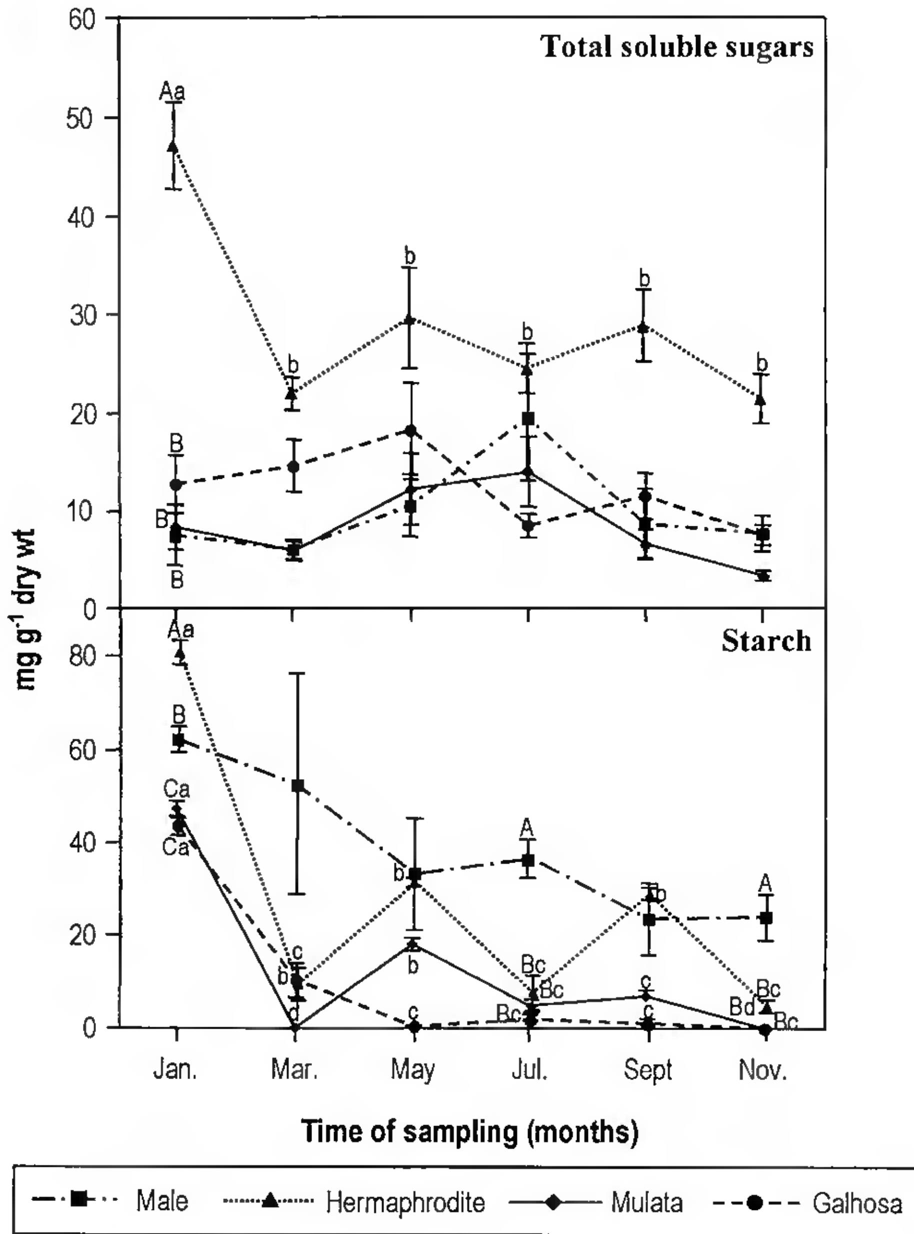

Figure 5.5. Seasonal variations in the leaf content of total soluble sugars (sucrose + glucose + fructose) and starch in the three sexual types and two female cultivars of carob tree. For the same type of sugar, sexual type and female cultivar (lower case letters) and for different sexual types and cultivars, for the same type of sugar and sampling date (upper case letters), values marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

From March to late spring (May), all sexual types tended to accumulate sugars, while from May to early summer (July) the levels of soluble sugars decreased in the

hermaphrodites and in Galhosa as previously reported (Cavaco et al. 2002), increasing however, in males and Mulata (Fig. 5.5). From July to September, when the flowering, fruit ripening and harvest occur, an opposite trend was observed, with hermaphrodites and Galhosa accumulating sugars, while males and Mulata exhibited a decreasing tendency (Fig. 5.5). Regardless the sexual type of trees, all exhibited a decrease in the leaf sugar levels from September to November, when the flowering period ends and the fruit initiation is starting.

Carbon sources for growth are pools of storage carbohydrates and currently produced photosynthate (Kramer and Kozlowski 1979), while carbon sinks are pools of respiratory metabolism, storage of carbohydrates, structural growth and import (Taiz and Zeiger 1991). The importance of source-sink relations in carbohydrate translocation has been demonstrated by experimentally altering the relative strengths of sources and sinks (Kramer and Kozlowski 1979). Removal of sources or potential sinks will cause corresponding changes of photosynthesis (*up*- or *down*-regulation) in remaining or newly formed leaves, and in concentrations of mobile carbohydrates, and finally, it will affect growth rate.

In general, it was observed an accumulation of starch in January and a decline throughout the growing season, reaching low levels in November (Fig. 5.5). This is contrary to other observations in the same species (Cavaco et al. 2002), where the levels of starch reached minimum levels in winter, and peaked in spring and early summer. There was no significant seasonal variation of starch in male leaves, while the hermaphrodites and females exhibited an accumulation in winter (January), followed by a decrease in early spring (March) (Fig. 5.5). The hermaphrodites and Mulata exhibited a similar trend in starch variation, increasing from early (March) to late spring (May), decreasing until early summer (July), with a minimum level in November (Fig. 5.5). In hermaphrodites, an increase was observed from July to September, and in Galhosa, starch decreased from March onwards, remaining in small traces or undetectable (Fig. 5.5). Differences where observed between sexual types in starch accumulation, for the same sampling date (Fig. 5.5). In winter (January), hermaphrodites accumulated more starch than the males and female cultivars, which had the lower levels. In July and November males exhibited the highest levels of starch. The highest accumulation of starch seems to be species dependent (Mandre et al. 1998). In the leaves of *Fagus sylvatica* the levels of starch peaked in early

winter before defoliation (Larcher 1995), but in the woody mass of *Populus canadensis* 'robusta' the maximum corresponds to the period from July to November (Mandre et al. 1998).

Annual patterns of carbohydrate accumulation differ among species and genotypes in accordance with their growth characteristics (Kozlowski 1992). The foliar carbohydrate reserves are very important for maintenance during and after periods of resource limitation (Amundson et al. 1995). Most, but not all, carbohydrates are translocated during the growing season, in accordance with the presence of strong sinks (Kozlowski 1992).

During spring and summer, several phenological events occur simultaneously in carob: branch and leaf growth, enlargement of fruits initiated during the previous season (Martins-Loução and Brito de Carvalho 1989), and probably, floral induction (Correia 1996). Therefore, from January to April the growth is mainly vegetative with the formation of new leaves, being the fruits in development stage I (vide chapter I, section 1.1.1). The development of these sinks (leaves and young fruits) could explain the general decreasing pattern of soluble and insoluble sugars observed from winter until March. During spring, the young leaves photosynthetically active contribute to the increase in the carbon pools, increasing the levels of sugars and starch. Growing cells act as carbohydrate sinks but later may act as sources by becoming photosynthetically active or releasing stored carbohydrates, and whether an organ is a source or sink at a specific time depends on the sum of the many sources and sinks represented by its individual cells (Kozlowski 1992). Flower buds and first inflorescences appear in late June, and a clear blooming occurs in late August and September, suggesting that various amounts of reserves are mobilized for vegetative and reproductive growth (Kozlowski 1992). Flowers are usually referred to as short-term (Kozlowski 1992). Stored carbohydrates are often used in flowering as shown by correlations between flowering and carbohydrate levels in sources (Kozlowski 1992). In addition, in hermaphrodite and female carob trees, development of flowers starts during fruit maturation. This situation has been reported to result in competition for carbohydrate reserves between developing flowers and maturing fruits, and has been pointed out as the main cause of alternate or biennial bearing in many fruit trees (Sedgley and Griffin 1989), such as carob (Batlle and Tous 1997).

In order to evaluate changes occurring within the soluble sugars, seasonal variations of sucrose, glucose and fructose were studied. Although no statistical significant

differences were observed in the seasonal variation of total soluble sugars (Fig. 5.5), significant differences were observed among patterns of the individual sugar that constitute that fraction (Fig. 5.6). In male trees no significant seasonal variations were observed in the levels of glucose, while sucrose reached the highest concentration in July, and decreased significantly until September (Fig. 5.6).

In hermaphrodites, glucose content peaked in January and minimums were observed in March and November (Fig. 5.6). Sucrose decreased from a maximum level in January throughout the year, reaching a minimum in November (Fig. 5.6). Slight differences in the patterns of sugars variation were observed between female cultivars (Fig. 5.6). In Mulata, the levels of glucose significantly decreased from winter to early spring, increased until May, when it peaked, followed by a plateau until summer, and decreasing gradually to a minimum level in November (Fig. 5.6). Sucrose reached a maximum in July (Fig. 5.6). In Galhosa, a plateau in the levels of glucose was observed from winter to early spring, followed by a significant increase until May, where it peaked. Glucose levels reached a minimum in early summer (July), increased significantly until September, decreasing to a minimum level in November (Fig. 5.6). A peak in the sucrose content sucrose was observed in March (Fig. 5.6). The sexual type significantly affected the accumulation of sugars ($P<0.05$), and differences were observed in all the sampling dates, except for July and September (Fig. 5.6). Fructose was only detected in the leaves of the hermaphrodite trees in January, and decreased to zero in March, remaining undetectable thereafter (Fig. 5.6).

The higher accumulation of total soluble sugars and sucrose observed in winter in the hermaphrodite trees may be indicative of a higher resistance to cold temperatures, since it is known that sugars accumulate during cold hardening in woody plants, when starch is hydrolyzed to low-molecular weight carbohydrates, and that sugar accumulation may be an important factor in increased cold hardiness (Palonen 1999, Strand et al. 1997, 1999).

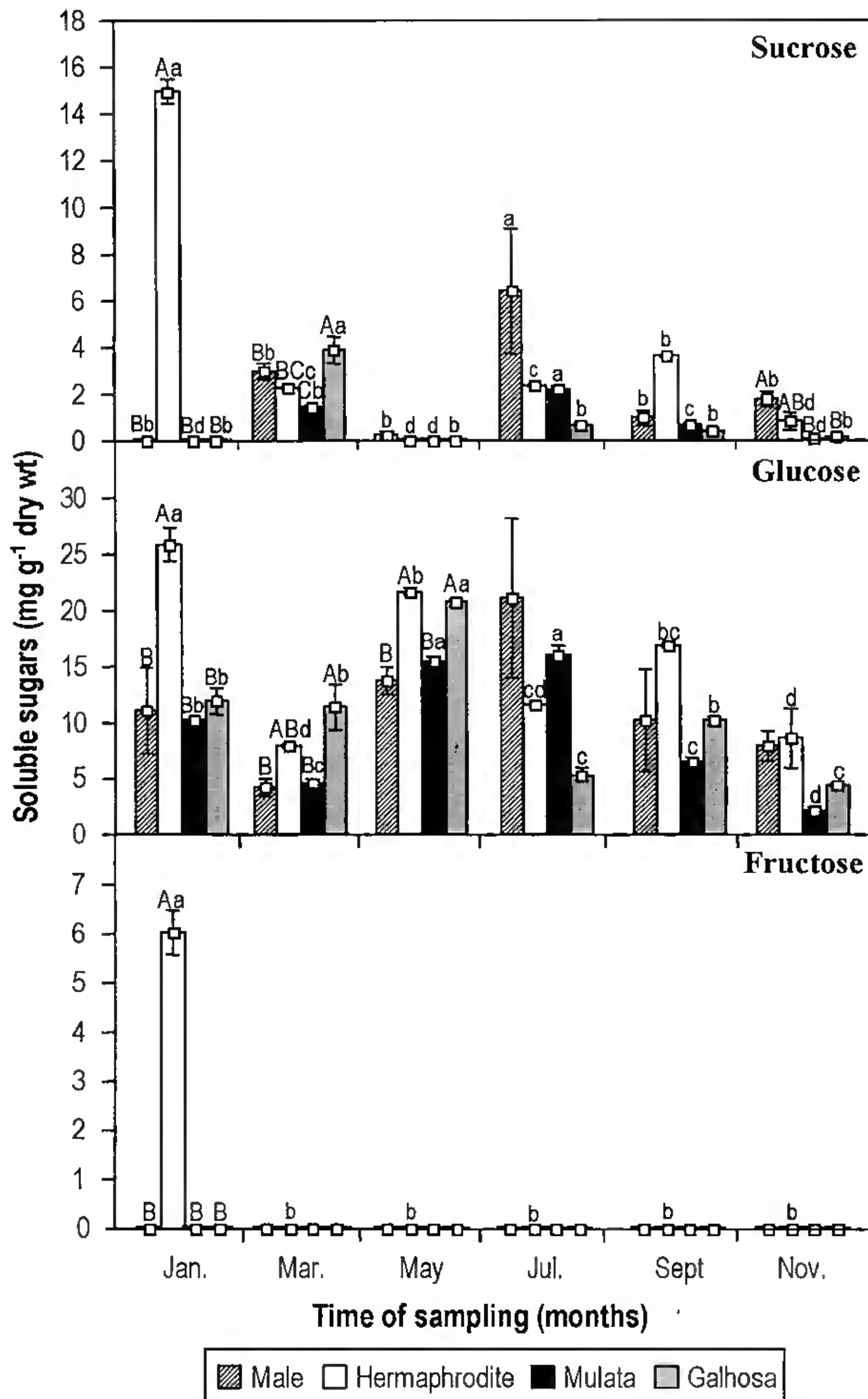

Figure 5.6. Seasonal changes in the content of sucrose, glucose and fructose on leaves from males, hermaphrodite, and female cvs. Mulata and Galhosa carob trees. For the same sexual type and female cultivar and different sampling dates (lower case letters) and for the same sugar and period of time and different sexual types and cultivars (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

However, in carob, the high content of soluble sugars occurring in January in the presence of a high content of starch might otherwise indicate a higher restriction imposed by cold on tree growth. For the time being it is not possible to assure which possibility is more plausible.

In cloudberry (*Rubus camaemorus* L.) a positive correlation between cold hardiness and the quantities of both soluble sugars and sucrose was observed (Kaurin et al. 1981), while in apple [*Malus sylvestris* (L.) Mill. var *domestica* Borkh (Mansf.)] (Ichiki and Yamaya 1982) and red raspberry (*Rubus idaeus* L.) cultivars (Palonen 1999) high levels of sucrose and total sugars are related to cold hardiness. Likewise, more soluble sugars accumulate in the shoots and flower buds of a hardy peach [*Prunus persica* (L.) Batsch] and apricot (*Prunus armeniaca* L.) cultivar than in tender ones (Tamassy and Zayan 1982). In grape (*Vitis vinifera* L.), accumulation of carbohydrates is related to both bud cold hardiness and dormancy status (Wample and Bary 1992). Carob tree is commonly accepted as a chilling-sensitive species, its growth being limited to warmer periods and areas (Mitrakos 1981, Correia et al. 2001). However, since leaves remain functional for about two years, carob tree must be able to cope with periods of chilling and night frost, without suffering injuries that would impair its normal development (Larcher 2000).

5.3.2. *Carbohydrates content in flowers*

Flowers are short-term sinks that produce nectar and/or superfluous pollen used by pollinating vectors (Kozlowski 1992). In this section the contents and variation of NSC in flowers of the three sexual types of carob and its variation along the stages of flower development (*vide* chapter II) were studied.

Overall, carbohydrates content in carob flowers were significantly affected by the stage of flower development ($P<0.001$), the type of carbohydrate ($P<0.01$), and by the interactions between stage of flower development and sexual type of tree ($P<0.05$), stage of flower development and type of sugar ($P<0.001$), and between the sexual type of tree and type of sugar ($P<0.01$).

Regardless of flower development stage, and contrary to what was observed in leaves, levels of total soluble sugars were significantly higher than those of starch in

flowers of the three sexual types, and the sexual type of the tree significantly affected the concentration of starch in flowers ($P<0.05$), which was higher in the males than in the female flowers (Fig. 5.7).

