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FLORAL SCENT EVALUATION OF ALSTROEMERIA

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY. CARDIFF UNIVERSITY, SCHOOL OF BIOSCIENCES

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THE FLOWER LOVER

In the Valkerie Mountains
among the strutting peacocks
I found a flower
as large as my head
and when I reached in to smell it

I lost an ear lobe
part of my nose
one eye
and half a pack of cigarettes.

I came back the next day
to hack the damned thing down
but found it so beautiful
I killed a peacock instead.

Charles Bukowski (1920-1994)

SUMMARY

Alstroemeria is an important cut flower and its breeding has been developed focused on aesthetic characteristics and vase life longevity, but little is known about its scent. Five different genotypes were assessed including the non scented cultivars 'Rebecca' and 'Samora' and the scented cultivars, 'Sweet Laura', 'Ajax' and the species *A. caryophyllaea*.

The scented Alstroemerias emitted the terpenoids: isocaryophyllene and ocimene as the major floral volatile compounds.

Characterization of an Alstroemeria TPS (*ALSTER*) was based on four ESTs previously found in *A.* cv 'Rebecca'. Rapid amplification of cDNA ends (RACE) was performed and the full length ORF was used for characterizations of the genomic organization and amino acid sequences (phylogenetic analysis). *ALSTER* genomic region contains five introns and six exons. This unique genomic organization classified *ALSTER* as a member of the class III with a merged 5-6 exon. The deduced amino acid sequence was classified into the subfamily TPS-b.

A functional analysis showed enzymatic activity of *ALSTER* with geranyl diphosphate (GPP) and the monoterpene myrcene was the only product obtained.

Gene expression evaluated through real time and semi q RT-PCR on eight different stages of development (S0 to S7) showed high expression of *ALSTER* at around S2 – S4 in the scented Alstroemerias, coinciding with high scent emission perceived and also with the maturation of reproductive organs.

Evaluations through surveys focused on level of liking of floral scent, were performed finding positive correlations between floral scent liking and floral appearance liking and between floral scent liking and floral scent intensity.

Finally, 17 new lines of *A. caryophyllaea* were evaluated in terms of their morphology, phenology and productivity. Although none of them were suitable for the market because of their low productivity, short stems and small flowers, they were all scented and identified as promising starting points for breeding purposes.

Keywords: Alstroemeria, Floral Scent, Terpene synthase, GC-MS, Real time PCR and Sensorial analysis.

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DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

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This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD (insert MCh, MD, MPhil, PhD etc, as appropriate)

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CHAPTER I.

GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

1.1. ALSTROEMERIA

1.1.1. General characteristics

The genus *Alstroemeria* belongs to the *Alstroemeriaceae* family and includes about 60 described species, all of them originating in South America, with Chile and Brazil as the main diversity centres (Bayer, 1987; Muñoz and Moreira, 2003). This family includes three genera: *Alstroemeria* L., with about 60 described species; *Bomarea* Mirb., with about 100 species (Sanso and Hunziker, 1998) and the monotype genus *Leontochir* Phil. (Bayer, 1987; Aker and Healy, 1990).

Alstroemeria are herbaceous, perennial and rhizomatous plants with big flowers, living in a wide range of habitats from rainy forest to desert areas and from the Andes Mountains to the coast (Muñoz and Moreira, 2003).

1.1.2. Evolution and breeding

Alstroemeria has been very attractive for breeders as a cut and pot flower and many commercial cultivars have been developed through interspecific hybridization (Burchi *et al.*, 1997), selection of mutants and polyploidization (Broertjes and Verboom, 1974).

Breeding programmes in Alstroemeria have focused on aesthetic characteristics, like size and colour of flowers; vase life; and productive characteristics like number of flowers per stem or all-year round production, but little is known about floral scent in Alstroemeria. Breeders have found some difficulties with this character because they cannot assure to the consumers quality of scent. This is due to its irregular emission during the day (depending on circadian rhythm, light and temperature) and vase life (strong scent for only short periods). Furthermore, the heredity of scent in Alstroemeria is not clear, making the development of breeding programmes focused on this character difficult (Meijles, 2006). The other problem identified by breeders is the fact that stems of Alstroemeria usually are included in bouquets and consumers lack familiarity with this species, so they are not able to identify which flower is emitting scent in a mixed bouquet. For this reason, floral scent has not been included as a selection criterion in new cultivars (Kuiper, 2006).

At present there are only two scented cultivars in the European-American market: 'Sweet Laura', obtained though an interspecific cross between Chilean non scented *A. aurea* and Brazilian scented *A. caryophyllea* (Bridgen, 2008) and 'Ajax', obtained from the breeding of *A. caryophyllea* with six or seven other lines (Meijles, 2008). Scent is one of the most attractive characters in flowers such as rose and lily, but not in Alstroemeria probably due to a lack of selection and also the use of a narrow genetic background in breeding programmes (Aros *et al.*, 2006) including almost only non scented species. Furthermore, floral scent has been usually unintentionally selected against in some flowers, for example, due to the negative correlation between longevity and fragrance. An example of this correlation is the common floral scent compounds jasmonic acid and methyl jasmonate, identified as promoters of ethylene production during floral senescence (Porat *et al.*, 1993).

Floral scent has been classified as a variable trait since in many taxa there are scented species closely related to non-scented ones, suggesting that this character has been frequently acquired and lost throughout evolution (Dudareva *et al.*, 1996). Moreover, specificity of the volatile compounds emitted, suggests that this character is highly evolved in plants since the chemical properties of floral scent are complex and it is almost impossible to find the same mixture of volatiles emitted even by two closely related species. However the basis of these evolutionary changes is not completely clear due to the complex nature of scent itself (Dudareva and Pichersky, 2000). Evolution of morphological characteristics is also related to floral scent, for example, Jürgens *et al.* (2003) found that flowers of some *Dianthus spp* that are whitish in colour and have a long calyx tube, were able to emit stronger scent than other species of the same genus with different floral morphology. On the other hand, no association was found by Kondo *et al.* (2006) between morphological characteristics of subspecies of *Petunia axillaris* and their floral scent properties, although they concluded that floral scent emission is closely related to the self incompatibility characteristic of this species.