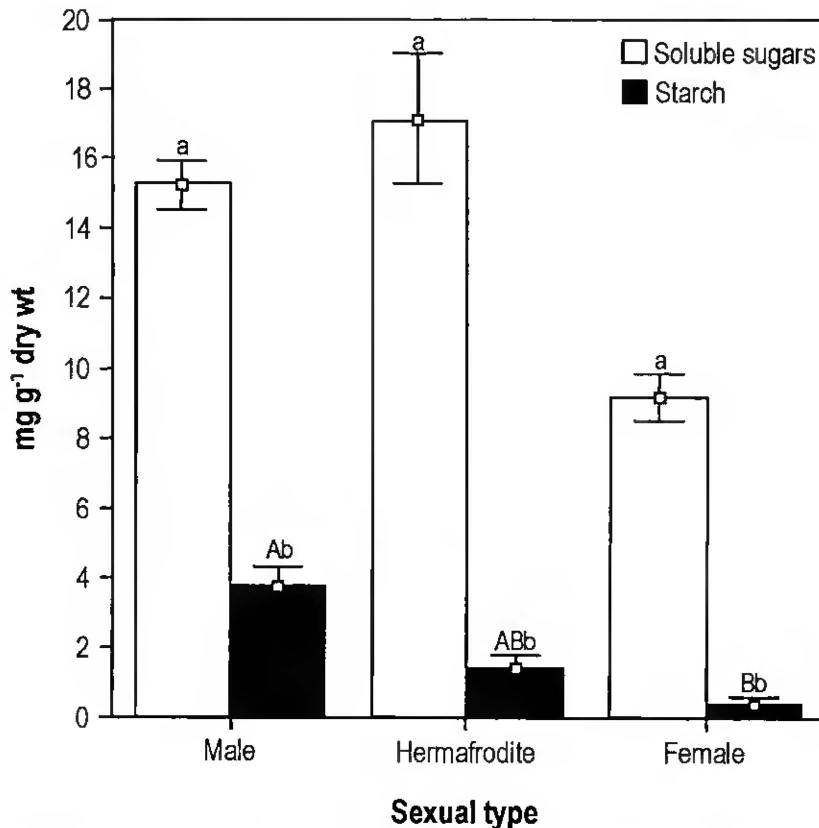

Figure 5.7. Concentrations of total soluble sugars (sucrose + glucose + fructose) and starch on the three sexual types of flowers. For each sexual type (lower case letters) and between sexual types (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

There were significant differences ($P<0.05$) among types of carbohydrates, and similarly to what was observed in leaves, glucose was the main soluble carbohydrate present in flowers of the three sexual types (Fig. 5.8). Levels of starch and sucrose were similar, and fructose was only detected in small amounts in male flowers (Fig. 5.8).

As already observed in leaves, changes in the content of soluble sugars along the stages of flower development were less pronounced than those taking place in starch (Fig.

5.9). Although no statistical differences were observed, in the male and hermaphrodite flowers, a similar pattern was observed in respect to soluble sugars, which decreased from developmental stage 0 to stage II, and increased until stages III + IV (Fig. 5.9). In female flowers, the content of total soluble sugars decreased from stage 0 to stage IV, and increased until stage V (Fig. 5.9). It was observed that the variation tendencies observed along the stages of flower development in the levels of total soluble sugars were mainly due to the variations in the glucose content (Fig. 5.10).

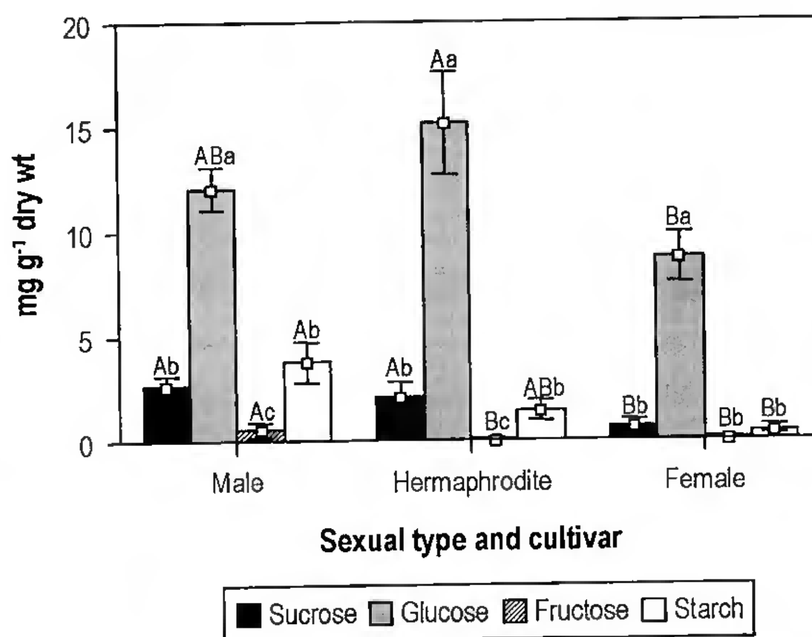

Figure 5.8. Concentrations of sucrose, glucose, fructose and starch in male, hermaphrodite and female flowers of carob tree. For different sugars of the same sexual type (lower case letters) and for the same sugar of different sexual types (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test).

The sexual type significantly affected the accumulation of soluble sugars, for the same stage of flower development, with the hermaphrodites being richer in glucose, in stages III + IV (Fig. 5.9). In male and hermaphrodite flowers, glucose followed a plateau from stage 0 to II, increasing to stages III + IV (Fig. 5.10), while in the females, it decreased from the first stage until stage IV, followed by a increase until the last stage of development (Fig. 5.10). Sucrose content in flowers was maximum in the last stage of

development, in males and hermaphrodites, while in females it peaked in stages II and III, remaining constant in low levels in the other stages of development (Fig. 5.10).

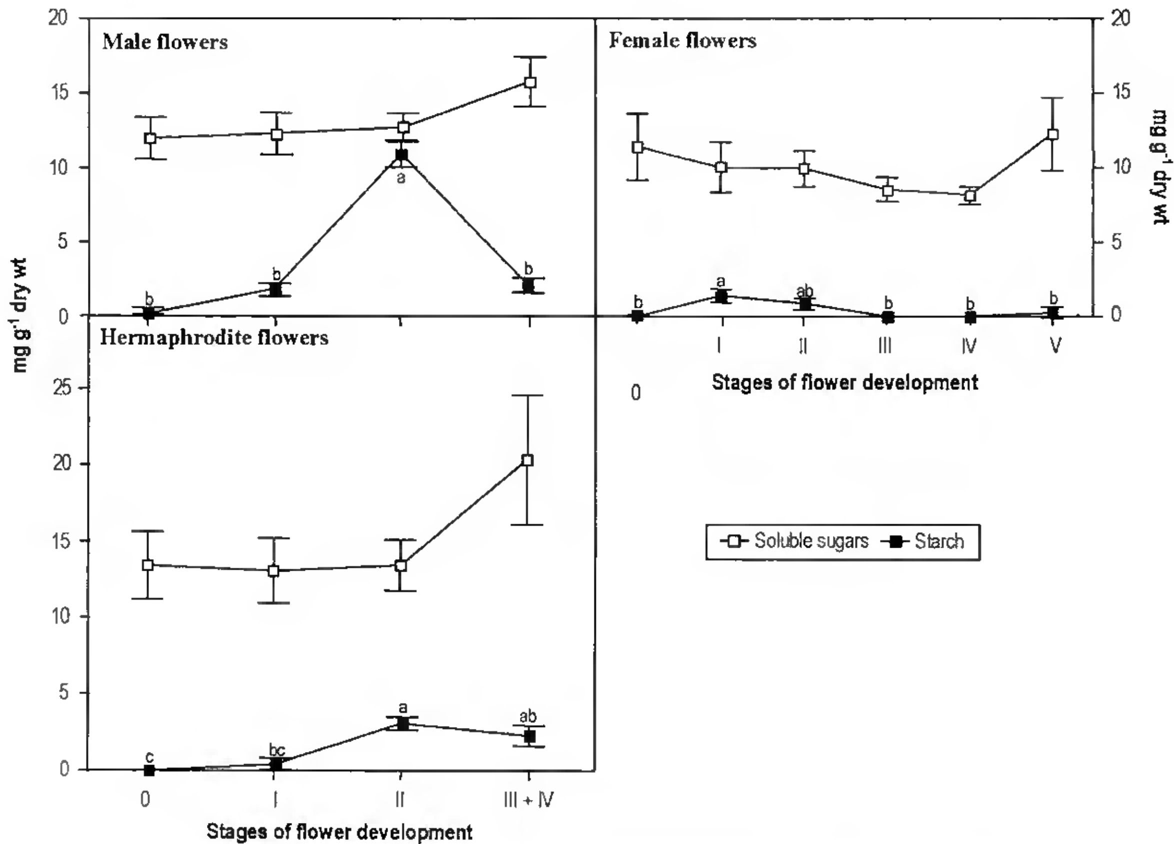

Figure 5.9. Changes in the content of total soluble sugars (sucrose + glucose + fructose) and starch along the stages of flower development in the three sexual types of carob tree. For the same sexual type (lower case letters) and for different sexual types and cultivars in the same sampling date (upper case letters), values marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

The sexual type significantly affected the accumulation of starch, for the same stage of flower development (Fig. 5.9), with males exhibiting the highest level in stage II. In male and hermaphrodite flowers, a significant increase in starch accumulation was observed from stage 0 to II, where it peaked (Fig. 5.9). From stage II to stages III + IV the level of starch decreased in the males, while in the hermaphrodites it remained constant. In

female flowers, a peak of starch accumulation was observed in stage I, significantly higher than at all the other stages, except for stage II (Fig. 10).

The increase in soluble sugars content, namely glucose and sucrose, paralleled by a decrease in starch concentration detected from stage III to III + IV observed in males, strongly suggests the mobilization of starch to soluble sugars in these trees (Fig. 5.9).

Instead, in hermaphrodites from stage III to III + IV and in females from stage IV to V, the increase of soluble sugars (glucose and sucrose in hermaphrodites and glucose in females) occurred without any change in starch content (Figs. 5.9 and 5.10). Based on the reported data, it is difficult to assess the exact reason underlying the increase in none of the cases. Indeed, even when sucrose was not increasing as in females, fructose remained constant, therefore suggesting a low sucrose degradation capacity (Figs. 5.9 and 5.10).

The referred peak of glucose observed in males and hermaphrodites in stages III + IV or in females in stage V is associated to different flower developmental stages, and therefore might be associated to different physiological and regulatory mechanisms. Therefore, in males and hermaphrodites, this peak is most probably involved in the production of pollen and nectar. Nectar is a multi-component aqueous solution, rich in carbohydrates (Tóth et al. 2003), in which preliminary studies on carob indicated that glucose is the major sugar regardless of the tree sexual type (personnal obs.). Moreover, glucose, fructose, sucrose and different types of polysaccharides are known to occur in the pollen grains of different species in different proportions (Nepi et al. 2001) and under different environmental conditions (Vesprini et al. 2002).

In females, the peak in the concentration of glucose from stage IV to V coincides to the stage of development when, after fertilization, the volume of carpel increases becoming flatly shaped, and the fruit development begins, which might suggest that in this case, the newly formed fruits act as strong sinks enhancing soluble sugars accumulation. However, as previously noted, the increase in glucose was not paralleled by the simultaneous decrease in the contents of starch or sucrose. Although very difficult to understand, based on the data set obtained in this study, a further analysis on the enzymatic control underlying partitioning of photoassimilates between choroplastidic and cytosolic pathways, might indicate that the increased glucose content, is indeed the result of a drift of carbon from the chloroplastic starch synthetic pathway to the cytosolic one, allowing the presence in the flowers of a very easily assimilated sugar by the growing fruit (Ho 1988).

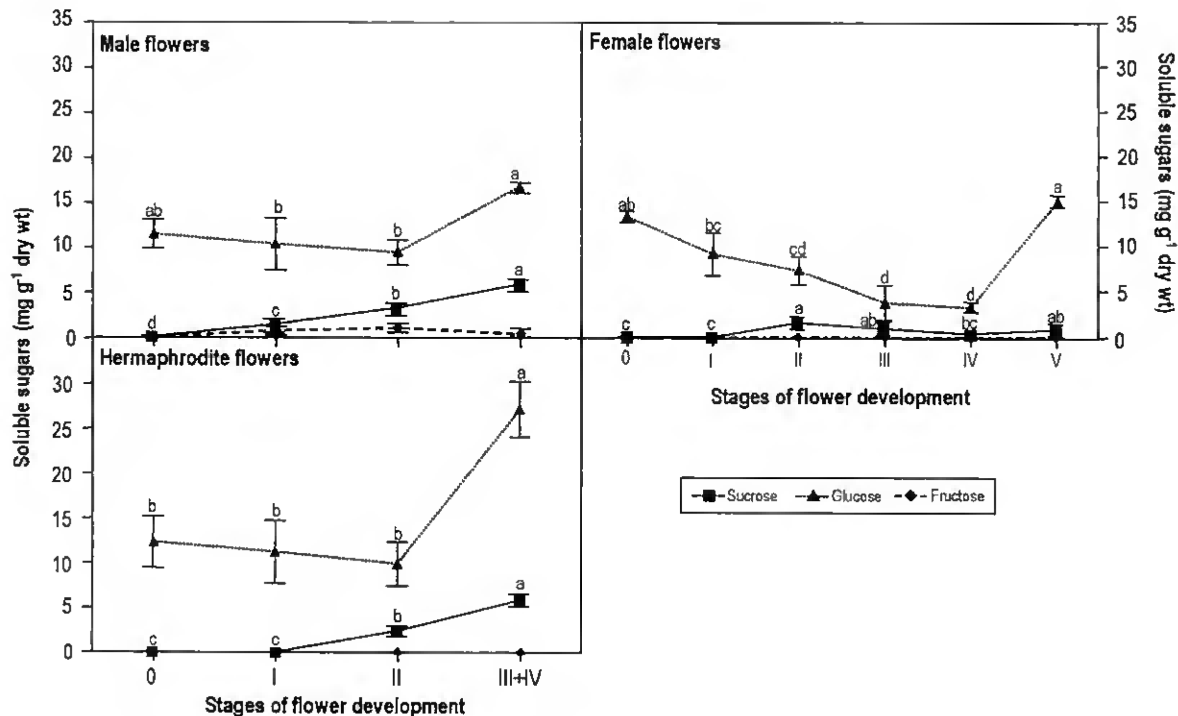

Figure 5.10. Seasonal variation of sucrose, glucose and fructose on flowers from males, hermaphrodite and female carob trees. For the same sexual type and different developmental stages (lower case letters) and between sexual types for the same stage (upper case letters) values marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

5.3.3. *Concentrations of carbohydrates in pulp and seeds during fruit development*

Fruit development is thought to be triggered by plant hormones and further sustained by the carbohydrate supply available in flowers (Gillaspy et al. 1993).

In carob, fruit growth follows a typical sigmoidal curve (Stephenson 1981). The earliest stage of fruit development, stage I, is a slow growth period characterized by cell division, including the change of ovary to fruit, and occurs from January to April. This stage is especially long in carob because in Mediterranean areas winter cold starts at the end of the carob blooming period, when flowers have wilted and fruit initiation is just starting (Bosch et al. 1996). The second stage (stage II), takes place from April to late

June, and is characterized by a rapid linear growth period, mainly due to cell enlargement. From late June to September fruit enters stage III of development, corresponding to a final period of declining growth. Generally, most fruits are ready to be harvested in August.

The two main carob pod constituents are (by weight): pulp (90%) and seed (10%); the constituents of the seed are (by weight): coat (30-33%), endosperm (42-46%) and embryo or germ (23-25%), and the endosperm is the galactomannan locust bean gum (LBG) (Batlle and Tous 1997).

In this section the differences in the contents of NSC in pulps and seeds, between hermaphrodites and female cultivars, along the stages of fruit development, was accessed. In addition, galactomannans content was further determined. It is known that the carbohydrate content of carob fruits depends on the cultivars, origin and climate (Calixto and Canellas 1982, Marakis 1996).

Contrary to other reports in the same species (Cavaco et al. 2002), starch content was where not always higher than the soluble sugars: in Galhosa, leaves sampled at stage I of fruit development were richer in total soluble sugars than starch ($P<0.05$). In females, the concentration of total soluble sugars were higher than starch in seeds from fruits at stages I and II, and in he hermaphrodites this was also true for seeds from fruits at stage I and pulp from fruits at stage II ($P<0.05$). A significant seasonal variation in the total soluble sugars ($P<0.05$) was observed in the pulps from females fruits, which accumulated the maximum levels in development stage III (Fig. 5.11).

Historically, due to its high content of sugars in the pulp (40–60%) (Batlle and Tous 1997), the brown pod was consumed as food, especially in ancient times as a candy for children or in emergency situations such as war. Nowadays, and as referred earlier in chapter I, the carob pods provide two important products: a) 'carob kernel', seeds from which LBG is extracted, and b) 'carob kibble', the remaining pulp obtained after the removal of the seeds, which can be used directly in animal and human nutrition or as a raw material for industrial processing (Batlle and Tous 1997, Martins-Loução and Brito de Carvalho 1989).

For the same organ at the same developmental stage of the fruit, there were no significant differences in the accumulation of total soluble sugars, between sexual types (Fig. 5.11).

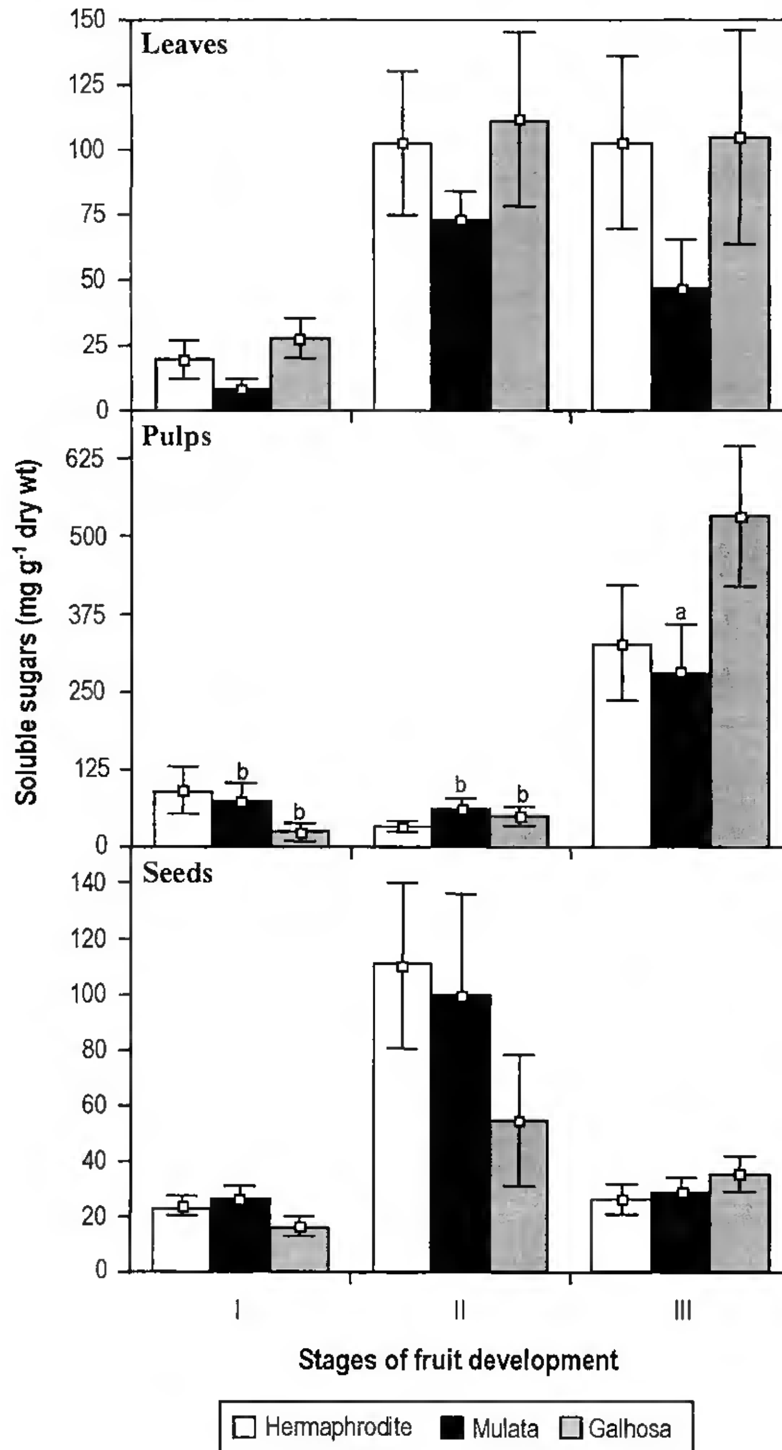

Figure 5.11. Seasonal changes in the content of total soluble sugars (sucrose + glucose + fructose) in leaves, pulp and seeds of hermaphrodite and female cvs. Mulata and Galhosa. For different stages of fruit development of the same organ of the same sexual type and female cultivar (lower case letters), values marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

As expected, females accumulated a higher level of total soluble sugars in the pulps of fruits at development stage III (corresponding to the stage of fruit harvest), than in leaves or seeds (Fig. 5.11), although contrary to other reports, hexoses, namely glucose were predominant relatively to sucrose (Albanell et al. 1991, Avallone et al. 1997, Batlle and Tous 1997) (Figs. 5.12 and 5.13).

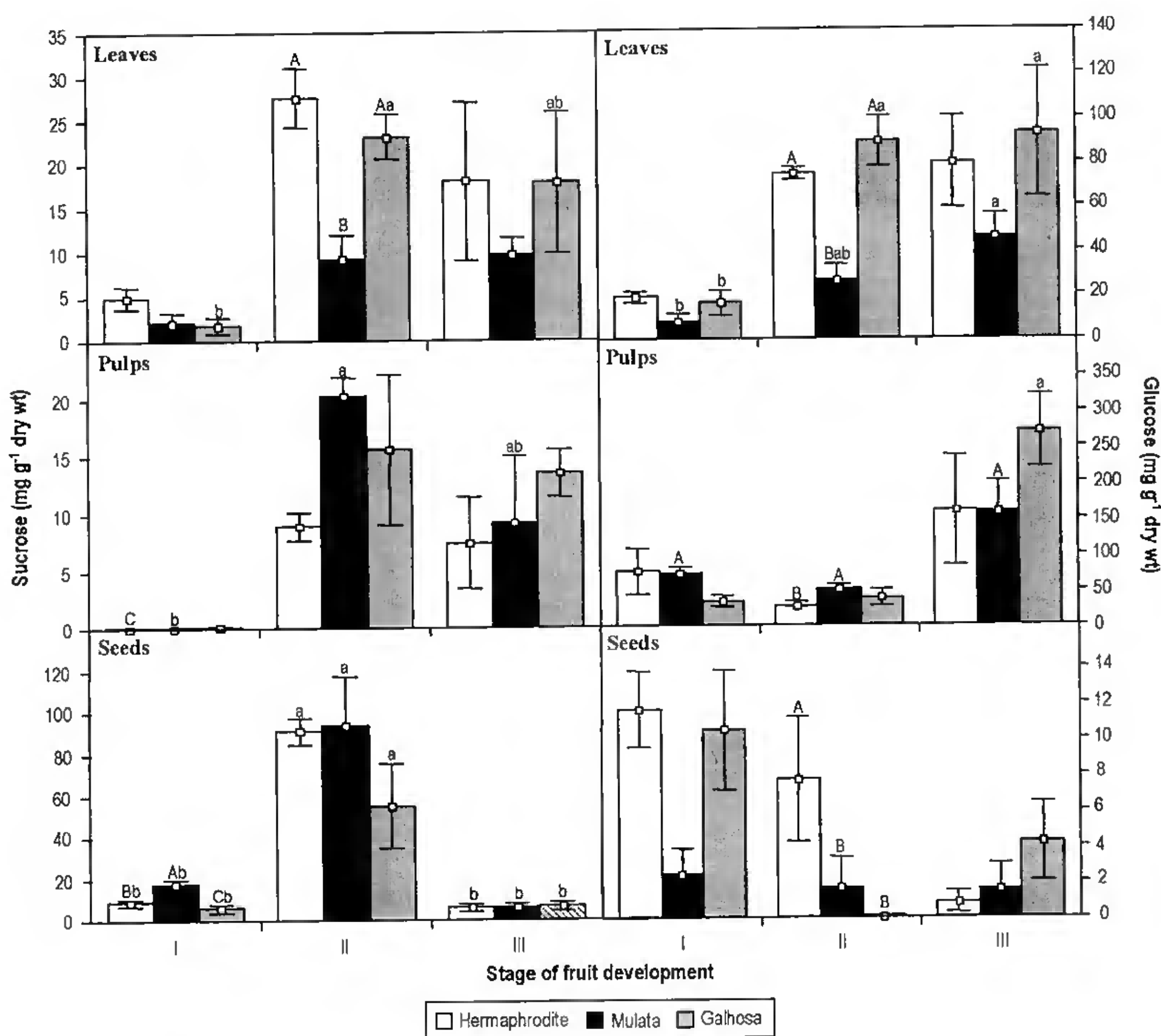

Figure 5.12. Variations in the content of sucrose and glucose on leaves, pulp and seeds of hermaphrodite and female cvs. Mulata and Galhosa, along the stages of fruit development. For the same organ and sexual type and different stages of fruit development (lower case letters) and for different sexual types and cultivars for the same organ and stage of fruit development (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

It is known that the carbohydrate content of carob fruits depends on the cultivars, origin and climate (Calixto and Canellas 1982, Marakis 1996). In mature fruits of females (stage III), glucose and fructose were the major sugars in the pulp, and fructose in seeds, while in hermaphrodites, fructose was the main sugar present in seeds ($P<0.05$) (Figs. 5.12 and 5.13).

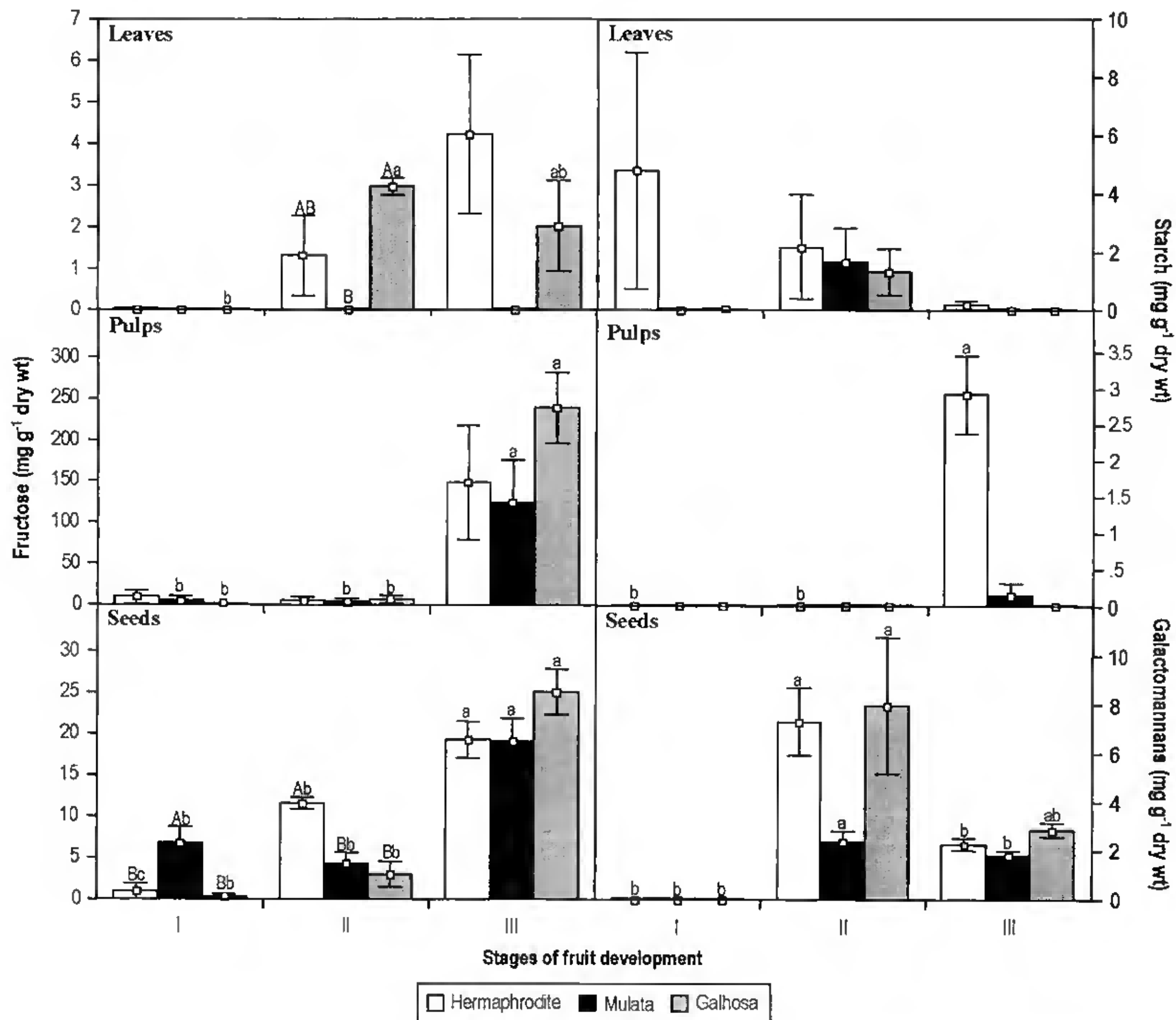

Figure 5.13. Concentrations of fructose and starch on leaves, pulp and seeds, and galatomannans on seeds of hermaphrodite and female cvs. Mulata and Galhosa, along the stages of fruit development. For the same organ and sexual type and different stages of fruit development (lower case letters) and for different sexual types and cultivars for the same organ and stage of fruit development (upper case letters), columns marked with different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test). Absence of letters indicates no significant differences at $P\geq0.05$.

Starch was detected in small amounts in pulps from fruits at stage III, from hermaphrodites and Mulata (Fig. 5.13), and was absent in seeds of either hermaphrodites or females. These results are similar to those obtained in the same species, by other authors (Avallone et al. 1997).

As expected (Batlle and Tous 1997), GM was the only insoluble carbohydrate present in carob seeds (Fig.5.13), and its content was not significantly affected by the sexual type or cultivar ($P \geq 0.05$). The GM content steadily increased from stage I to stages II and III of fruit development in females, and in the hermaphrodite, its level peaked in stage II (Fig. 5.13).

Overall, results concerning the pattern of carbohydrates partitioning and allocation in source leaves and fruit in carob tree depicted in Figures 5.11-5.13, clearly indicate that these are processes highly dependent on the sexual type and cultivar considered. In general, the variation of the respective stage when a predominant content in soluble sugars was observed in leaves, pulps and seeds, strongly supports the expected relation underlying an export of assimilate from source leaves which sustains fruit development and maturation (Ho 1988, 1992). Furthermore, in hermaphrodites and Galhosa, fruit development and maturation seemed to be supported by an initial leaf starch store, which later remobilization was most probably induced by the increasing sink strength exerted by fruits growth. Also, the simultaneous increment of glucose and fructose observed in seeds suggests that it is most probably resulting from the enhancement of sucrose and galactomananns degradation, since their levels presented an inverse relation at the various stages (Figs. 5.12 and 5.13). All these suspicions could be further confirmed through enzymatic studies concerning the various pathways involved in the process.

5.4. Conclusions

In this work glucose was the main soluble sugar present in leaves, flowers and fruits of carob tree. In leaves, the starch content was higher than the total soluble sugars. The sexual type of the tree affected the levels of carbohydrates, and the hermaphrodites were richer than females in total soluble sugars, while starch was more abundant in the leaves of male trees, than in the females, and in the hermaphrodites compared to Galhosa. It was observed

a higher accumulation of total soluble sugars and sucrose in winter, in leaves of the hermaphrodite trees, which may indicate a higher resistance to cold temperatures.

In contrast to other trees in temperate areas, in carob dynamics of partitioning and allocation of NSC seems to be very complex. The patterns of carbohydrates and their seasonal and ontological variations are dependent on the sexual type and cultivar. Moreover, the impacts of vegetative and reproductive growth on the partitioning and allocation of NSC are clearly difficult to evaluate, although it seems for instance that a certain level of sugars must be achieved in the leaves in order to support pods development. Furthermore, these trees are typically located in Climatic Mediterranean areas, which comprehend to strongly limiting seasons, namely winter and summer, through chilling and low water availability, respectively. Both vegetative and reproductive growth is initiated immediately after winter restrictions and is finished in late summer when water availability is very low.

In flowers, the total soluble sugars content was higher than starch, which was present in higher amounts in the male flowers, than in the female ones. The levels of glucose peaked in the last stage of flower development, which in the female flowers suggest that the newly formed fruits act as strong sinks enhancing soluble sugars accumulation.

In fruits, the females accumulated a higher level of total soluble sugars in the pulp in stage III of fruit development. Starch was detected in small amounts in pulps from fruits at stage III, and was absent in seeds. GM was the only insoluble sugar present in seeds, and there were no differences in its content, between the sexual types and female cultivars. The results concerning the pattern of carbohydrates partitioning and allocation in source leaves and fruit clearly indicate that these are processes highly dependent on the sexual type and cultivar considered.

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CHAPTER VI

CRYOPRESERVATION OF POLLEN

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6.1. INTRODUCTION

It is well known that the biological diversity is crucial for the development and survival of plant species. The conservation of plant genetic resources for food and agriculture, one sector of biodiversity, is an issue of common global concern owing to the rapid erosion of plant genetic diversity often associated with modern agriculture (Withers and Engelmann 1997, Hawkes et al. 2000).

According to each particular situation different conservation strategies are available, being generally divided into *ex situ* and *in situ* methods (Withers and Engelmann 1997). The *in situ* methods involve the conservation of germplasm in wilderness and/or protected areas, reserves, or as field collections (field genebanks) (Reed et al. 2001). *Ex situ* conservation consists of the removal of the plant genetic resources from their natural habitat and placing them under artificial storage conditions (Withers and Engelmann 1997).

The most common approach to *ex situ* conservation is seed storage (Withers and Engelmann 1997, Reed et al. 2001). There is a large proportion of agricultural crops that produce 'orthodox' seeds, i.e. amenable to drying and cold storage (Withers and Engelmann 1997). However, three categories of crop present problems for seed storage (Withers and Engelmann 1997): those that do not produce seeds (ex. *Musa* spp.), crops that have some sterile genotypes and others that although producing orthodox seeds, are highly heterozygous and, therefore, of limited usefulness for the conservation of gene combinations (ex. *Solanum tuberosum*, *Dioscorea* spp., *Manihot esculenta*, *Ipomoea batatas* and *Saccharum* spp.), and finally species producing 'recalcitrant' seeds, that is, seeds that cannot tolerate desiccation to moisture contents that would allow exposure to low temperatures (ex. *Cocos nucifera*, *Persea americana*, *Mangifera indica* and *Theobroma cacao*).

Nowadays the classical approaches to conservation are complemented by *in vitro* conservation methods that can be used in combination with traditional practices, offering added security for field genebank conservation (Ashmore 1997). In fact, the effective integration of contemporary technologies with traditional conservation strategies is crucial for the successful preservation of plant biodiversity (Benson 1999a).

In vitro conservation comprises two inter-dependent techniques: tissue culture and cryopreservation (Reed et al. 2001). Cryopreservation is the conservation of biological material at ultra-low temperature (-196°C), usually in liquid nitrogen (LN). It is considered the best option for the long time storage of germplasm, since at that temperature the metabolic processes and cellular divisions stop (Withers and Engelmann 1997, Reed et al. 2001). Cryopreservation has become a promising tool for long-term conservation of plant genetic resources, and remarkable progress has been made in the past few years. At the beginning of the 1990's, the cryopreservation techniques were applied to more than 100 species using cell suspensions, *calli*, protoplasts, embryos and meristems, and the number of cryopreserved species is rapidly increasing (Bajaj 1995, Takagi 2000). Furthermore, in the last decades cryopreservation has assumed relevant importance in biotechnological studies (Zhang et al. 2001).