1.2. FLORAL SCENT

1.2.1. Function

Many functions are attributed to floral scent, especially those involved in interactions between plants and their environment. However it is clear that the main function of floral scent is to attract and guide pollinators. This finding has been confirmed in many species (Ando *et al.*, 2001; Reinhard *et al.*, 2004; Dudareva *et al.*, 2004; Jürgens *et al.*, 2003). Floral scent thus plays a crucial role in pollination and consequently in fertilization and production of seeds and fruits, therefore the success of plants emitting floral scent to attract pollinators is essential in the yield of important

crops (Free, 1970) such as almonds (Abrol, 1988), kiwifruit (Marucci, 1977) and sunflower (Krause and Wilson, 1981). Those plants that have been introduced artificially into a new environment may have some disadvantages in this regard, because there has not been co-evolution between them and pollinators living in this new environment (Traub *et al.*, 1942).

Despite the fact that the main function of floral scent has been elucidated, it is not completely clear how exactly each individual substance has the ability to attract specific pollinators (Dudareva *et al.*, 2004). It has been found that olfactory receptors of insect antennae have the ability to discriminate among diverse types of specific volatile compounds emitted from flowers of different species. Thus insects are able to find and pollinate the flower of choice (Pham-Delegue *et al.*, 1993; Raguso and Pichersky., 1995). However, Henning *et al.* (1992) suggest that little is still known about how insects detect specific volatiles in flowers and how they have developed responses (attraction, repulsion, or indifference) to such compounds.

Some floral scent compounds have been identified as specific for some insects, even in plants belonging to different taxonomical groups. For example, aromatic esters and esters of salicylic acid were dominant during an evaluation performed on *Dianthus spp* and *Saponaria spp*, both clearly adapted to moths or hawkmoths (Jürgens *et al.*, 2003). Additionally, small amounts of nitrogen-containing compounds were detected in the floral scent of *Saponaria* suggesting that nitrogen-containing fragrances are a specialization of tropical species pollinated by hawkmoths (Nilsson, 1985).

Dudareva and Pichersky (2000) suggest that flowers can also emit certain compounds with the aim of repelling non-beneficial insects, for example pollen or nectar 'thieves' or destructive insects. Furthermore flowers are able to emit some volatile compounds in order to protect reproductive organs from enemies through the

anti-microbial or anti-herbivore activity attributed to some of these compounds (DeMoraes *et al.*, 2001; Friedman *et al.*, 2002; Hammer *et al.*, 2003). Also vegetative organs of most of the plants have the ability to release volatile compounds after damage in order to avoid further attack caused by herbivores. The volatiles act as direct defences, repelling (DeMoraes *et al.*, 2001; Kessler and Baldwin, 2001) or intoxicating (Vancanneyt *et al.*, 2001) herbivores and pathogens. Physiological roles have been also attributed to herbivore-induced volatiles changing for example membrane solubility and also provoking some oxygen-related reactions to avoid internal oxidative damage (Delfine *et al.*, 2000; Loreto *et al.*, 2004). Another way in which volatiles repel herbivores is the capacity of some compounds to attract arthropods able to prey upon or parasitize herbivores (Pare and Tumlinson, 1999; Dicke and Van Loon, 2000). The possibility that these substances also act in plant-plant communication has been discussed (Arimura *et al.*, 2000; Dicke and Bruin, 2001; Engelberth *et al.*, 2004).

1.2.2. Temporal and physiological variation of emission

Many variations in floral scent profiles have been detected during the lifespan of the flowers including variations in both total amount and specific composition. This variation has been related to the ability of plants to produce and emit higher levels of scent only when their reproductive organs are mature and ready to be pollinated (Dudareva and Pichersky, 2000). For example, both a marked decrease and changes in composition of scent have been observed in flowers soon after pollination. Following this decrease, scent emission normally ceases with the senescence of the flowers since the source of emission of floral scent, mainly petals, wilt and cease to function (Dudareva and Pichersky, 2000).

Emission of scent is also variable during circadian rhythms. For example, emission of myrcene and ocimene from snapdragon flowers reached the highest peaks during the afternoon even when the flowers were exposed to continuous light or continuous dark, showing that emission was not light-dependent but controlled by a circadian rhythm (Dudareva *et al.*, 2003). Flowers of petunia also followed a circadian rhythm and production of benzaldehyde, methylbenzoate, benzylbenzoate and isoeugenol reached maximum output during the afternoon (Verdonk *et al.*, 2005).

Flowers pollinated during the day will show higher scent output at this time and just the opposite for flowers pollinated by nocturnal insects such as moths (Loughrin *et al.*, 1990). Besides the fact that plants can save energy emitting scent only during pollination, there is also an advantage to the plant by avoiding dispersal and waste of pollen by non-specific pollinators. However, visits by generalist pollinators are not always avoided as they might be useful for plant reproduction when specific pollinators are rare or absent (Dudareva and Pichersky, 2000).

Temperature is also important in relation to fragrance emission affecting mainly the quantity of volatile compounds. For example, an increase of 58% in the fragrance emission of *Trifolium repens* flowers was observed when they were exposed to 20 °C instead of 10 °C. This increase involved all the compounds released (Jakobsen and Olsen, 1994). Temperature could be involved in both volatility of compounds and biological processes, including an increase in the biosynthesis of these compounds.

1.2.3. Location of emission

Determination of which part of the flower is emitting volatile compounds is technically difficult because detection of specific compounds inside a floral organ does

not assure that this organ is actually the source of the volatiles. Some specialized morphological structures called "scent glands" have been developed by some plants for the production of floral scent. However the non-specialized floral epidermal cells have also been identified as a direct source of fragrance emission in some species (Dudareva and Pichersky, 2000).

Headspace analysis of flowers from which specific parts have been excised is the closest approach to determining from which organ of the flower the scent is emitted. Results from this type of analysis have found some differences in the specific compounds emitted from different organs, although normally the same components of floral scent are emitted by all the floral organs, varying only in the amount and rate of production (Dobson *et al.*, 1990; Pichersky *et al.*, 1994). Nevertheless, these findings are not completely reliable because these experiments also involve injury to the flower when the parts are excised and this could change emission profiles. Appropriate controls and comparisons with whole-flower emission profiles are needed to avoid these problems (Dudareva *et al.*, 1998).

Evaluation of the expression of genes related to the biosynthesis of volatile compounds in different organs of the flowers has also been useful to address this issue. Dudareva *et al.* (2003) found that a monoterpene synthase involved in snapdragon floral scent biosynthesis showed the highest expression in upper and lower lobes of the flowers. Expression of this gene in the tube and stamens was very low and no detectable signals were found in pistils, sepals, or leaf tissues. In a similar experiment performed by D'Auria *et al.* (2002) on *Clarkia breweri* evaluating the expression of an enzyme which catalyzes the formation of benzylbenzoate (BEBT), the pistil showed the highest expression, stamens, sepals and petals had approximately the same amount of transcripts, lower than pistil. Leaves showed little discernible expression of BEBT.

1.2.4. Hedonic attributes of floral scent

The admiration and the sensual pleasure caused by flower fragrance to mankind has developed a new commercial commodity, even though it is known that floral scent has evolved in plants for other reasons (Vainstein *et al.*, 2001). Volatiles produced mainly synthetically, but also naturally, are widely used in the industry for the production of perfume, cosmetics, air fresheners, laundry detergents, and the food and drink industry (Burdock, 1995). However, the same volatile compound present in different scents emitted by flowers can be perceived by human beings as completely different fragrances. For example, the monoterpene geraniol is the main volatile compound in rose flowers, but is also present in the characteristic scent of jasmine flowers (Croteau and Karp, 1991); furthermore, this compound is also present in the scents of more than 250 different plant species (Knudsen *et al.*, 1993).

Both the composition and amount of volatiles in the fragrance determine a scent's appeal to humans (Burdock, 1995) and can provoke big differences in perception. For example, a high level of indole has a disgusting odour, but at low concentrations it is perceived as floral and pleasant.

1.2.5. Composition

Many investigations have been performed in order to determine the composition of floral scent through collection of headspace and then GC-MS analysis by which different volatile compounds are separated and identified. These studies have described floral scents as a complex mixture of small (approximately 100–250 D) volatile molecules composed mainly of monoterpenoid and sesquiterpenoid, phenylpropanoid, and benzenoid compounds. There are also fatty acid derivatives and

some other chemicals, especially those containing nitrogen or sulfur (Dudareva and Pichersky, 2000).

Knudsen *et al.*, (1993) carried out a checklist of plant volatile compounds isolated by the head-space technique, and a total of more than 700 compounds observed in 441 taxa in 118 studies were compiled in this review. The floral scent components are divided into three different groups: fatty acid derivatives, benzenoids and isoprenoids. The compounds found in the greatest number of taxa are benzenoids and isoprenoids. Most common benzenoids are: methyl-2 hydroxybenzoate, benzaldehyde, benzyl alcohol, benzylacetate, 2-phenylethanol and methyl benzoate, and among the isoprenoids: limonene, myrcene, linalool, pinenes, ocimenes and 1,8-cineole.

Based on functionality the most common compound type is the ester. Among the fatty acid derivatives both saturated and unsaturated hydrocarbons are fairly common. Aldehydes, alcohols and ketones are also frequent. Free acids are less common, whereas esters include the largest number of different chemical structures. Apart from some of the very common benzenoids mentioned above, some other compounds should be noted, like the distinct group of phenylpropanoids (eugenols and cinnamic acid derivatives) (Dudareva and Pichersky, 2000).

1.2.6. Biosynthesis

Biosynthesis of volatile compounds starts with a carbon skeleton and continues then with further modifications, or simple modifications of this basic structure. Terpene pathways are essentially biosynthetic and end up in compounds that are already fairly volatile, in contrast with other compounds (Dudareva *et al.*, 2004).

Modifications of carbon skeletons include reduction or removal of carboxyl groups, addition of hydroxyl groups, and formation of esters and ethers. Catalysis of these modifications is performed by a group, or several groups, of related enzymes clustered in protein families. Previous biochemical studies have identified some of these protein families participating in the biosynthesis of non volatile compounds, however recently some of them related to the biosynthesis of plant volatile compounds have been also identified (Table 1.1) (Dudareva *et al.*, 2004). Some of the modifications and enzymatic reactions listed above are described below.

1.2.6.1. Oxidation by cytochrome P450 Enzymes

The P450 cytochrome oxidases are involved in numerous metabolic pathways and have been identified in many plant and animal species (Schuler, 1996). These enzymes are also involved in many of the reactions required for the biosynthesis of volatile compounds.

Both hydroxylation and cleavage are regulated by these enzymes (Figure 1.1). Hydroxylation is normally found in monoterpene and sesquiterpene biosynthesis, for example, P450 has been found to be involved in the conversion of the sesquiterpene 5-epi-aristolochene to capsidiol, which is a dihydroxylated volatile compound (Ralston *et al.*, 2001).

Table 1.1. List of some of the genes identified in some previous investigations and involved in the biosynthesis of volatile compounds.

Authors	Volatile compound	Enzyme	Gene	Species
Ross et al., 1999 Pott *et al.*, 2004	Methyl salycilate; Methyl benzoate	(Salicylic Acid / Benzoic acid) Methyl Transferase	SAMT and BSMT	S. floribunda, Clarkia, Petunia, Antirrhinum
Underwood *et al.*, 2005	Methyl benzoate; Methyl salycilate	(Salicylic Acid / Benzoic acid) Methyl Transferase	PhBSMT	Petunia
Koeduka *et al.*, 2006	Isoeugenol	IFR, PCBER, PLR	IGS1 isoeugenol synthase 1	*Medicago sativa; P. trichocorpa; Thuja plicata*
Verdonk *et al.*, 2005	Benzenoid		ODO1	Petunia
Shalit *et al.*, 2003	Ester	Acyltransferase	RhAAT1	Rose
Verdonk *et al.*, 2003	Benzenoid: benzaldeyhde, phenylacetaldehyde, methylbenzoate, phenylethylalcohol, isoeugenol and benzylbenzoate	Phenyl ammonia Lyase (PAL) S-adenosyl methionine (SAM)	PAL and SAM	Petunia
Wu *et al.*, 2004	1,3,5-Trimethoxybenzene (TMB)	Phloroglucinol 0-Methyltransferase	POMT	Rose
Scalliet *et al.*, 2006	Phenolic methyl ether 3,5-dimethoxytoluene (DMT)	Orcinol O-Methyltransferase(OOMT)	OOMT	Rose
Wang *et al.*,1997	Methyleugenol Isomethyleugenol	S-Adenosyl-L-Methionine:(iso)eugenol O-Methyltransferase (IEMT)	IEMT	*Clarkia sp*
D'Auria *et al.*, 2002	Benzylbenzoate	Benzoyl-coenzyme A (CoA):benzyl alcohol benzoyl transferase	BEBT	*Clarkia sp*
Dudareva *et al.*, 2000	Methyl benzoate	S-Adenosyl-L-Methionine: benzoic acid carboxyl methyltransferase (BAMT)	BAMT	Snapdragon