The classical techniques of cryopreservation are based on chemical protection by the use of cryoprotectants, and in the rate of dehydrative cooling, namely rapid and controlled cooling (Withers and Engelmann 1997). The majority of higher plant somatic cells are not inherently freeze-tolerant, and the transition of extra and intracellular water into ice causes damage of a physical or biochemical nature (Reed 2001). The dynamics of the freezing process are particularly important: extracellular freezing commonly occurs first, causing a flow of water from the cytoplasm and vacuole to the extracellular space where it freezes (Taylor 1987). Modifications of the pregrowth medium used for the passage before cryopreservation by, for example, the addition of osmotically active compounds, such as dimethyl sulfoxide (DMSO), glycerol mannitol or sucrose, increases the resistance of the cells to ultra-low temperatures, either by their capacity of linkage to the exposed surface of the bio-membranes (Popova and Busheva 2001), to reduce the temperature at which freezing initiates (Watanabe et al. 1999) or by the reduction in cell size, thus increasing the freeze tolerance (Withers and Engelmann 1997).

Depending on the rate of cooling, different amounts of water will leave the cell before the intracellular contents solidify (Pitt 1992). Rapid cooling will result in more water remaining within the cell and causing potentially damaging ice than in slow cooling. Ice causes damage when formed in the freezing process *per se*. It can also cause damage during rewarming owing to the phenomenon of recrystalization, in which ice melts and reforms at a thermodynamically favourable, larger, and more damaging crystal size. This

can be mitigated by rapid thawing (Meryman and Williams 1985). Slow cooling reduces this risk, but can incur different damaging events owing to the concentration of intracellular salts and changes in the cell membrane (Meryman et al. 1977 *in* Withers and Engelmann 1997). Shrinkage of the protoplast and loss of surface area in the plasmalemma can render the protoplast incapable of resuming its original volume and surface area after thawing, resulting in rupture (Steponkus 1984).

Controlled freezing involves the application of chemical cryoprotectants followed by exposure of plant tissues to a low temperature gradient, which is optimized for a critical rate of cooling to a terminal sub-zero transfer temperature (Withers and Engelmann 1997, Reed et al. 2001). On reaching this point, the tissues are transferred to LN. The precise control of cooling rates and extracellular ice nucleation is critical for the success of cryopreservation using controlled-freezing methods, and such control can only be reliably achieved by using controlled rate, programmable freezers (Withers and Engelmann 1997, Reed et al. 2001). Techniques of controlled freezing may prove difficult to achieve, mainly due to the complex and expensive equipment required.

In classic cryopreservation techniques, the removal of cellular water and the behaviour of the remaining cellular water during the freezing and thawing processes are critical to success (Withers and Engelmann 1997). In contrast with these freezing-based techniques, new cryopreservation techniques are based on the phenomenon of vitrification (Withers and Engelmann 1997, Reed et al. 2001).

Vitrification is cryopreservation in the absence of ice, since it can be defined as the transition of water directly from the liquid phase into an amorphous phase or glass, while avoiding the formation of crystalline ice (Withers and Engelmann 1997, Reed et al. 2001). In vitrification based procedures, cell dehydratation is performed before freezing by the pretreatment of explants with highly concentrated chemical cryoprotectant mixtures (Sakai et al. 1991, Niino et al. 1992, Towill and Jarret 1992, Sakai 1995, Withers and Engelmann 1997, Zhang et al. 2001). Four different procedures based on the phenomenon of vitrification can be identified: encapsulation-dehydration, dessication, pre-growth-dessication and vitrification (Withers and Engelmann 1997). Techniques of vitrification, encapsulation/dehydration and combination of these are now available to be applied to hundred of plant species (Reed 2001).

The encapsulation-dehydration technique is based on the technology developed for the production of synthetic seeds (Redenbaugh et al. 1991), involving the encapsulation of the explants in alginate beads and their exposure to osmotic and evaporative dehydration to a critical moisture leve (Dereudre et al. 1990, Fabre and Dereudre 1990, Plessis et al. 1993). Before the cryopreservation procedure itself, plant material is often submitted to various treatments that increases survival potential, such as exposure to low temperatures or to sucrose enriched medium (Withers and Engelmann 1997). The stages of the process after encapsulation are: pregrowth, usually by osmotic conditioning with sugars; desiccation, either in the air current of a laminar airflow cabinet or by using silica gel; cooling, usually by plugging samples directly into LN; storage normally performed at -196°C and rapid warming, generally in a water bath and recovery under standard culture conditions (Withers and Engelmann 1997).

The dessication technique is very simple, consisting of the dehydration of the plant material, usually by placing it in the air current of a laminar airflow cabinet, and then freezing it rapidly by direct immersion in LN, and has been applied mainly to zygotic embryos (Withers and Engelmann 1997).

Cryopreservation using a pregrowth-dessication procedure comprises a pregrowth treatment with cryoprotectants, namely carbohydrates, dessication, in the air current of a laminar airflow chamber or with silica gel, rapid cooling by immersion in LN, storage at -80 or -196°C and rapid warming (Withers and Engelmann 1997).

The vitrification technique involves the pretreatment ('loading') of samples with highly concentrated, chemical cryoprotectant mixtures (Sakai et al. 1991, Niino et al. 1992; Towill and Jarret 1992, Withers and Engelmann 1997), rapid cooling and warming, and removal ('unloading') of the vitrification solution.

The vitrification may be the only freeze-avoidance mechanism that enables hydrated cells, tissues and organs to survive at the temperature of LN (Sakai 1995), and is one of the most often applied cryopreservative strategies, requiring simple equipment and resulting in high rates of recovery of the cryopreserved material (Pennycooke and Towill 2000, Matsumoto et al. 2001, Verleysen et al. 2004).

The glasses formed during vitrification are highly unstable and great care must be taken to prevent the occurrence of damaging glass relaxation and de-vitrification events upon re-warming (Reed et al. 2001). Furthermore, vitrification solutions can be toxic to

cells, so their application and removal must be precisely controlled in order to avoid cell damage and death (Reed 2001, Reed et al. 2001). The osmotic conditioning with sugars, such as sucrose or sorbitol, is often used for cryopreservation by vitrification (Verleysen et al. 2004).

During the cryopreservation of plant material, alterations at the genetic, physical and biochemical stability of the cells can occur (Reed 2001). The modifications in the genetic stability can cause somaclonal variations, which are phenotypic changes arising in plants grown in tissue culture (Reed 2001). These variations are most common in plants regenerated from single cells or *callus*, and less common in micropropagated or meristems propagated plants derived from existing meristems (Reed 2001). Thus, genetic analyses are needed to determine whether instability is a problem, although several studies have shown little evidence for concern (Towill 1990, Häggman et al. 1998). The physical and biochemical stability of the cryopreserved material can be altered, in the particular case of vitrification, due to the cracking of vitrified solutions, in response to physical shocks or to some warming procedures, and thermal-stress induced fractures of biological materials may cause serious damage to stored samples (Reed 2001, Reed et al. 2001).

Pollen grains are stored to make easier crosses in breeding programmes, distributing and exchanging germplasm among locations, preserving nuclear genes of germplasm, and for the performance of studies concerning basic physiology, biochemistry, fertility, biotechnology involving gene expression, transformation and *in vitro* fertilisation (Towill and Walters 2000). On the other hand, stored pollen can be used for haploid generation through pollen embryogenesis (Bajaj 1987). For this purpose, storage of mature pollen is of minor importance since in most systems pollen at the uninucleate stage is more responsive to embryogenesis (Bhojwani and Razdan 1983, Towill and Walters 2000). This may herald a need for long term storage of immature pollen, but such storage has not been addressed yet (Towill and Walters 2000). Furthermore, it is known that cultures of haploid cell are highly unstable, and that they revert easily to the diploid status. The cryopreservation of haploid material, like microspores, is therefore of utmost importance for the conservation of genetically stable germplasm, to be used in breeding programs.

For the collection of pollen from many species flowers must be gathered just before anthesis, and the stamens or anthers excised. Then, the pollen or the anthers are dissected to a pre-determined required moisture level. In some species, the pollen must be separated

from the anther before desiccation, while in others drying of the whole anther followed by gentle crushing is sufficient for storage and use (Towill and Walters 2000).

The ultimate goal when preserving pollen is to retain high viability and functionality. In fact, viability is an essential parameter in the evaluation of adaptation to various stresses, such as freezing tolerance (Leborgne et al. 1995). Thus, the determination of pollen viability is crucial for any conservation study (Towill and Walters 2000). Pollen viability can be determined by measurement of the metabolic activity (usually respiration rate), membrane semi-permeability and germination (Towill and Walters 2000). Metabolic activity is rarely used as a method, probably due to the difficulty in the quantification, requiring a high amount of pollen, besides being time consuming (Towill and Walters 2000). Simple measures of membrane semi-permeability are much commonly applied (Towill and Walters 2000). Potassium leakage into an osmotically adjusted medium is one test (Towill and Walters 2000). Tetrazolium dyes can be used to determine the ability of the membrane to retain some reducing agent (ex. nicotinamide adenine dinucleotide - NADH) such that the tetrazolium becomes reduced upon permeation, usually forming an insoluble, coloured formazan (Towill and Walters 2000). Another common method to access pollen viability is the fluorescein diacetate (FDA) test (Widholm 1972, Heslop-Harrison et al. 1984, Towill and Walters 2000), which is generally considered the most rapid and accurate staining test for viability (Towill and Walters 2000).

The storage of immature pollen has been rarely reported (Chen and Beversdorf 1992), and there are a few reports on the cryopreservation of pollen of different species, namely *Simmondsia chinensis* (Lee et al. 1985), *Panax ginseng* (Zhang et al. 1993), *Dioscorea rotundata* (Ng and Daniel 2000), *Hevea* (Hamzah and Leene 1986) and pine species (Lanteri et al. 1992). However, in carob, there are no reports on the cryopreservation of any type of explant.

In this chapter it was compared the capacity of different types of cryoprotectants, namely carbohydrates (sorbitol, mannitol, sucrose and glucose), and other chemical substances (DMSO, glycerol, proline, ethyleneglycol and polyetileneglycol, applied alone or in mixtures), to induce freeze tolerance in immature pollen of carob. Those cryoprotectants were applied at different concentrations as a pretreatment to anthers excised from male and hermaphrodite flowers at developmental phases I and II. Two storage periods were assayed when carbohydrates were used as cryoprotectants. It was also

assayed if the presence of the cryoprotectant during the storage period was beneficial for pollen viability.

6.2. Materials and methods

6.2.1. *Plant material*

For many plant species the most suitable microspore stage for the induction of androgenesis is the late uninucleate to early binucleate stage (Bhojwani and Razdan 1983). The cytological studies done during this work (*vide* Chapter II) indicate that in carob, for the induction of pollen embryogenesis, anthers should be excised from flowers in developmental stages I and II, since in both phases we have observed uni- and binucleate microspores. As a consequence, in this chapter we used male and female inflorescences of carob with flowers at developmental stages I and II, having in mind its potential for androgenesis induction.

6.2.2. *Pollen cryopreservation*

6.2.2.1. Use of cryoprotectants

Inflorescences with flowers at developmental stages I and II (*vide* chapter II) were collected and surface sterilized with a commercial bleach solution (10%, v/v) for 10 min, followed by tree washes in sterile distilled water. The anthers were isolated, placed in eppendorf tubes on ice and dehydrated with six types of cryoprotectants at different concentrations: DMSO (3.5, 5, 7 and 10%, v/v), glycerol (5, 10 and 15%, v/v), glycerol + DMSO (2.5% glycerol + 2.5% DMSO, 1% glycerol + 5% DMSO and 5% glycerol + 5% DMSO, v/v), proline (10%, p/v), ethyleneglycol (2.5%, v/v) and polyetileneglycol (10%, v/v). Dehydration was made by the addition of 1 ml of the cryoprotective solution to each tube, divided in 4 applications of 0.25 ml each, at 5 min intervals. This gradual addition of the cryoprotectant is very important, because a sudden addition causes plasmolysis of the cells (Bajaj and Reinert 1977). During this time the tubes containing the anthers were maintained on ice, since the exposure of the cells to the cryoprotectant at room temperature

adversely affects their viability (Bajaj and Reinert 1977). After 20 min, the tubes were plunged into LN for 1 h, and stored at –80°C during 5 months. In other assay, the anthers were cryopreserved without the addition of the cryoprotective solution.

6.2.2.2. Use of carbohydrates as cryoprotectants

Inflorescences with flowers at developmental stage II (*vide* chapter II) were collected and surface sterilized as described on section 6.2.2.1. The flowers were picked from the inflorescences, the anthers isolated, placed on test tubes and pre-treated with four types of carbohydrates, used as cryoprotectants, at different concentrations: sorbitol (0.5, 1 and 2 M), mannitol (0.5 and 1 M), sucrose (0.5, 1 and 2 M) and glucose (0.5, 1 and 2 M). The pretreatment consisted of the addition of 10 ml of the cryoprotective solution to test tubes containing the anthers, and their maintenance in a roller at 25 rpm for 24h. After the pretreatment period, the cryoprotective solution was drained off, the anthers were placed in eppendorf tubes on ice, dehydrated by the addition of 500 ml of the cryoprotective solution to each tube, immediately plunged into LN for 1 h and stored at –80°C during 5 and 8 months. In other assay, the anthers were cryopreserved without the addition of the cryoprotective solution.

6.2.3. *Evaluation of microspore viability*

After storage, the samples were rewarmed by a 15 min immersion in a 40°C water bath, and the cryoprotectant solution was drained off. The anthers were washed with liquid basal medium MS, supplemented with 2% sucrose, 2 mg l^{-1} BA and 1 mg l^{-1} of 2,4-D. For each treatment 2 eppendorf tubes were used with 50 anthers each. As a control, 100 anthers per sexual type and stage of flower development were used. Those anthers were treated with the respective treatment, except that no cryoprotectant solution was added.

After recovery from LN plant tissues will contain living, weakened and dead cells. For an efficient screening of a wide range of cryopreservation protocols and techniques, it is essential to have several or at least one good test(s) available, to allow a quick and

accurate prognosis of the efficiency of the cryoprotection and to forecast the chances of a quick recovery of a considerable number of explants (Verleysen et al. 2004).

Microspore viability was assessed by using the fluorosceín diacetate (FDA) test (Widholm 1972, Heslop-Harrison et al. 1984). This test uses the FDA, which is permeable and is cleaved within the cell by non-specific esterases to form fluoresceín (Widholm 1972, Heslop-Harrison et al. 1984, Towill and Walters 2000). Fluoresceín is impermeable and can be detected by cellular fluorescence. If the cell has lost membrane semi-permeability, the cleavage is slow and the product is not concentrated, otherwise it will appear fluorescent (green). The FDA test is generally considered the most rapid and accurate staining test for viability (Towill and Walters 2000).

A stock solution of FDA in a concentration of 0.5% (w/v) was prepared in acetone (100%, v/v), and kept in the dark at -20°C. For determination of microspore viability, a fresh solution was prepared, by diluting 0.1 ml of the stock solution of FDA in 5 ml of liquid MS basal medium, without growth regulators. For staining, 3 anthers were squashed on a microscope slide in 40 µl of liquid MS basal medium without growth regulators and 40 µl of the diluted FDA solution. The anther debris where removed, a cover slip was added, and after 5-10 min the samples were observed in a microscope equipped with fluorescence. Photographs were taken, scanned and subsequently prepared with Adobe Photoshop. For each treatment, at least 2 slides were prepared and stained, and the viable microspores were counted in at least 10 optical fields *per* slide.

6.2.4. *Statistical analysis*

The results presented are the mean ± SE of at least 20 observations and all the experiments were carried out at least 2 times. The data were subjected to analysis of variance (ANOVA) to assess treatment differences and interactions using the SPSS statistical package for Windows (release 11.0, SPSS INC). Significance between means was tested by Duncan's New Multiple Range Test (P=0.05). Arcsin square root transformation was used prior to analysing data.

6.3. RESULTS AND DISCUSSION

6.3.1. *Use of cryoprotectants*

The application of the cryoprotectants as pretreatment, and its presence during the storage period generally increased pollen viability, comparatively to the control (Tables 6.1 and 6.2). It is known that the cryoprotectants can reduce the temperature at which freezing initiates and alter the crystal state of ice, and also minimise the deleterious action of electrolyte concentration resulting from the conversion of water in ice (Watanabe et al. 1999). Cryoprotectants facilitate the flow of water across the cell membrane, and protect both molecular and gross structures through a range of modes of action, including colligative effects and free radical scaveging (Finkle et al. 1985). Furthermore, the addition of osmotically active compounds can lead to a reduction in cell size and thus, in an increase in freeze tolerance (Withers and Engelmann 1997).

The analysis of variance of the percentage of viable cryopreserved pollen after pretreatment with DMSO, indicates that this pollen viability was significantly affected ($P<0.001$) by the concentration of the cryoprotectant, the sexual type of the tree, the stage of flower development and by the possible interactions between those variables, except for sexual type *vs* stage of flower development (Table 6.1).

The viability of pollen in anthers from male flowers at stage I was statistically higher than the obtained with anthers in flowers at stage II, except for the control and DMSO 7% (Table 6.1). That fact can be related with specific characteristics of the anthers, which are younger, with smaller cells, less vacuoles, a denser cytoplasm and a higher nucleus/cytoplasm ratio, which make them more appropriate for cryopreservation (Bajaj 1995). Furthermore, it is known that the cryoprotectant solutions can cause severe damages in the cryopreserved material, and that those damages are more frequent in larger organs (Reed 2001, Reed et al. 2001).