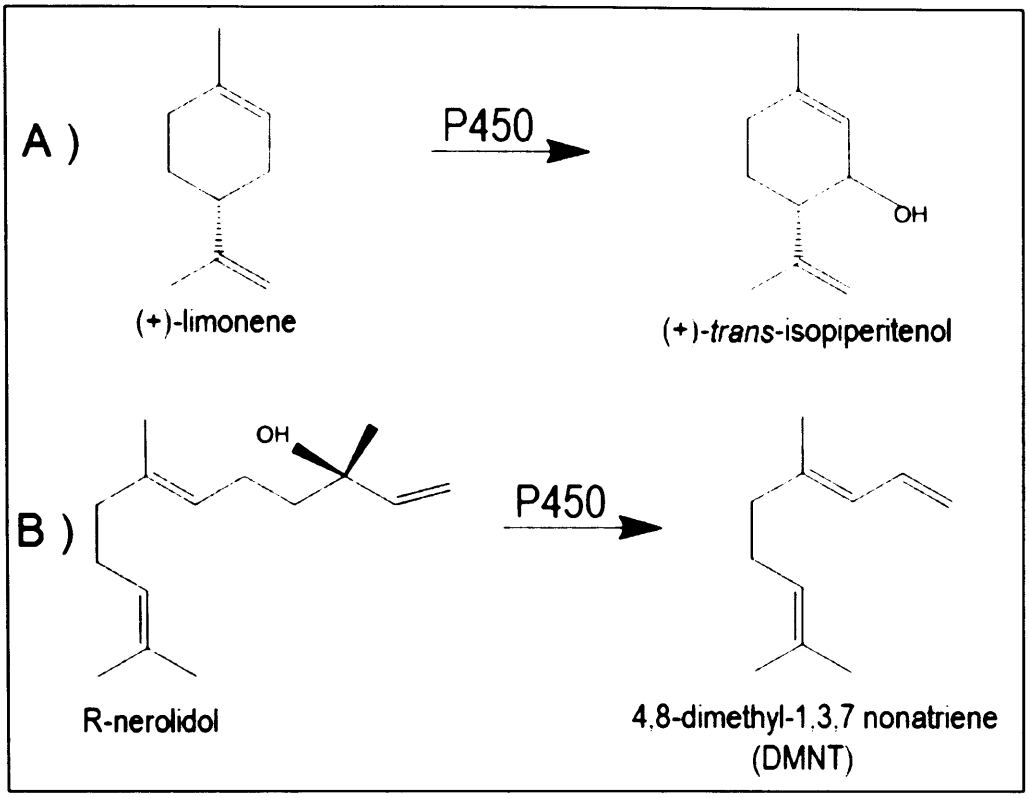


Figure 1.1. P450 oxidation modification reaction leading to the biosynthesis of volatile compounds through A) Hydroxylation and B) Cleavage (Modified from Dudareva *et al.*, 2004).

Cytochrome P450 enzymes are very important in the biosynthesis of phenylpropenes derived from phenylalanine, such as eugenol and the benzenoid vanillin (Frank *et al.*, 1996) and also play an essential role in the octodecanoic pathway, which leads to the biosynthesis of volatiles derived from fatty acids (Howe and Schilmiller, 2002).

1.2.6.2. Oxidation by dehydrogenases

Another important family of enzymes involved in the biosynthesis of volatiles are NADP/NAD-dependent oxidoreductases. Conversion of volatile alcohols and aldehydes has been related to the activity of these enzymes (Figure 1.2), for example, changing

short-chain aldehydes such as hexanal and 3-cis-hexenal to hexenol and 3-cishexenol (Bate *et al.*, 1998).

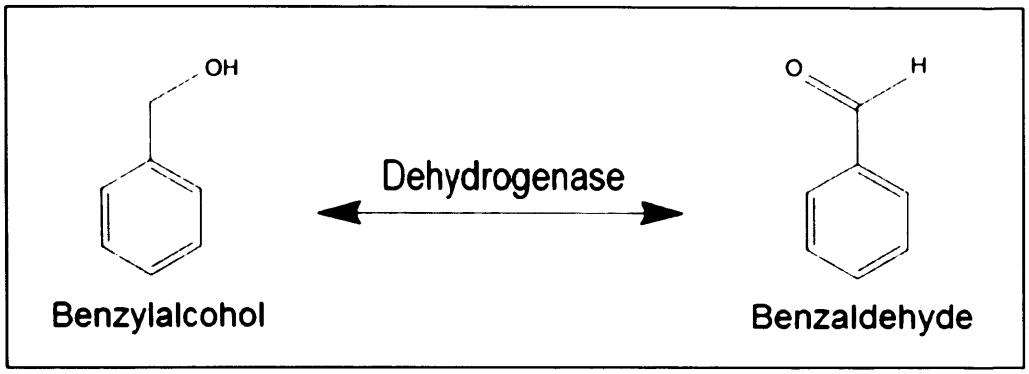


Figure 1.2. Oxidation by dehydrogenases leading to the biosynthesis of volatile compounds (Modified from Dudareva *et al.*, 2004).

Since some of these changes are controlled by nonspecific alcohol dehydrogenases, this lack of specificity has been used for example in ripe tomato fruits in order to alter their aroma profile (Prestage *et al.*, 1999).

Other nonspecific dehydrogenases also regulate synthesis of aldehydes from some terpene alcohols such as geraniol and carveol to aldehydes (Bouwmeester *et al.*, 1998). Some other compounds are also synthesized through this kind of modification such as the lemony-scented geranial and neral, and benzyl alcohol, derived from benzaldehyde in a reversible reaction catalyzed by a member of the NADP/NAD-dependent oxidoreductase family (Boatright *et al.*, 2004).