Table 6.1. Effect of DMSO on pollen viability in anthers from male and hermaphrodite flowers at developmental stages I and II.

DMSO (%, v/v)	Viability (%)			
	Male flowers		Hermaphrodite flowers	
	Stage I	**Stage II**	**Stage I**	**Stage II**
0	B17 b	A33 b	A44 d	B17 b
3.5	A46 a	B28 bc	A68 a	B37 a
5	A45 a	B7 d	A58 bc	B37 a
7	44 a	45 a	A61 b	B37 a
10	A45 a	B23 c	55 c	41 a
Significance of three-way ANOVA				
Concentration (A)			***	
Sexual type (B)			***	
Stage of flower development (C)			***	
A × B			***	
A × C			***	
B × C			ns	
A × B × C			***	

Values represent means ± SE of a minimum of 20 observations. In each column, statistical comparisons were made between different concentrations of DMSO for the same stage of flower development, and are shown in lower case letters. In each row, statistical comparisons were made for each sexual type between different stages of flower development, for the same concentration of DMSO, and are shown in capital letters. Values followed by different letters are significantly different at $P<0.05$. Absence of letters indicates no differences $P\geq0.05$. ns, *, **, ***: non significant, significant at $P<0.05$, significant at $P<0.01$ and significant at $P<0.001$, respectively (Three-way ANOVA, Duncan's New Multiple Range Test).

With anthers taken from male flowers at stage I, the viability of pollen was similar between DMSO concentrations, and statistically higher than the observed for control (Table 6.1). With anthers taken from flowers at stage II the best results were obtained with DMSO 7% (Table 6.1). In the hermaphrodite flowers, the highest pollen viability was observed with DMSO 3.5% applied to anthers taken from flowers at stage I, while in stage II the viability of pollen was similar between DMSO concentrations (Table 6.1).

Comparing the same concentrations and stages of flower development for both sexual types, we generally observed higher pollen viability in the hermaphrodite flowers (Table 6.1). This may suggest that in carob, pollen viability is affected not only by the stage of

flower development from which the anthers are taken, but also by the sexual type of the donor plant.

DMSO is normally used in the cryopreservation of different types of plant material. When applied alone it allowed high survival rates in different species, namely in embryogenic *calli* of citrus (Pérez et al. 1999), shoot tips of *Solanum tuberosum* (Harding and Staines 2001), meristems of *Ribes* spp (Reed et al. 2001), and *calli* and cellular suspensions of *Populus* spp (Lambardi 2002).

The results of the effect of the application of different types and concentrations of cryoprotectants on pollen viability in anthers excised from male flowers at stage II are summarized on Table 6.2.

Table 6.2. Effect of the application of different types and concentrations of cryoprotectants on pollen viability in anthers excised from male flowers at developmental stage II.

Cryoprotectant solution		Viability (%)
Control		33 fg
DMSO	3.5%	28 h
	5%	7 j
	7%	45 e
	10%	23 i
Glycerol	5%	74 b
	10%	62 d
	15%	47 e
Glycerol 2.5% + DMSO 2.5%		88 a
Glycerol 1% + DMSO 5%		37 f
Glycerol 5% + DMSO 5%		69 cd
Proline 10%		66 cd
Ethyleneglycol 2.5%		43 e
Polyetileneglycol 10%		31 gh

Values represent means ± SE of a minimum of 20 observations. Statistical comparisons were made in the column, between the different types and concentrations of cryoprotectors. Values followed by different letters are significantly different at $P<0.05$ (one-way ANOVA, Duncan's New Multiple Range Test).

When glycerol was used as cryoprotectant we obtained high percentages of viability, corresponding the higher value (74%) to the lower concentration tested (Table 6.2). Glycerol rarely produces positive results when applied alone, and is usually used in combination with other cryoprotectants (Bajaj 1984, de Carlo et al. 2000, Matsumoto et al. 2001, Reed et al. 2001). However, it was successfully used in *calli* and cellular suspensions of *Populus* spp (Lambardi 2002).

When applying the solution containing both glycerol and DMSO, the best results were obtained with the lower concentrations tested (2.5% glycerol + 2.5% DMSO), which led to the maximum viability obtained in this work (88%) (Table 6.2).

Doubling the concentrations of both glycerol and DMSO (5% glycerol + 5% DMSO), resulted in a significant decrease of pollen viability, from 88% to 69% respectively (Table 6.2). Comparing the solutions containing 1% glycerol + 5% DMSO and 5% glycerol + 5% DMSO, it was observed that increasing concentrations of glycerol resulted in a significant increase in pollen viability, clearly confirming the importance of this substance as cryoprotectant. These results are in agreement with previous reports, which attribute a higher cryoprotective efficacy to mixtures of cryoprotectants, when compared to the use of the same substances alone (Withers and Engelmann 1997). A cryoprotectant solution consisting of glycerol + DMSO was also successfully used in cereals, in cryopreservation of segments of androgenic anthers (Bajaj 1984), and in *calli* and cellular suspensions of *Populus* spp (Lambardi 2002).

We observed that the application of the remaining cryoprotectants increased pollen viability comparatively to the control, except for polyetileneglycol (Table 6.2). Polyetileneglycol is generally used in combination with other cryoprotectants (Reed 1990, Häggman et al. 1998), and our results confirm its inefficacy when applied alone.

The use of proline conduced to good results, contrary to the reported for other species, where its use did not result in tolerance of the cells to freezing (Göldner et al. 1991). Eyhyleneglicol is a cryoprotectant generally used in vitrification, in mixtures of cryoprotectants (de Carlo et al. 2000, Matsumoto et al. 2001, Reed et al. 2001, Lambardi 2002).

6.3.2. *Use of carbohydrates as cryoprotectants*

In the previous section it was observed that the viability of pollen in anthers from male flowers at stage I was higher than the obtained with anthers in flowers at stage II. However, due to the unavailability of enough material to perform all the assays, the results presented in this section refer to the experiments done with anthers excised from flowers at stage II.

Analysis of variance of the percentage of viable cryopreserved pollen in anthers from flowers at stage II, after pretreatment with equimolar concentrations of different types of cryoprotectants, indicates that this pollen viability was significantly affected by the type and concentration of the carbohydrate ($P<0.001$), the addition of the cryoprotector during storage ($P<0.001$), the length of the storage period ($P<0.001$), and by some of the possible interactions between these variables (Table 6.3).

As observed earlier (*vide* section 6.3.1), the application of the cryoprotectants as pretreatment was in generall beneficial for pollen viability (Table 6.3). Regardless of the concentration of the cryoprotectant and the length of the storage period, a significant promotion of pollen viability was observed when sorbitol ($P<0.05$) and glucose ($P<0.01$) were added during storage (Table 6.3). The osmotic conditioning with sugars, such as sucrose or sorbitol, is often used for cryopreservation by vitrification (Verleysen et al. 2004). However, there are cases where the viability of the cryopreserved sample is considerably lower than that of the uncooled sample, and reasons for this reduced viability are not apparent (Engelmann 2000).

Regardless of the concentration of the carbohydrate used as cryoprotectant, its addition during storage and the length of the storage period, the best results were obtained with sucrose ($P<0.001$). Sucrose is one of the most commonly employed cryoprotective substance in pretreatments (Engelmann 1992). Pre-culturing with sucrose has shown to be very important in improving the survival of cryopreserved shoot tips of several species, namely *Ipomea batatas* (Pennycooke and Towill 2000), *Populus* spp (Lambardi 2002), *Prunus domestica* (de Carlo et al. 2000), *Diospyros kaki* (Matsumoto et al. 2001), *Malus domestica* (Niino et al. 1992) and *Holostemma annulare* (Decruse et al. 2004).

Table 6.3. Effect of the application of different concentrations of sorbitol, mannitol, sucrose and glucose to anthers excised from flowers at stage II, influence of the presence of cryoprotective solution during the storage period and effect of the length of the storage period, on the viability of pollen from male flowers of carob.

Concentration (M)	Addition of cryoprotector during storage	Storage period (months)	Sorbitol	Mannitol	Sucrose	Glucose
Control		5	33 ab	33 b	33 cde	33 cd
		8	8 e	8 c	8 h	8 g
0.5	+	5	C37a	B48 a	A64 a	A65 a
		8	B6 ef	B10 c	A31 de	A25 e
	−	5	C29 bc	B46 a	A62 a	B46 bc
		8	B7 e	B8 c	A22 f	nd
1	+	5	C3 c	nd	A48 b	B43 b
		8	13 d	nd	16 fg	14 f
	−	5	B14 d	nd	A37 cd	A37 bcd
		8	B7 e	nd	A16 fg	B12 g
2	+	5	B8 e	nd	A38 c	A37 bcd
		8	C5 f	nd	A16 g	B9 g
	−	5	B2 e	nd	A28 e	A30 de
		8	C3 f	nd	A16 fg	B7 g

Significance of four-way ANOVA	
Cryoprotectant (A)	***
Concentration (B)	***
Addition of cryoprotector during storage (C)	***
Duration of storage (D)	***
A × B	***
A × C	***
A × D	***
B × C	ns
B × D	***
C × D	***
A × B × C	***
A × C × D	ns
A × B × C × D	***
B × C × D	ns

Values represent means ± SE of a minimum of 20 observations. In each column, statistical comparisons were made between all the variables, for the same cryoprotector, and are shown in lower case letters. In each row, statistical comparisons were made between all the cryoprotectors, and are shown in capital letters. Values followed by different letters are significantly different at $P<0.05$. ns, *, **, ***: non significant, significant at $P<0.05$, significant at $P<0.01$ and significant at $P<0.001$, respectively (four-way ANOVA, Duncan's New Multiple Range Test). *nd: not determined.*

Furthermore, sucrose has been successfully used in the cryopreservation of microspores of *Brassica napus* (Chen and Beversdorf 1992), embryogenic suspension cells of *Oryza sativa* (Zhang et al. 2001) and embryogenic cultures of *Quercus suber* (Valladares et al. 2004). Two major hypotheses exist concerning the mode of action of sucrose in dessication tolerance. First, it could replace the water molecules involved in the maintenance of macromolecular structure (Crowe et al. 1988); secondly, it could indunce vitrification of the intracellular medium at biological temperatures (Koster 1991).

Sorbitol was the less effective cryoprotectant in inducing freezing tolerance in carob pollen (Table 6.1), similarly to the observed by Göldner et al. (1991) in cell cultures of *Digitalis lanata*. Nevertheless, it has been successfully used as pre-treatment in the cryopreservation of embryogenic cultures of larch and black spruce (Klimazewska et al. 1992), *Oryza sativa* (Zhang et al. 2001), and *Pinus patula* (Ford et al. 2000). Sorbitol is widely applicable as a pre-freezing additive (Withers 1986). It operates by a combined dehydrative and physiological effect upon individual cells, rather than by providing an environment within which cells grow to a higher degree of freeze tolerance (Withers 1986).

There were no statistically significant differences between pollen viability induced by mannitol and glucose (Table 6.1). In cryopreservation of cell cultures, mannitol has been successfully used in *Digitalis lanata* (Göldner et al. 1991), while others species do not tolerate the treatment with this carbohydrate (Chen et al. 1984). Glucose is another carbohydrate often employed as a pretreatment in cryopreservation studies (Whithers and Engelmann 1997), although it is more commonly used as a component of cryoprotectant solutions (Yamada et al. 1991, Zhang et al. 2001).

Regardless of the type of cryoprotectant, its addition during storage and the length of the storage period, we observed that pollen viability decreased with increasing concentrations of the cryoptotector (P<0.001) (Table 6.1). It is known that cryoprotectants can be toxic (Reed 2001, Reed et al. 2001), and high concentrations have been reported to have deleterious effects on cell viability (Göldner et al. 1991, Pennycooke and Towill 2000). However, high levels of sugar or sorbitol accumulated during pre-culture have been reported to be crucial in improving the survival of cryopreserved cells and meristems of different species (Uragami et al. 1990, Dereuddre et al. 1991).

It was observed that the pollen viability decreased significantly with the increase of the storage period (Table 6.1). There are several works reporting a decline in pollen viability with increasing storage periods (Hamzah and Leene 1986, Lanteri et al. 1992). Cryopreservation has the potential to suspend metabolism and, to all intents and purposes, suspending time (Withers and Engelmann 1997). Thus, theoretically cells can retain viability during hundred of years (Towill and Walters 2000). However, during cryopreservation, alterations of the physical and biochemical stability of the system can occur (Reed 2001), which may lead to significant losses in cell viability.

6.4. Conclusions

In this chapter it was observed that the both the application of the cryoprotectants as pretreatment and their presence during the period of storage generally promoted pollen viability compared with the control, regardless of the type of cryoprotectants used. The anthers excised from flower at developmental stage I were more suitable for cryopreservation. Furthermore, pollen viability decreased significantly with the increase of the storage period. When we used carbohydrates as cryoprotectants, pollen viability was not significantly affected by the developmental stage of the flowers from which the anthers were taken, and the most effective treatment consisted of a pretreatment with 0.5 M sucrose and a storage period of 5 months.

When DMSO was used as cryoprotectant we observed that pollen viability was affected not only by flower development stage but also by the sexual type of the donor plant, with the best results observed with anthers taken from hermaphrodite flowers at stage I. When we tested the use of other chemical substances, we observed that the use of solutions containing combination of cryoprotectants was more effective than the use of cryoprotectants alone, and that the most effective in inducing resistance to freezing tolerance was the one containing glycerol (2.5%) + DMSO (2.5%). With the use of different carbohydrates as cryoprotectants, we observed that the maximum percentages of viability were lower than those observed with the application of other chemical substances.

Although there is a need to increase the viability levels, and to evaluate the response of cryopreserved microspores to induction androgenesis, our results show that the

cryopreservation of immature pollen of carob is possible, by using a relatively simple technique and maintaining high viability.

Pollen cryopreservation may indicate the way forward for the conservation of genetic diversity. It has the advantage of being a relative low-cost option, but the disadvantage that only paternal material would be conserved and regenerated. It may be of use in certain cases, and where there are serious difficulties with the application of other methods. Nevertheless, pollen storage is not a widely-used method of *in vitro* conservation. Since pollen is haploid it would need to be used for crossing with living material, thought it might in the future be used to regenerate haploid plants. No generalised schedules have been developed as yet and much more research is needed, since the development of cryopreservation procedures implies the evaluation of the unique characteristics of each particular type of species and explant.

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CHAPTER VII

IN VITRO CULTURE OF ANTHERS

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7.1. INTRODUCTION

Embryogenesis has evolved as a successful strategy for the reproduction of higher multicellular organisms, and in plant species is a unique process in the sense that it can be initiated from a wide range of cells other than the zygote (Fig. 7.1) (Maraschin et al. 2005).

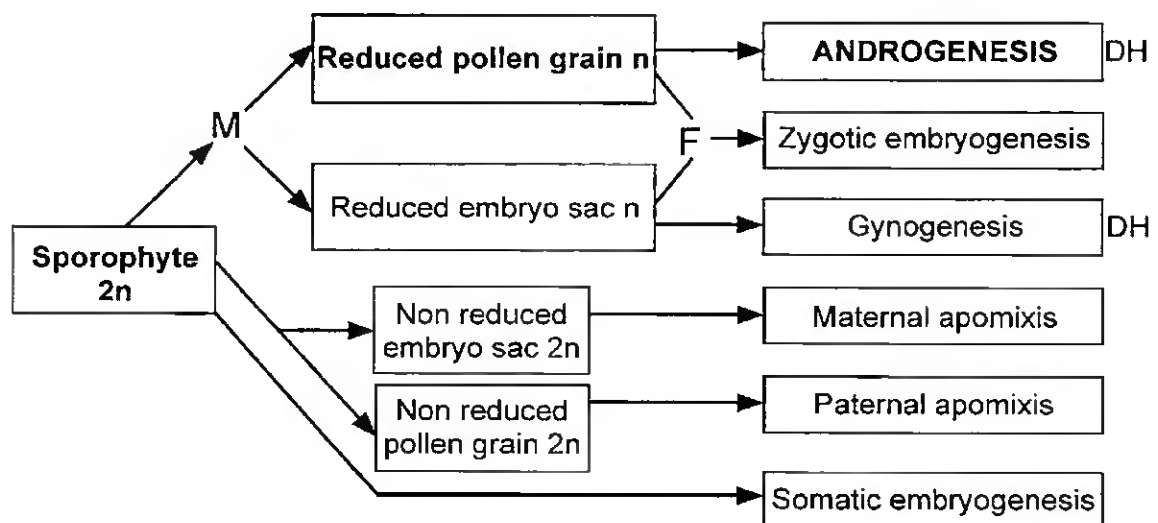

Figure 7.1. Overview of the different types of cell structures that can undergo embryogenic development in higher plants. F, fertilization; DH, double haploid; M, mitosis (Maraschin et al. 2005).

Clonal propagation is usually achieved via the induction of *in vitro* somatic embryogenesis, a process that is defined as the regeneration of a whole plant from undifferentiated somatic cells in culture. Depending on the donor tissue and the induction treatment conditions, embryos may develop either directly from single cells or indirectly through an intermediary *callus* phase (Zimmerman 1993). Additional routes to *in vitro* embryogenesis are defined by the ability of male or female gametophytes to irreversibly switch from their gametophytic pathway towards an embryogenic route.