1.2.6.3. Methylation of hydroxyl groups

A methylated hydroxyl group is a very common side group in a large number of plant volatiles (i.e. a methoxyl group). In some reactions it is catalyzed by a methyltransferase (MT), normally a methyl group is added from S-adenosyl-L-

methionnine (SAM) (Figure 1.3). All plant methyltransferases seem to have a common SAM-binding domain; nevertheless there are diverse families sharing only little primary sequence similarity (Noel *et al.*, 2003).

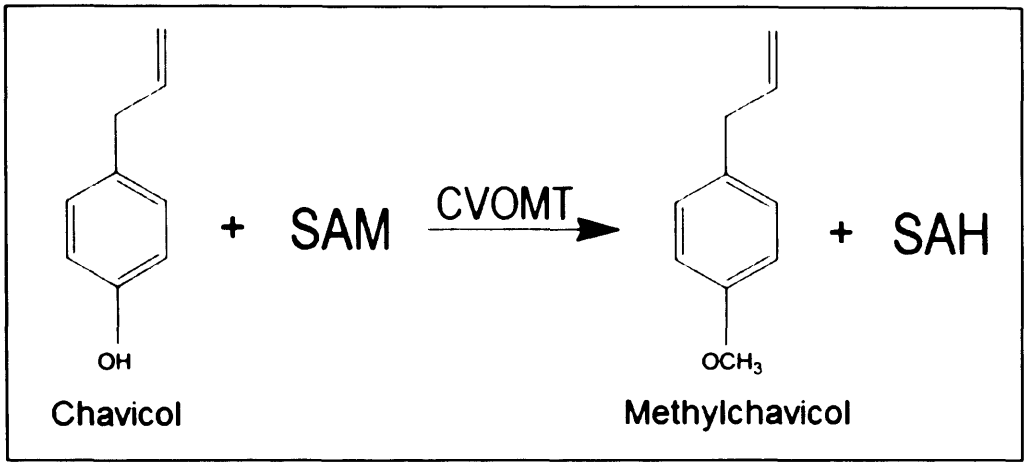


Figure 1.3. Methylation of hydroxyl groups leading to the biosynthesis of volatile compounds (Modified from Dudareva *et al.*, 2004).

Some methyltransferases involved in metabolic pathways of volatile and non-volatile biosynthesis have been identified and included in the Type I methyltransferase family (Noel *et al.*, 2003). Enzymes of this family have been identified in flowers of *Clarkia breweri* and glands of basil (*Ocimum basilicum*) catalyzing the 4-hydroxyl methylation of eugenol to form methyleugenol and controlling methylation of chavicol to methylchavicol respectively (Wang *et al.*, 1997; Lewinsohn *et al.*, 2000).

Another important methylation reaction has been found in the transformation of orcinol (3,5-dihydroxytoluene) to 3,5-dimethoxytoluene, an important scent compound present in many hybrids of roses, through two consecutive methylation reactions catalyzed by two related MTs identified as orcinol OMTs (OOMT1 and OOMT2; Lavid *et al.*, 2002; Scalliet *et al.*, 2002).

The action of another Type I MT was observed in the production of 2,5-dimethyl-4-methoxy-3(2H)-furanone, a major scent compound in strawberry (*Fragaria* x *ananassa*). A large range of substrates are also methylated by this MT, for example coniferal aldehyde and coniferal alcohol included as intermediates in the lignin biosynthetic pathway (Wein *et al.*, 2002).

1.2.6.4. Methylation of carboxyl groups

Methyl esters have been found in many different plants, and have been described in both numerous floral scents (Knudsen *et al.*, 1993) and vegetative tissues under attack by insects or parasites (Chen *et al.*, 2003a). The enzyme salicylic acid carboxyl methyltransferase (SAMT) (Table 1.1) was first reported by Ross *et al.*, (1999) in *C. breweri* flowers and is capable of methylating salicylic acid (SA) (Figure 1.4).

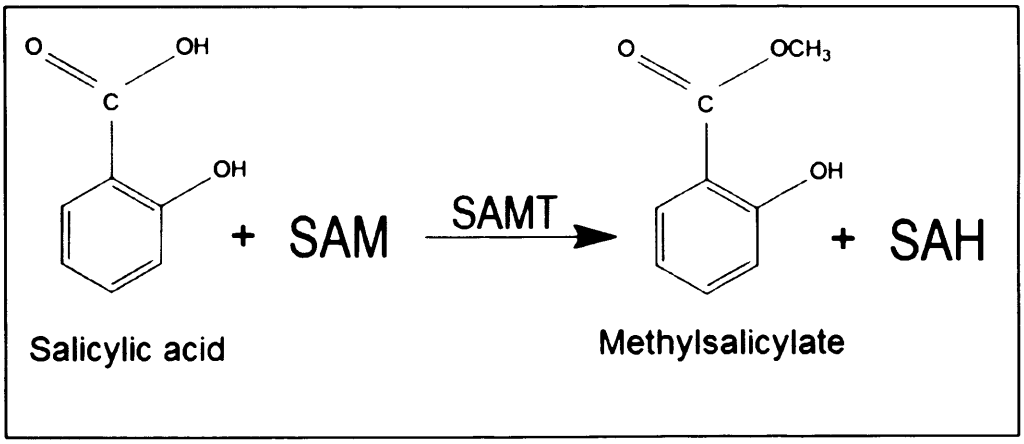


Figure 1.4. Methylation of carboxyl groups leading to the biosynthesis of volatile compounds (Modified from Dudareva *et al.*, 2004).

Type III or SABATH MT are a novel type of plant MT known and includes enzymes which use SAM as the methyl donor. Some SAMT enzymes control the methylation of benzoic acid (BA), a compound very similar to SA (Pott *et al.*, 2004). On the other hand, Benzoic acid carboxyl methyltransferase (BAMT) is the enzyme

responsible of the synthesis of methylbenzoate, a volatile present in snapdragon scent, but is unable to methylate SA (Dudareva *et al.*, 2000).

1.2.6.5. Acylation

Another important modification leading to biosynthesis of volatile compounds is acylation, mainly with an acetyl moiety and also with larger acyls such butanoyl or benzoyl acyls. Certain volatile esters are synthesized by acyltransferases included in a superfamily of enzymes denominated BAHD after the first letter of the first four enzymes identified (St-Pierre and De Luca, 2000). These enzymes catalyze the transfer of an acyl group from an acyl-CoA intermediate to the hydroxyl group of an alcohol (Figure 1.5).