While androgenesis refers to the development of embryos from microspores or immature pollen grains (Touraev et al. 1997), gynogenesis refers to the *in vitro* or *in vivo* development of embryos from unfertilized ovaries (Musial et al. 2001). By contrast to

apomixis and somatic embryogenesis, which lead to clonal propagation of a specific genotype, androgenic and gynogenic plants reflect the product of meiotic segregation. Thus, they have the remarkable characteristic of possessing only one set of chromosomes, and therefore are haploid plants.

Doubled haploid plants (DH) are derived from haploid cells or tissues (microspore, androgenic structures, *callus*) by doubling its chromosome number. This occurs either spontaneously or after a treatment with doubling agents, such as colchicine, oryzalin, pronamide or amiprophosmethyl (Schmidhalter-Saisingtong 1998). The main advantages of using DH plants in plant breeding includes the rapid development of completely homozygous lines within one generation, and efficient means of genotypic selection (Nitch 1981, Dunwell 1985, Guo et al. 1999, Maraschin et al. 2005). Such a rapid route to homozygosity has great potential in plant breeding as an alternative to repeated cycles of inbreeding in self-pollinating crops (Dunwell 1985). In cross-pollinating species, DH are more likely to be used as parents in the production of single or double cross hybrids (Dunwell 1985). Furthermore, haploidy may be of considerable use in studies on the quantitative genetics of crop species. Such studies include the detection of gene interaction, estimation of genetic variances, detection of linkage, estimation of the number of genes affecting a quantitative character and the location of polygenes (Dunwell 1985, Marachin et al. 2005).

The technique of *in vitro* androgenesis to produce DH plants came into existence in 1964, when Guha and Maheshwari's discovered that pollen grains from isolated anthers of *Datura* can switch from their normal gametophytic developmental mode to an embryogenic pathway and develop into haploid embryos. Since then, haploid plants obtained by *in vitro* embryogenesis from haploid male gametophytes have been reported in more than 50 genera (Nägeli 1998), and the greatest effort has been given to economically important plants such as the vegetable crops and cereals (Veilleux 1994, Cao et al. 1995).

The underlying principle of androgenesis is to stop the development of the microspore, whose fate is to become a sexual cell, and to force its embryogenic development directly into a plant, as it is done with somatic cells (Nitsch 1981). In culture, microspores undergo various modes of androgenesis which can lead to the formation of haploids either directly by embryogenesis, or indirectly via *callus* formation (Reinert and Bajaj 1977) (Fig. 7.2). During *in vitro* androgenesis the embryogenic development can be

divided in three main characteristic, overlapping phases: I) acquisition of embryogenic potential by stress, involving repression of gametophytic development and leading to the dedifferentiation of the cells; II) cell divisions lead to the formation of multicellular structures (MCSs) contained by the exine wall; and III) embryo-like structures (ELS) are released out of the exine wall and pattern formation takes place (Maraschin et al. 2005).

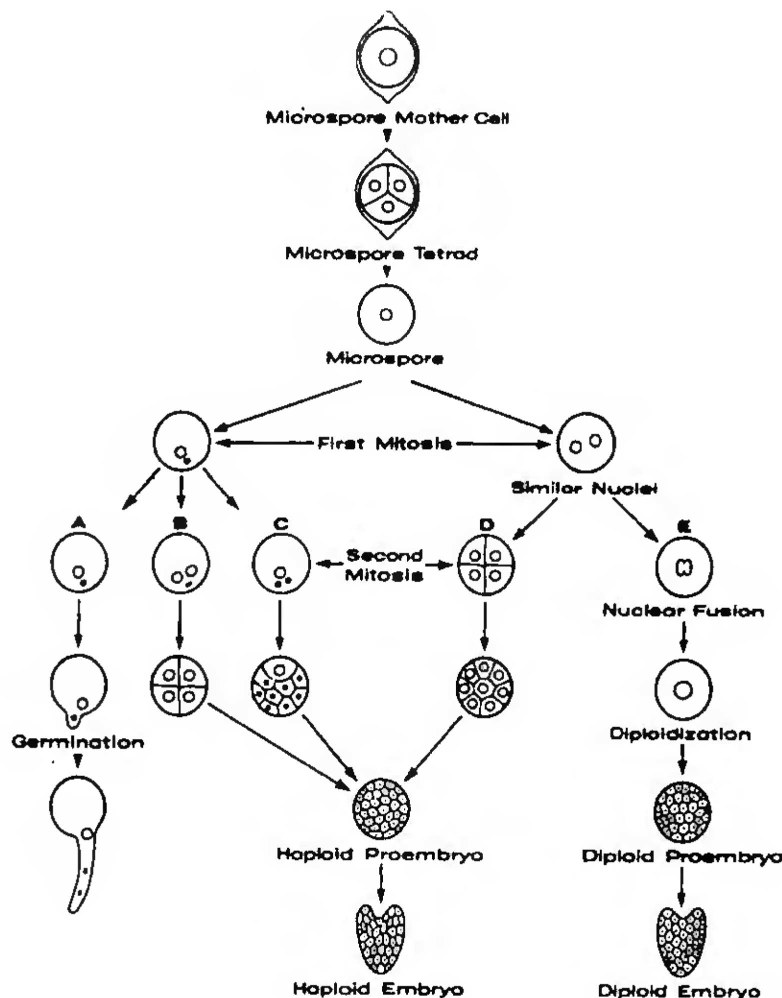

Figure 7.2. Schematic representation of microsporogenesis, and various modes of development of pollen under *in vivo* and *in vitro* conditions. (A) Normal second mitosis of pollen *in vivo* forming two sperms and the germination of pollen to form a pollen tube; (B-E) *In vitro* behaviour of pollen; (B) Repeated division of the vegetative nucleus and the abortion of the generative nucleus; (C) Formation of a haploid embryo as a result of repeated division of the generative nucleus, while the vegetative nucleus aborts; (D) Haploid embryo formation as a result of repeated division of two similar nuclei of a pollen; (E) Homozygous embryo formation as a result of repeated division of two similar nuclei of a pollen; and (E) Homozygous diploid embryo formed by fusion of two similar nuclei of the pollen after first mitosis (Reinert and Bajaj 1977).

There are two techniques that can be used to induce androgenesis: anther culture and culture of isolated microspores (or pollen culture) (Nitsch 1974, 1981, Bhojwani and Razdan 1983, Dunwell 1985, Sangwan and Sangwan-Norreel 1990) (Fig. 7.3).

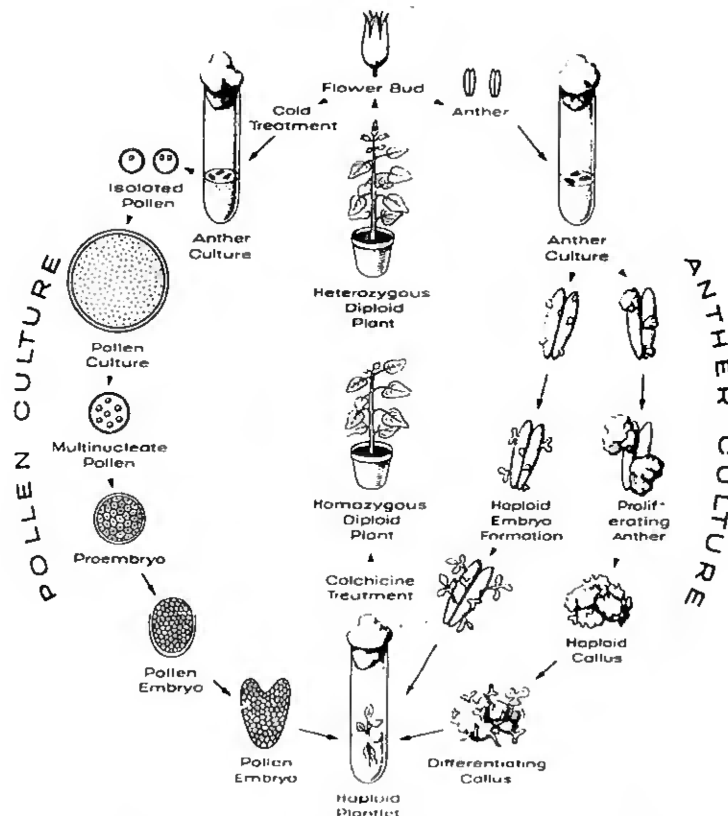

Figure 7.3. Schematic representation of anther and pollen culture and the development of haploid plants directly by embryo formation, or through haploid *callus* (Reinert and Bajaj 1977).

Anther culture is the most important and commonly used technique, and consists of plating the entire anthers on a culture medium. Although anther culture has proved to be quite efficient for the induction of haploids, it has one main disadvantage: the plants do not only originate from pollen but also from various other parts of the anther, generally resulting in a population of plants with various ploidy levels (Dunwell 1985, Sangwan and Sangwan-Norreel 1990). If the proliferation of anther wall cells occurs concomitant with the callusing of the microspores, the tissue finally derived would not be of pure gametophytic origin (Bhojwani and Razdan 1983, Dunwell 1985, Sangwan and Sangwan -

Norrell 1990). In some cases *calli* can be initiated only from anther tissues, with no division of the microspores (Faure et al. 1996). Even when the embryoids formed are from microspore origin, the occurrence of secondary embryogenesis can result in the development of dihaploid plants (Hidaka 1984a). On this basis, diploid plants obtained through anther culture can be divided in three groups, depending on their origin: plants regenerated from normal reduced gametes (microspores) followed by chromosome doubling; plants regenerated from somatic cells from the anther; and plants regenerated from unreduced gametes (Chani et al. 2000).

The culture of isolated microspores consists of the mechanical extraction of the microspores from the anthers and their inoculation on an appropriate induction medium. There are several methods for the culture of isolated microspores, namely nurse culture (Sharp 1972) and shed pollen (Sunderland and Roberts 1977). Although anther culture is much simpler in handling, the culture of isolated micropores shows several important advantages, namely the fact that the formation of *calli* and embryos from somatic tissues of the anther is avoided, and the elimination of unknown effects of somatic tissues (Říhová and Tupý 1999). Since this technique allows the direct access to the microspores there is the possibility to manipulate their developmental pathway (Říhová and Tupý 1999), and to speed up the optimization of culture conditions (Touraev et al. 1996a). Moreover, in certain plant species, the number of embryos per anther obtained in isolated microspore culture is higher than in anther culture (Höfer 2004). Furthermore, the culture of isolated microspores provides an accessible haploid system for biochemical and molecular analyses and for *in vitro* selection for desirable traits (Jähne and Lörz 1995) without the interference of the anther wall, and also supplies a target for genetic transformation (Touraev et al. 1997). The main disadvantage of this technique is that the isolation of the microspores from the anthers generally reduces its viability and thus their survival and development in culture (Dunwell 1985).

There are several factors that influence the *in vitro* androgenesis. Genotype is in certain cases the major factor affecting both anther culture response and regeneration rates (Chani et al. 2000, Germanà and Chiancone 2003). The donor plant must be in an optimum state of nutrition, irrigation and environmental conditions, and the seasonal effects are known to greatly influence the number of haploid embryos obtained by *in vitro* androgenesis (Höfer 1997, Chani et al. 2000, Ritala et al. 2001). The stage of pollen

development at the time of inoculation is critical for the success of androgenesis (Nitsch 1981, Bhojwani and Razdan 1983, Dunwell 1985, Sangwan and Sangwan-Norreel 1990). This stage is genotypic dependent, and for many plant species it is generally accepted that the most suitable for the induction of androgenesis is the late uninucleate to early binucleate stage (Nitsch 1981, Sangwan and Sangwan-Norreel 1990, Chani et al. 2000, Ritala et al. 2001). It is widely accepted that when the vegetative cytoplasm of binucleate pollen starts to accumulate starch, androgenesis can no longer be triggered (Touraev et al. 1997).

A number of stress treatments, which are needed to trigger androgenesis, can be applied to excised floral buds, inflorescences or anthers before culture, namely temperature (Germanà and Chiancone 2003, Höfer 2004), sugar starvation (Höfer 2004), auxin (Hoekstra et al. 1996), chemicals, gamma irradiation (Zheng et al. 2001), and colchicine (Redha et al. 1998) applied as single or combined treatments. However, there are no standard recommended pre-treatments, and for each plant species the exact type and duration of treatment must be determined (Nitsch 1981, Sangwan and Sangwan-Norreel 1990, Maraschin et al. 2005). Since so many stress factors can trigger the reprogramming of microspores into embryos, it is likely that the initiation of androgenesis is induced by converging signalling pathways, although, of course, different stress signals may trigger the same downstream pathways (Maraschin et al. 2005).

For *in vitro* androgenesis the most commonly used basal media is Murashige and Skoog (1962) (Dunwell 1985), and several different components of the medium influence the success of anther or the culture of isolated microspores, namely the physical state, the osmotic pressure, mineral salts, vitamins, hormones and organic and other nitrogen supplements (Novotný et al. 2000, Rimberia et al. 2004). Furthermore, the incubation conditions (temperature, light, culture vessel, explant orientation and density of anthers/microspores) are also known to affect the embryogenic response (Dunwell 1985).

Besides the factors that affect the *in vitro* androgenesis, the environmental conditions in which the cultures are placed after the achievement of embryos or embryogenic *calli* can enhance the differentiation of the globular embryos into normal plantlets (Nitsch 1981, Dunwell 1985). Generally, for plant development and/or embryo conversion, the *calli* and/or embryos must be transferred to a medium with different formulation, namely with reduced concentration of carbohydrates and growth regulators (Dunwell 1985).

The improvement of carob tree is hampered by its highly heterozygous nature and long reproductive cycle. The *in vitro* production of haploids is extremely valuable in plant breeding and genetics, and with haploids, the establishment of homozygous lines of new varieties is possible in a short period of time, resulting in an important reduction in the number of selection generations. This is particularly important for a highly heterozygous, long-generation tree species such as carob.

There is to date no report of the induction of androgenesis in carob. Therefore, in this chapter we studied some factors that may influence the callogenesis, in order to induce *in vitro* androgenesis by anther culture. The culture of isolated microspores was also attempted.

7.2. MATERIALS AND METHODS

7.2.1. *Plant material*

This work was done during the flowering period of 2001 (June to December). Young inflorescences were collected from one of the male trees referred to in chapter II (tree M8.1), thoroughly washed under running tap water, surface pre-sterilized by immersion in a benlate solution (3 g l^{-1}, w/v), for 10 min, washed 3 times with sterile distilled water, immersed in a commercial bleach solution (10%, v/v) for 10 min, and finally rinsed three times with sterile distilled water. Flowers at developmental stages I and II, with microspores at the late uninucleate to early binucleate stage (*vide* chapter II), were taken to the laminar air-flow cabinet, and further operations were carried out under aseptic conditions.

In order to control contaminants, different sterilizing agents at different concentrations and time of exposure were tested, namely commercial bleach and calcium hypochloride ($Ca(ClO)_2$ [5, 10 and 15% (v/v), during 2, 5, 10, 15 and 20 min] and mercuric chloride ($HgCl_2$) [0.1, 0.25 and 0.5% (w/v), during 2, 5, 10 and 20 min], in a total of 35 treatments tested. After surface disinfection, flowers were rinsed three times in sterile distilled water.

7.2.2. *Anther culture*

After disinfection, the filaments were removed and the anthers were plated horizontally, with the connective in contact with the medium, in Petri dishes (100 × 100 mm) containing 30 ml of induction medium. Petri dishes, each containing 10 anthers, were then sealed with stretch film. After eight weeks of culture the number of anthers showing browning and the number of anthers producing *calli* was registered. The nature of the *calli* produced (embryogenic or non-embryogenic) was assessed by acetocarmine (4%) staining and subsequent light microscopic observation. The *calli* were then transferred to regeneration medium.

The induction medium consisted of Murashige and Skoog (MS) mineral salts and vitamins, 800 mg l^{-1} L- glutamine, 100 mg l^{-1} serine, 4% (w/v) sucrose, 0.2% (w/v) gelrite, and it was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (0.2 and 0.5 mg l^{-1}) combined with 1, 2 or 3 mg l^{-1} of the following cytokinins: N^6-benzyladenine (BA), kinetin (Kin), zeatin (Zea) and thidiazuron (TDZ). The regeneration medium had the same composition of the induction one, but was devoid of the auxin. The pH of the medium was adjusted to 5.6 before autoclaving at 121°C and 1.1 Kg cm^{-2} for 20 min. Cultures were maintained continuously in the dark at 25 ± 2°C.

7.2.3. *Culture of isolated microspores*

After disinfection with the method that allowed better results during the *in vitro* culture of anthers (0.1% $HgCl_2$ during 10 min), anthers were aseptically removed from male flowers at developmental stages I and II. The extraction of microspores was made according to Nitsch (1974): 50 anthers were placed in a small beaker containing 5 ml of extraction solution, and gentle squeezed against the side of the beaker with the piston of a syringe. Different extraction solutions were used: distilled water, a 0.3 M mannitol solution and liquid MS basal medium, with mineral salts and vitamins.

The anther tissues debris was removed by filtering the suspension thus obtained by a nylon mesh with a 60 µm pore diameter. The microspore suspension was centrifuged 3 times: first at 2500 rpm during 10 min, and then 2 times at 2500 rpm during 5 min. After each centrifugation, the supernatant was discarded and the microspore suspension was

resuspended in 1 ml of induction medium. Aliquots of 100 µl of the microspore suspension were placed in Petri dishes (60 × 60 mm) containing 2.5 ml of induction medium. Each dish was sealed with stretch film to avoid dehydration, and 14 dishes were placed together in a 200 mm-wide dish.