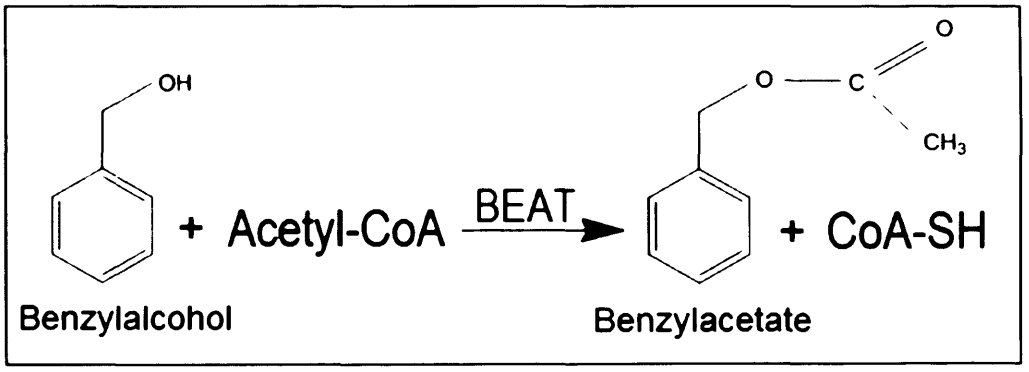


Figure 1.5. Acylation modification leading to the biosynthesis of volatile compounds (Modified from Dudareva *et al.*, 2004).

This group of enzymes has been also observed in the biosynthesis of volatile compounds such as eugenol, participating in reactions at the top of some pathways (Gang *et al.*, 2002). Some BAHD enzymes are also directly involved in the synthesis of other enzymes such as benzyl alcohol acetyl-CoA transferase (BEAT) which produces benzyl acetate (Figure 1.5) and benzyl alcohol benzoyl-CoA transferase which

produces benzylbenzoate in flowers of *Clarkia breweri* (D'Auria *et al.*, 2002) and petunia (Boatright *et al.*, 2004) respectively.

A common characteristic of most of the BAHD enzymes is their wide substrate specificity for both the acyl moiety and the alcohol moiety. For example, the benzyl alcohol benzoyl-CoA transferase enzyme present in petunia flowers is also able to generate phenylethylacetate by transferring an acetyl moiety to the alcohol phenylethanol (Boatright *et al.*, 2004). Furthermore, in rose flowers a BAHD enzyme is able to acetylate both geraniol and citronellol (Shalit *et al.*, 2003).

1.3. TERPENOID COMPOUNDS

1.3.1. General characteristics

Terpenoids represent the largest and most diverse family of natural products with more than 30,000 individual compounds, half of them synthesized by plants (Buckingham, 1998). A small number are involved in primary plant metabolism, for instance in the formation of the phytol side chain of chlorophyll, carotenoid pigments, phytosterols of cellular membranes, and gibberellin plant hormones (Trapp and Croteau, 2001).

However, most terpenoids are not necessary for plant growth and development and probably participate only in ecological functions. These compounds are identified as secondary metabolites (Harborne, 1991) and have been classified mainly into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}) (Dudareva and Pichersky, 2000). Terpenenoids are usually volatile compounds due to their high vapour pressures at normal atmospheric conditions, allowing significant release into the air. Some terpenoids are of pharmacological importance such as limonene which is implicated as a dietary anticarcinogen (Crowell and Gould, 1994), artemisin which acts as an antimalarial (Van Geldre *et al.*, 1997), and the diterpenoid taxol, used as an anticancer drug (Holmes *et al.*, 1995).

1.3.2. Biosynthesis of terpenoid compounds

All terpenoids are derived from the central precursor isopentenyl diphosphate (IDP) and from dimethylallyl diphosphate (DMADP). These basic C_5 units are synthesized from acetyl-CoA in the cytosol via the acetate/mevalonate pathway (Qureshi and Porter, 1981) or from pyruvate and glyceraldehyde-3-phosphate through the alternative methylerythritol phosphate pathway which has been recently discovered (Rodriguez-Concepcion and Boronat, 2002) and is located in the chloroplast. The cytosol-localized acetate/mevalonate pathway may provide C_5 units for sesquiterpene biosynthesis, whilst the plastid-located methylerythritol phosphate pathway, probably supplies IDP and DMADP for hemiterpene, monoterpene, and diterpene biosynthesis (Figure 1.6).

During the second phase of terpene biosynthesis there is a condensation of IDP and DMADP catalyzed by short-chain prenyltransferases (Koyama and Ogura, 1999; Liang *et al.*, 2002) to generate geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP), the precursors of monoterpenes, sesquiterpenes and diterpenes, respectively. While the formation of GDP is not completely clear, it is known that FDP is synthesized by a large family of homodimeric prenyltransferases called FDP synthases (Trapp and Croteau, 2001) (Figure 1.6).

Some more recent studies have contradicted the idea of a strict compartmentalization where GDP and GGDP are present only in plastids and FDP only in the cytoplasm. Davidovich-Rikanati *et al.* (2008) showed that a transgenic line of tomato, expressing a cytoplasm-specific signal sequence linked to a sesquiterpene synthase, was able to produce monoterpenes. This suggests that a pool of GDP, and not only FDP, is also available in this compartment. Furthermore, some TPS with a

dual targeting signal sequence have been reported (Lee and Chapel, 2008; Davidovich-Rikanati *et al.* 2008). Thus a TPS could utilize both monoterpene (GDP) and sesquiterpene (FDP) substrates.