The induction medium consisted of MS basal medium, with the macronutrients reduced to ½, and with 2% (w/v) sucrose, 800 mg l^{-1} L- glutamine, 100 mg l^{-1} serine, without growing regulators or supplemented with 1 mg l^{-1} 2,4-D combined with 1 or 2 mg l^{-1} of BA. Cultures were maintained continuously in the dark, or with a 16- photoperiod at 60 µmol m^{-2} s^{-1} provided by cool-white fluorescent lights.

7.2.4. *Cytological observations*

For cytological observations of the *calli*-cells, an acetocarmine 4% (w/v) stain was used. The preparation of the acetocarmine stain is described on Chapter II, section 2.2.4.

For observation, a small portion of *calli* was squashed on a microscope slide in a drop of acetocarmine stain. After removing the debris, a cover slip was added, and the microscope slide heated on a flame. Drops of stain where then placed in an extreme of the covers slide, and removed carefully with a filter paper in the other extreme (this procedure was repeated several times, not allowing the sample to boil). The samples were immediately observed using conventional brightfield microscopy. Photographs were taken, scanned and subsequently prepared with Adobe Photoshop.

7.2.5. *Statistical analysis*

The results presented are the mean ± SE of 10 anthers per experiment, and all the experiments were carried out 8 times. The data were subjected to analysis of variance (ANOVA) to assess treatment differences and interactions using the SPSS statistical package for Windows (release 11.0, SPSS INC). Significance between means was tested by Duncan's New Multiple Range Test (P=0.05). Arcsin square root transformation was used prior to analysing data.

7.3. RESULTS AND DISCUSSION

7.3.1. *Anther culture*

The establishment of the cultures was very difficult due to the high level of contaminations, which appeared few days after inoculation. Most of the disinfection methods were unsuccessful (~ 100% contamination), and from the 35 methods tested only seven allowed positive results (Table 7.1). This may be due to the growing conditions of the donor-plant, which was a field-grown adult tree, and by the peculiar fact of carob male flowers being apetalous, and the anthers more exposed to the external agents of contamination.

The percentage of sterile anthers obtained was very low, ranging between 1.0% and 9.5% (Table 7.1). Since the higher percentage of callogenic anthers was observed with 0.1% $HgCl_2$ during 10 min, this disinfection method was used during the following assays.

Table 7.1. Percentage of surface sterile anthers and anthers with *calli* formation observed in the disinfection methods where positive results were obtained. The anthers were taken from flowers at developmental stages I and II, from an adult male tree.

Disinfection method	Anthers surface sterile (%)	Anthers with *calli* (%)
NaOCl (5%, v/v), 15 min.	4.5	0
NaOCl (15%, v/v), 5 min.	1.0	0
NaOCl (15%, v/v), 15 min	5.5	9
$HgCl_2$ (0.1%, v/v), 2 min	7.5	0
$HgCl_2$ (0.1%, v/v), 10 min	7.5	20
$HgCl_2$ (0.1%, v/v), 20 min	9.5	0
$HgCl_2$ (0.5%, v/v), 15 min	5.0	0

In spite of being very toxic, $HgCl_2$ is sometimes used as a disinfection agent in flower buds taken from outgrowing trees (Chaturvedi et al. 2003). It is known that less strong disinfection methods allow a higher viability of the explants, but usually they are

not effective in controlling contaminations. On the other hand, more efficient disinfection agents result in a decrease in the viability of the explants.

When using anthers from flowers at stage II, we observed that after a few days of cultivation anthers turned brown. In some cases, the oxidation of the anther wall totally inhibited *calli* formation. This problem could not be circumvented by transferring the anthers onto fresh medium whenever exudation was observed, or by the use of antioxidants. The inhibitory effect of browning on *calli* formation was also observed in anther cultures of *Fagus sylvatica* (Jörgensen 1988), while in other species browning of the anther wall did not hinder callogenesis (Saji and Sujatha 1998).

The cytological studies done during this work (*vide* chapter II) indicated that for androgenesis we could use anthers excised either from flowers in stages I or II, since in both stages uni- and binucleate microspores were observed. The browning incidence made impossible the use of anthers excised from flowers at stage II, and therefore all the subsequent experiments were done with anthers excised from flowers at stage I.

The results of the anther culture experiments are summarized in Table 7.2. The anthers with browning and *calli* formation varied significantly with the type and concentration of the growth regulators tested (Table 7.2). After 1 week of culture most of the anthers were swollen, and after 3–4 weeks they started to produce *calli* asynchronously.

Analysis of variance of the percentage of anthers showing browning indicates that this phenomenon was significantly affected by the cytokinin type ($P<0.01$), and by the interactions between auxin concentration and cytokinin type, and cytokinin type *vs* concentration ($P<0.01$). When using TDZ we obtained the lower percentage of browning, independent of its concentration and auxin concentration ($P<0.01$).

The percentage of anthers producing *calli* was significantly affected by the auxin concentration ($P<0.05$), the type and concentration of cytokinin ($P<0.001$), and by the interaction between the auxin concentration and the type of cytokinin ($P<0.01$) (Table 7.2). The interactions between the concentration of auxin and cytokinin ($P<0.05$), and between cytokinin type and concentration ($P<0.01$), also influenced callogenesis (Table 7.2).

Table 7.2. Effect of auxin (2,4-D) concentration combined with different concentrations of BA, Kin, Zea and TDZ on browning and callusing in anther cultures. Growth period: 8 weeks.

Growth regulators (mg l^{-1})		% browning	% callusing	Degree of callusing [a]
2,4-D (0.2)	+ BA (1)	70 ±8 a	30 ± 8 cde	++
	+ BA (2)	36 ± 4 bc	36 ± 5 bc	++
	+ BA (4)	19 ± 5 cd	32 ± 5 cd	+++
	+ Zea (1)	27 ± 15 cd	13 ± 11 de	++
	+ Zea (2)	30 ± 10 cd	63 ± 9 ab	+++
	+ Zea (4)	63 ± 9 ab	23 ± 7 cde	++
	+ TDZ (1)	19 ± 6 cd	81 ± 6 a	++
	+ TDZ (2)	13 ± 4 cd	85 ± 5 a	++
	+ TDZ (4)	13 ± 7 d	79 ± 6 a	++
	+ Kin (1)	25 ± 9 cd	58 ± 10 bc	++
	+ Kin (2)	16 ± 6 cd	38 ± 9 bc	++
	+ Kin (4)	13 ± 7 d	9 ± 4 e	++
2,4-D (0.5)	+ BA (1)	24 ± 8 abc	55 ± 9 bc	++
	+ BA (2)	21 ± 6 abc	68 ± 7 b	+++
	+ BA (4)	7 ± 4 bc	80 ± 3 b	+
	+ Zea (1)	25 ± 14 abc	0 ± 0 e	-
	+ Zea (2)	38 ± 22 ab	76 ± 4 b	+++[b]
	+ Zea (4)	47 ± 24 a	34 ± 22 cd	++
	+ TDZ (1)	20 ± 9 abc	53 ± 16 bc	++
	+ TDZ (2)	21 ± 9 abc	63 ± 12 bc	++
	+ TDZ (4)	0 ± 0 c	100 ± 0 a	+++
	+ Kin (1)	30 ± 5 ab	61 ± 5 bc	+++
	+ Kin (2)	19 ± 5 abc	58 ± 10 bc	+++[c]
	+ Kin (4)	43 ± 12 ab	10 ± 5 de	+[c]
Significance of three-way ANOVA				
Auxin concentration (A)		ns	*	
Cytokinin type (B)		**	***	
Cytokinin concentration (C)		ns	***	
A × B		**	**	
A × C		ns	*	
B × C		**	**	
A × B × C		ns	ns	

[a] The size of *callus* is directly related to the number of plus signs. +: *microcallus*, located in small spots along the anther surface; ++: *callus* covering 50-75% of the anther surface, and +++: *callus* covering the entire anther surface; [b] *callus* showing root differentiation; [c] non-embryogenic *callus*. Values represent means ± SE of 8 replicates with 10 anthers each. For each auxin concentration, values followed by the same letter are not significantly different at $P \geq 0.05$. ns, *, **, ***: non significant, significant at $P<0.05$, significant at $P<0.01$ and significant at $P<0.001$, respectively (three-way ANOVA, Duncan's New Multiple Range Test).

Regardless of the type and cytokinin concentration used in the induction medium, the best results in terms of *calli* induction were obtained with the higher concentration of 2,4-D (0.5 mg l^{-1}) ($P<0.05$). Culture on medium containing 0.2 mg l^{-1} 2,4-D produced a lower percentage of callusing, indicative of insufficient auxin for *callus* induction. Among various types of auxins, 2,4-D is employed in most anther culture systems, and the optimum concentration required for callusing induction and androgenesis is species dependent (Jörgensen 1988, Faure et al. 1996, Radojevic et al. 1989, 2000, Chaturvedi et al. 2003).

Although BA (Jörgensen 1988, Faure et al. 1996, Chaturvedi et al. 2003, Rimberia et al. 2004) and Kin (Radojevic et al. 1989, 2000) were successfully used in anther culture of several woody species, in our work TDZ was the most effective cytokinin for *calli* induction (Table 7.2). The higher concentration of TDZ (4 mg l^{-1}) combined with 0.5 mg l^{-1} of 2,4-D induced the higher degree of callusing (100%) (Table 7.2). TDZ is one of the most active cytokinins used in woody plant tissue culture and enables the micropropagation of the most recalcitrant species, especially when used at concentrations ≥ 0.1 μM (0.022 mg l^{-1}) (Huetteman and Preece 1993). TDZ has also been employed in the anther culture of some woody species (Harst-Langenbucher and Alleweldt 1993, Höfer 1995).

Except for the combinations 0.5 mg l^{-1} 2,4-D + 2 mg l^{-1} Kin and 0.5 mg l^{-1} 2,4-D + 4 mg l^{-1} Kin, all the developed *calli* were embryogenic (Table 7.2). The *calli* generally covered 50-100% of the anther surface, and different types of *calli* were observed, namely compact, semi-compact, and friable and whitish *callus* (Plate 7.1A-G). Sometimes *calli* were observed developing in the distal ends of the anthers (Plate 7.1B). The non-embryogenic *calli* presented cells with an intense starch deposition (Plate 7.2E). The presence of starch accumulations usually indicates the development of a gamethophytic pollen, rather than sporophytic (Říhová and Tupý 1999).

Under brightfield microscopy we observed multicellular structures and proembryos (Plate 7.2A-D), suggesting the androgenic nature of these *calli*. The *calli* induced on medium supplemented with 0.5 mg l^{-1} 2,4-D + 2 mg l^{-1} Zea differentiated roots (Table 7.2, Plate 7.1D).

An efficient system for the production of DH through microspore embryogenesis requires the artificial manipulation of immature pollen and subsequent success in plant regeneration (Touraev et al. 1996a, b, 1997, Zheng et al. 2001). Such manipulations often consist of two critical steps, the first involving attempts to switch from the naturally determined pollen-forming developmental program of microspores to the inherent alternative developmental program, leading to the induction of their embryogenic competence; and the second step, aiming to provide an adequate environment for the induced micropores to continue their embryogenic programme (Zheng et al. 2001). If the conditions are sub-optimal many competent microspores may either fail to divide or stop their divisions, hence aborting the embryogenic process (Zheng et al. 2001).

In an attempt to induce the development of the multicelular and proembryos observed, the *calli* were transferred to media without auxin. However, these *calli* failed to produce shoots or embryos, and the multicellular structures and proembryos showed no further development. Thus, the conditions for the development of the induced microspores need further improvement.

Anther culture has proven to be an efficient method to produce homozygous lines from gametic cells in several important crop species, although efficient protocols are only well established in model systems, such as *Brassica, Nicotiana* and *Hordeum* (Kyo and Harada 1986, Hoekstra et al. 1992, Custers et al. 1994). This technique is strongly species and genotype dependent, and there are some reports of its application to woody plants (Gresshoff and Doy 1974, Hirabayashi et al. 1976, Hidaka et al. 1979, Chen et al. 1980, Nair et al. 1983, Hidaka 1984a, b, Jörgensen 1988, Radojevic et al. 1989, 2000, Bueno et al. 1997, 2000a, b, Chaturvedi et al. 2003, Höfer 1997, 2004, Kadota and Niimi 2004, Rimberia et al. 2004). However, despite considerable efforts, androgenesis in woody plants is difficult, and there are still many recalcitrant species or genotypes (Říhová and Tupý 1999, Marachin et al. 2005).

In the normal gametophyte development, microspores divide asymmetrically to produce pollen grains. The male gametophyte is characterized by a small condensed generative and large diffused vegetative nucleus. It is known that some stress factors, like heat or cold shock treatment or sugar starvation are the launching mechanism for redirection of microspores to develop sporophytically (Touraev et al. 1997, Höfer 2004, Marachin et al. 2005). In this work some different pretreatments were tried, namely the

maintenance of the inflorescences at 4°C and 30°C for 24, 48 and 72h, and the inoculation of the anthers in MS basal medium with 7 M of mannitol or sucrose. However, neither of the pretreatments allowed positive results, mainly due to the high incidence of contaminations.

In spite of the flowering period in carob being relatively long (4-5 months), it is recommended that for *in vitro* androgenesis buds must be harvested from plants at the beginning of the flowering period (Dunwell 1985). If it is necessary to continue experiments over an extended period, the flower buds that contains microspores at an appropriate stage of development must be removed from the plant, and not allowed to mature. However, in carob the removal of the inflorescences from the donor plant implies its dehidratation in a very short time, even when stored at low temperatures. Furthermore, for the success of anther culture, the donor plant must be in an optimum state of nutrition, irrigation and environmental conditions (controlled temperature, photoperiods and light intensities) from the time of flower induction to the sampling of anthers, and without such environments, massive fluctuations in response must be expected (Nitsch 1981, Dunwell 1985, Sangwan and Sangwan-Norreel 1990). Those optimal conditions are very difficult or impossible to achieve in outgrowth trees.

Moreover, the carob flowers exhibits a peculiar feature: they are apetalous and thus, in the flower development stages (stages I and II) where anthers have microspores suitable for anther culture the anthers are completely exposed to the external agents of contamination. This makes the disinfection of the anthers extremely difficult, since for a significant reduction in the incidence of contaminations, we must use disinfection agents that are extremely toxic in handling, and that can damage the anther tissues, compromising their reactivity to the *in vitro* conditions.

7.3.2. *Culture of isolated microspores*

One possible way to circumvent the difficulties of anther culture could be the culture of isolated microspores (Nitsch 1974, 1981, Dunwell 1985, Sangwan and Sangwan-Norreel 1990). Similarly to what was referred in anther culture, procedures for haploid plant production from isolated microspores are based on culture media enabling survival and

development of microspores outside of the anthers, on factors triggering the switch from gametophytic to sporophytic pathay and on suitable culture conditions for embryo formation and plant regeneration (Říhová and Tupý 1999).

We attempted the induction of androgenesis by the culture of isolated microspores, by using different extraction solutions and growth regulators in the induction medium. However, the high degree of contaminations (100%) completely hindered the application of this technique. This problem was not circumvented by the reduction of the number of anthers used for the microspore extraction, since one contaminated anther is sufficient for the contamination of the microspore suspension.

7.4. Conclusions

Doubled-haploid (DH) production through anther culture is a potentially efficient means to generate homozygous true-breeding progeny lines in plant breeding programmes. However, especially in woody species, there are important constraints to overcome before the full potential of this technology can be realized. In the particular case of carob tree, the availability of efficient protocols for DH production, either by anther or isolated culture of microspores, could greatly enhance basic research in carob genetics and accelerate carob breeding programmes.

In this chapter it was observed that the first constraint to the *in vitro* culture of anthers of carob tree is the establishment of the cultures. This first step was extremely difficult due to the high level of contaminations, which appeared few days after inoculation, reaching ~ 100% in the majority of the disinfection methods tested. In fact, this problem completely hindered the culture of isolated microspores. In spite of the high level of contaminations, we were able to obtain some surface sterile anthers with high frequencies of callusing, most of it embryogenic. Thus, research has to be made to circumvent the high level of contaminations, namely by testing other disinfection agents and/or times of exposure or by the addition of antibiotics to the culture media.

The second constraint in this work was the regeneration of embryos and/or plants, since although embryogenic *calli* was obtained, after transference to the regeneration medium they failed to produce shoots or embryos. Thus, research is needed to redefine the

stress treatment needed to trigger the sporophytic development of microspores in culture, in order to improve the frequencies of proembryos formed, and also the conditions for the normal development of those proembryos.