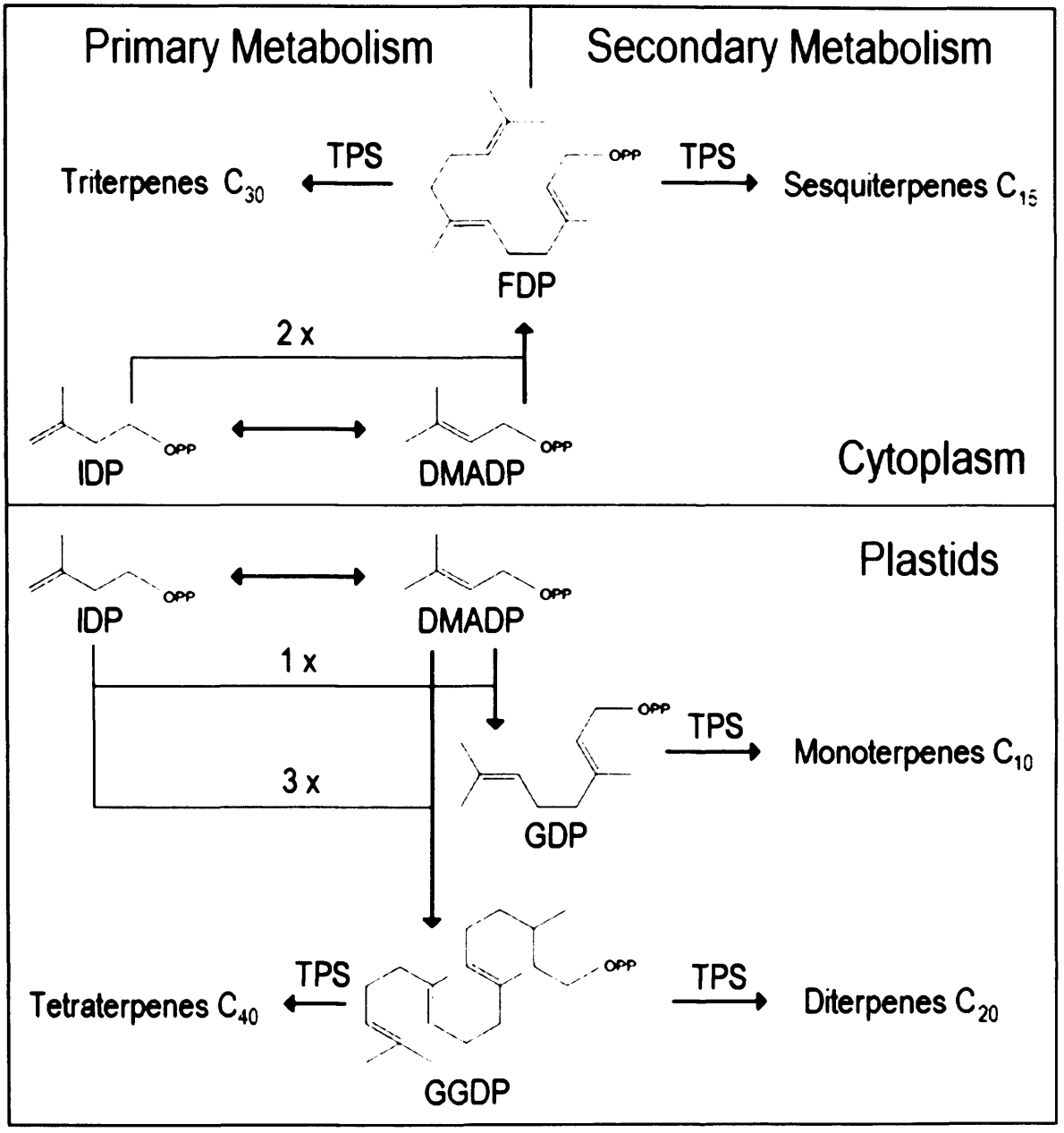


Figure 1.6. Overview of terpene biosynthesis in plants, starting with the condensation of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) to form the main precursors of all terpenoid compounds: geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP). The final product is controlled by specific enzymes called terpene synthases (TPS) (Modified from Douglas *et al.*, 1995).

In the third phase, the intermediate products GDP (C_{10}), FDP (C_{15}), and GGDP (C_{20}) can follow three different routes. One is the self condensation to C_{30} and C_{40}, precursors of triterpenes (i.e. sterols) and diterpenes (i.e. carotenoids) respectively. Alternatively these intermediate products can be utilized in alkylation reactions to produce non-terpenoid compounds (including proteins) providing prenyl side chains. Finally GDP, FDP and GGDP can create the basic parent skeleton of a wide range of terpenoid compounds by internal rearrangements (e.g. cyclization) (Douglas *et al.*, 1995).

The last phase includes modifications of the parent skeleton through oxidation, reduction, isomerization, conjugation, or other secondary transformations, to form an even greater structural variety. These processes are controlled by terpene synthases (TPS), a family including a very large number of enzymes (Trapp and Croteau, 2001).

1.3.3. Conserved domains present in terpene synthases

Little is known about the structural features of plant TPS in terms of how they mediate substrate binding and catalysis (Degenhardt *et al.*, 2009). Despite this fact, two well conserved motifs have been identified and characterized: the aspartate-rich region DDXXD and the arginine-rich region R(R)X_8W (Figure 1.7). The well known aspartate-rich motif has been found in almost all the plant TPS and also in isoprenyl diphosphate synthases and some microbial terpene synthases. Its function has been studied through site-directed mutagenesis and X-ray structural analysis, finding a relation of this motif with the binding of divalent metal ions which in turn interact with the diphosphate moiety of the substrate (Starks *et al.*, 1997; Cane *et al.*, 1996). Thus, mutations in this region could lead to decrease catalytic activity and the synthesis of

abnormal products which can be attributed to altered substrate binding (Seemann *et al.*, 2002; Little and Croteau, 2002; Prosser *et al.*, 2004).

The $R(R)X_8W$ motif is located approximately 60 amino acids from the N-terminus of many monoterpene and sesquiterpene synthases (Figure 1.7). The function of the tandem arginine (RR) is presumably related to the isomerisation of GPP to a cyclizable intermediate since the lack of this RR results in a protein unable to accept GPP as substrate but able to covert a cyclizable intermediate to a final cyclic product (Williams *et al.*, 1998).

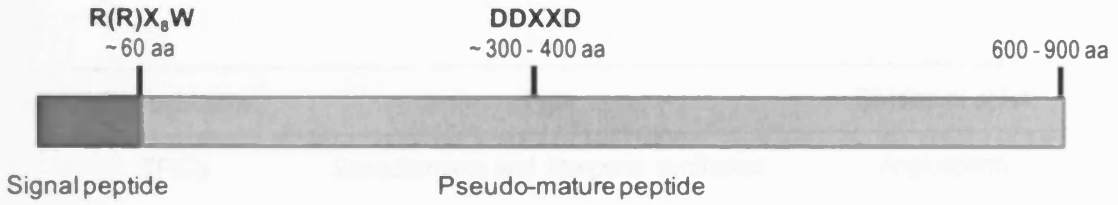


Figure 1.7. Scheme of a general TPS showing the two conserved domains $R(R)X_8W$ and DDXXD and their relative position in the amino acid sequence.