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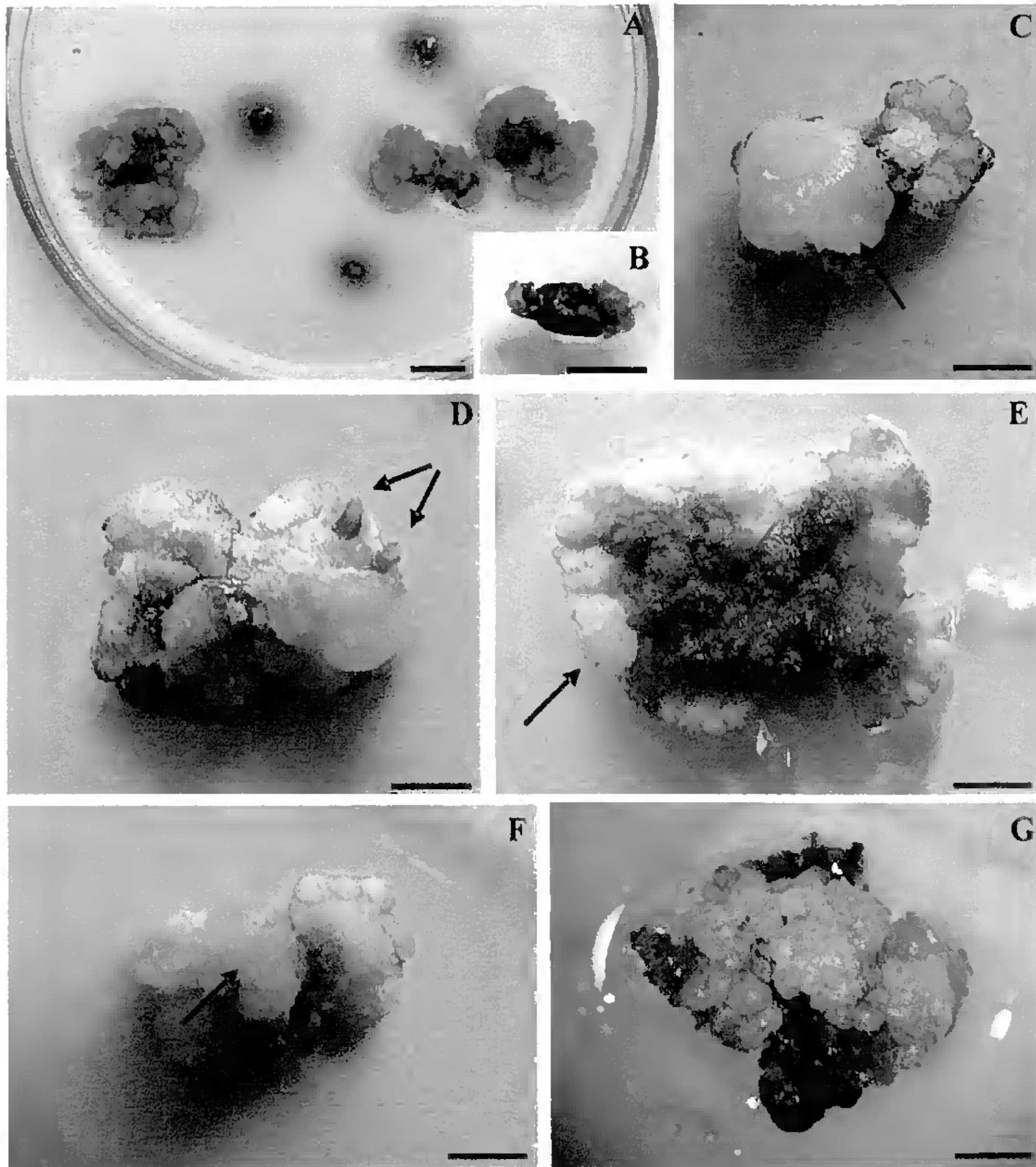

Plate 7.1. Different aspects of anther culture in carob tree. (A) Loose *calli* observed in anthers after 4 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 4 mg l^{-1} TDZ (bar = 5 mm); (B) anther with *calli* formation in the distal ends, after 4 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 4mg l^{-1} BA (bar = 17 mm); (C) non embryogenic compact *calli* observed in anthers after 8 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 2 mg l^{-1} Kin, with watery *calli* on the surface (arrow) (bar = 21 mm); (D) *calli* observed in anthers after 7 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 2 mg l^{-1} Zea with root differentiation (arrows) (bar = 17 mm); (E) compact pre-embryogenic *calli* observed in anthers after 8 weeks on medium supplemented with 0.2 mg l^{-1} 2,4-D + 4 mg l^{-1} BA, with watery *calli* developing in the surface (arrow) (bar = 17 mm); (F) compact pre-embryogenic *calli* observed in anthers after 8 weeks on medium supplemented with 0.2 mg l^{-1} 2,4-D + 4mg l^{-1} BA, with watery *calli* developing in the surface (arrow) (bar = 21 mm); (G) compact embryogenic *calli* observed in anthers after 8 weeks on medium supplemented with 0.5 mg l-*1* 2,4-D + 1mg l-*1* Kin (bar = 17 mm). Basal medium: MS.

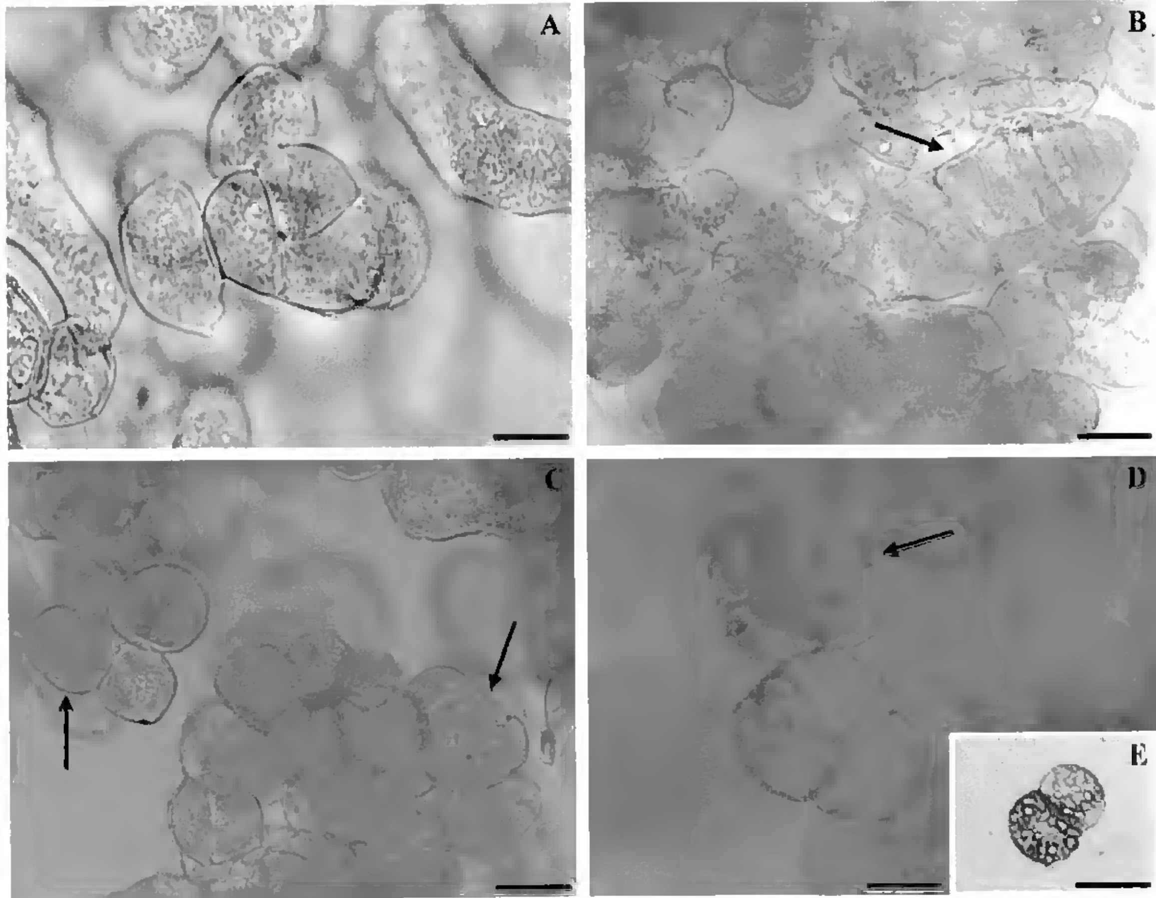

Plate 7.2. Different aspects of microspore development in anther culture in carob tree. (A) Pro-embryo with 3 cells observed in *calli* after 8 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin; (B) multicellular pro-embryo (arrow) observed in *calli* after 8 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 1 mg l^{-1} Kin; (C, D) pro-embryos (arrows) observed in *calli* after 8 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 4 mg l^{-1} TDZ; (D) cell with starch deposition observed in non embryogenic *calli* after 8 weeks on medium supplemented with 0.5 mg l^{-1} 2,4-D + 4 mg l^{-1} Kin. Basal medium: MS. Bars = 27 µm on Figs. A, B, D and E; 34 µm on Fig. C).

GENERAL CONCLUSIONS AND FUTURE REMARKS

Most studies on carob have focused on applied aspects, such as agricultural, industrial and commercial, and it is clear that there are several areas of carob biology where more research is needed. The general purpose of this work was to provide new approaches for the understanding of some aspects of the floral biology of carob, particularly those related with the reproductive process. Although the main purposes of this work can be considered achieved, since new information was obtained on the biology of the three sexual types of carob tree, it remains a feeling of incompleteness and a need for further studies. This work includes the first reports about the volatiles emitted by flowers of carob, the first development of a protocol for pollen cryopreservation, and the first approaches to the induction of androgenesis, having in mind the production of homozygous plants.

An overview of the main aspects of flowering, namely inflorescence and floral morphology and the study of the major events occurring during micro and megasporogenesis and their relation with the male, hermaphrodite and female flower developmental stages, is made in chapter II. The elucidation of some aspects of the reproductive biology of carob are still missing, namely those regarding the processes of micro and macrosporogenesis. Based on the results obtained in this work, a more detailed histological studied is needed, in material infiltrated and embedded in plastic resin, or in other support that permits sectioning in ultramicrotome. Furthermore, the reproductive organs of carob make this species a suitable object for further embryological investigations, due to the numerous and large ovules that are present in the developing pods.

Carob tree has been variously described as polygamous, dioecious or trioecious. The maintenance of diverse sexual forms in this species has not yet been studied. In natural populations however, male and hermaphrodite individuals may compete for seed siring and paternity analysis is needed to determine the effective male reproductive success of male vs. hermaphrodite individuals. An important aspect of male success of pollen from male vs. hermaphrodite individuals could be seed siring ability in competition. To date no

paternity analyses of different (i.e., male vs. hermaphrodite) pollen donors in controlled crosses have been reported in carob.

The flowers of the three sexual types of carob are initially bisexual, and differentiation of functional male or female flowers occurs when buds are less than 0.5 cm in size. However, it is still not known what are the factors triggering that sexual differentiation. The use of sexual markers could be helpful in determining, in an early stage of development, the sexual type of carob plants.

The volatile compounds of whole flowers and isolated floral parts of the three sexual types and female cvs. Mulata and Galhosa were analysed *in vivo* by HS-SPME-CGC/MSD. Linalool and its derivates were the dominant volatiles in whole flowers. Both the qualitative profile and the relative abundances of the volatiles of whole flowers and isolated floral parts were dependent on the sexual type and cultivar. The fragrances from the whole flowers depend mainly on the volatiles emitted from nectarial disks.

The seasonal variation in the leaf nutrient concentrations, the nutrient content of flowers and the differences in nutrient concentrations between flowers and leaves at full bloom, were assessed in male, female and hermaphrodite carob trees. The differences in nutrient concentrations between vegetative and reproductive organs are influenced not only by the phenological stage, but also by the sexual type. The knowledge of the nutritional demand of the different sexual types of carob and the variation in leaf nutritional concentration along the season can have practical implications for fertilisation guidance, namely the optimization of the amounts and the timing of fertiliser's application.

In carob tree, the dynamics of partitioning and allocation of NSC seems to be very complex, and dependent on the sexual type and cultivar, being the impacts of vegetative and reproductive growth on the partitioning and allocation of NSC clearly difficult to evaluate. Further research is needed to elucidate the patterns of carbohydrates and seasonal variations, namely the determination of growth parameters, and of the enzymatic and photosynthetic activities.

A cryopreservation protocol for the storage of immature pollen was developed by using a relatively simple technique and maintaining high viability. The cryopreservation of pollen may indicate the way forward for the conservation of genetic diversity. It has the advantage of being a relative low-cost option, but the disadvantage that only paternal material would be conserved and regenerated. Cryopreserved pollen could be used for

crossing with living material, or in programmes aiming the regeneration of haploid plants. In carob, there is a need to increase the viability levels, and to evaluate the response of cryopreserved microspores to the induction of androgenesis.

Doubled-haploid (DH) production through anther culture is a potentially efficient means to generate homozygous true-breeding progeny lines in plant breeding programmes. However, especially in woody species, there are important constraints to overcome before the full potential of this technology can be realized. In the particular case of carob tree, the availability of efficient protocols for DH production, either by anther or isolated culture of microspores, could greatly enhance basic research in carob genetics and accelerate carob breeding programmes. In chapter VII, high percentages of embryogenic *calli* were, for the first time, achieved by anther culture of carob tree. Research has to be made to circumvent the high level of contaminations observed during the establishment of aseptic cultures of anthers, namely by testing other disinfection agents and/or times of exposure or by the addition of antibiotics to the culture media. Furthermore, the stress treatments needed to trigger the sporophytic development of microspores in culture have to be redefined, in order to improve the frequencies of the proembryos formed.

Carob has been traditionally neglected by Research & Development programmes. Centuries of carob cultivation have given rise to a number of cultivars differing in agronomic characters, but knowledge about the existing cultivars in the Mediterranean region in general, and in the Algarve, in particular, is still poor. Thus research to compare and characterize the most useful cultivars and types, in the wild and in the existing orchards, is much needed, not only regarding the currently industrial application of this species, but also having in mind its growing valorisation in the pharmaceutical and medical area. Therefore, detailed studies on the chemical composition and bioactive properties of extracts from different sources, aiming the development of new health-enhancing food and medical products from Portuguese carob cultivars, is much needed. All of the above mentioned could result not only in an important contribution for the knowledge about this valuable species, but also in an increase of the economical value of the Portuguese cultivars.

LIST OF ERRORS

Page	*§*	*Line*	*How it reads*	*How it should be read*
Account		27	Nutricão Mineral das Plantas	Nutrição Mineral de las Plantas
Aknowl.	6	1	Dr. Maria-Amélia Loução	Prof. Dr. MA Martins-Loução
List of Abbrev.	column 2	8	double haploids	double haploid
		24	capillary	capillary gas chromatography
		27	capillary gas chromatography coupled to mass spectometry detection	headspace solid phase microextraction followed by capillary gas chromatography coupled to mass spectometry detection
Table of contents		2	List of abreviations	List of abbreviations
		46	Analysis of the volatiles of carob tree flower	Analysis of the volatiles of carob tree flowers
		12	Before «5.4. Conclusions» it should be read «5.3.3. Concentration of carbohydrates in pulp and seeds during fruit development ....152»	
Resumo	4	4	Para alem disso	Para além disso
	5	5	único açúcar solúvel	único açúcar insolúvel
			Keywords	Palavras chave
5	2	5	bissexual	bisexual
7	2	2	Bosh et al. 1996	Bosch et al. 1996
8	2	1	it does not nodulate	does not nodulate
8	4	5	Blondel an Aronson 1999	Blondel and Aronson 1999
11	1	2	and the same origin	and of the same origin
17	1	2	information for the male	information of the male
19	1	1	access	assess the seasonal
28	Fig. 2.2	caption	At the end of the caption it should be read (McCormick 2004)	
30	3	2	(Fig. 2.4)	(Figs. 2.3, 2.4 and 2.5.)
33	4	6	The average weight of 25	The average weight of 30
33	5		The sentence: «For the flowering ... were used» should not be read.	
38	4	4	epidermis with short, trichomes,	epidermis with short trichomes,
46	3	2	2.9D	2.9E
46	3	4	Plate 2.9D	Plate 2.9E
46	3	9	2.9E	2.9D
52	1	4	with and marginal placentation.	with a marginal placentation
63	Plate 2.4	Fig.B	Following the } it should be read "pi"	
85	6	1	Arthur and Pawliszyn	Arthur and Pawlizyn
92	Fig. 3.4	caption	by SPME/CGC-MSD	by HS-SPME-CGC/MSD.
94	Sub-title		3.3.2. Volatiles of isolated floral parts	3.3.2. Volatiles of isolated floral organs
96	Fig. 3.5	caption	by HS-SPME-GC/MS.	by HS-SPME-CGC/MSD.
99	1	7	(Tollsten and Bergstrom 1993)	(Tollsten and Bergström 1993)
103	1	7	from emitted by	from the emitted by
105	1	3	access	assess
119	2	3	between the sexual type	between the sexual types
120	1	7	in the opposite was true	the opposite was true
120	3	7	(Buwalda and Meekgins 1990)	(Buwalda and Meekings 1990)

Page	§	Line	How it reads	How it should be read
124	2	4	higher nutrient	lower or similar nutrient
137	2	1	carbohydrate	carbohydrates
140	2	4	(Fig. 5.3	(Fig. 5.3).
144	3	16	short-term	short-term sinks
145	2	11	sucrose content sucrose	sucrose content
149	1	2	which decreased from	with a plateau from
149	2	3	(Fig. 5.9)	(P<0.05, Fig. 5.10)
150	Fig. 5.9	caption	The sentence «and for different…upper case letters)» should not be read	
150	2	2	(Fig. 5.9)	(P<0.05, Fig. 5.9)
151	1	2	(Fig. 10).	(Fig. 5.9).
152	Fig. 5.10	caption	The sentence «and between sexual…upper case letters)» should not be read	
153	3	2	accessed	assessed
153	4	2	was where not	was not
154	Fig. 5.11	caption	Following «… (lower case letters), » it should be read « and for different sexual type and cultivars for the same organ and stage of fruit development (upper case letters)»	
169	1	4	leve	level
172	2	6	male and female	male and hermaphrodite
175	2	2	this pollen	that pollen
181	1	6	indunce	induce
182	2	1	that the both the	that both the
182	2	4	flower	flowers
182	4	2	induction androgenesis	induction of androgenesis
193	1	2	Following «Nitsch 1974, 1981» it should be read «Reinert and Bajaj 1977»	
203	2	1	multicelular	multicellular
204	2	7	dehidratation	dehydratation
205	1	2	pathay	pathway