1.3.4. Classification of terpene synthases

Many investigations on TPS have suggested that they share a common evolutionary origin based both on their mechanism of reaction, and their well conserved gene structure and sequence (Bohlmann *et al.*, 1998 and Cseke *et al.*, 1998). Bohlmann *et al.*, (1998) have identified six gene subfamilies within the plant TPS gene family, through a phylogenetic evaluation of 33 terpenoid synthases present in angiosperms and gymnosperms. From this study, the angiosperm terpene synthases: copalyl diphosphate synthase, kaurene synthase, and a *Clarkia breweri* linalool synthase were identified as single members of the subfamilies Tps-c, Tps-e, and Tps-f

respectively. The rest of the terpene synthases were clustered in three other subfamilies; Tps-a clustering sesquiterpene and diterpene synthases from angiosperms; Tps-b, clustering monoterpene synthases from angiosperms; and Tps-d clustering gymnosperm monoterpene, sesquiterpene, and diterpene synthases. An additional subfamily TPS-g has been suggested later by Dudareva *et al.* (2003), including two myrcene synthases (ama1e20 and ama0c15) and an (E)-β-ocimene synthase (ama0a23) found in *Anthirrinum majus* (Table 1.2).

Table 1.2. Classification of terpene synthases in 6 subfamilies proposed by Bohlmann *et al.* (1998) (TPS-a, b, c, d, e, and f) and the additional subfamily TPS-g suggested by Dudareva *et al.*, (2003).

TPS subfamily	Type	Division or order
TPS-a	Sesquiterpene and diterpene synthases	Angiosperm
TPS-b	Monoterpene synthases	Angiosperm
TPS-c	Copalyl diphosphate synthase	Angiosperm
TPS-d	Mono and sesqui and diterpene synthases	Gymnosperm
TPS-e	Kaurene synthase	*Stevia rebaudiana*
TPS-f	Linalool synthase	*Clarkia breweri*
TPS-g	Myrcene and Ocimene synthases	*Anthirrinum majus*

1.3.5. Genomic organization of terpene synthases

The study of the evolution of a gene family can be analyzed by considering only exons, that is only the protein structure, or by considering both exons and introns and their organization within the genome. Even though low sequence similarities sometimes are found in exons, the genomic organization remains highly conserved

(Betts *et al.*, 2001). On the other hand, introns are dynamic structures throughout evolution. Thus introns can quickly change their size and sequence, and slowly throughout evolution their position in the genes. For this reason introns become an interesting parameter to consider in evolutionary studies (Lecharny *et al.*, 2003). While differences in size and sequence of the introns are useful to understand changes in recently duplicated genes (Liss *et al.*, 1997) or evolution of isolated single species, differences in the intron-exon organization could explain more ancient duplications or inter species relationships (Lecharny *et al.*, 2003).

Although non coding DNA or introns are a major component of all eukaryotic genomes, little is known about their size changes, evolution and for example how they spread within and among genes (Lynch and Richardson, 2002). Some of the factors described controlling intron size are: control of gene expression by the presence of regulatory elements (Bergman and Kreitman 2001), selection for reducing the energetic cost of transcription (Carvalho and Clark 1999) and selection for keeping active relatively small chromosomal domains (Prachumwat *et al.*, 2004). Furthermore, Marais *et al.* (2005) studied the introns in Drosophila genes, finding a negative correlation between intron size and expression level of the genes containing these introns. A minority of these Drosophila introns studied did not follow this trend, probably because they were enriched in regulatory elements and possibly because they were also more frequent in highly expressed genes. The regulatory function of introns has been confirmed experimentally (Fu *et al.*, 1995; Bolle *et al.*, 1996) demonstrating that these structures are not all neutral elements.

Regarding the dynamic of losses and gains of introns, the first clear evidence of an intron gain was discovered by Rogers (1985) who described an intron insertion in a serine protease gene. Although there is recent evidence of gained introns in some genomes (Coghlan and Wolfe, 2004), current studies have shown more frequent

examples of losses rather than gains (Cho *et al.*, 2004; Zhan *et al.*, 2008), especially in plant families like *Brassicaceae* (Charlesworth, *et al.*, 1998) and *Fabaceae* (Häger *et al.*, 1996). Moreover, when the insertion or deletion of an intron takes place between two codons (i.e. phase 0 introns), although no changes in the expression of the protein occur, this event may trigger some subfunctionalization either directly by introducing a difference in regulatory elements or by helping exon shuffling (Lecharny *et al.*, 2003).

1.4. OBJECTIVES

The main objective of this project was to evaluate the floral scent of Alstroemeria from two main starting points:

1.4.1. Scented cultivars of Alstroemeria

To my knowledge only three scented genotypes of Alstroemeria are currently available on the market as this character has been selected against in this species in commercial breeding programmes. Thus, the fragrance of these three scented genotypes was characterized using GC-MS and these profiles were later on used as a starting point to investigate genes related to scent production. Furthermore, the floral scent of Alstroemeria was evaluated through sensorial analysis first to assess the appreciation of this character by consumers and its importance as a subject of study, and then to investigate interactions of scent liking with visual stimuli and scent intensity. Finally, some new lines of inbred *Alstroemeria caryophyllaea* were generated in order to evaluate their potential for future breeding purposes.

1.4.2. A putative Alstroemeria TPS

Based on the information obtained from the GC-MS analysis where individual volatile compounds were identified, the full length ORF of a putative Alstroemeria TPS was obtained and then compared with other TPS, in order to classify this enzyme in one of the TPS subfamilies previously proposed, and also to investigate the putative evolution of its genomic organization. Moreover, gene expression studies were performed in order to evaluate the spatial and temporal expression of this gene in flowers of Alstroemeria. Finally this putative Alstroemeria TPS was expressed in *E.coli* in order to functionally described this enzyme and obtain evidence for its participation in the biosynthesis of some of the terpenoid compounds emitted by the flowers.

CHAPTER II.

GENERAL MATERIAL AND METHODS

II. GENERAL MATERIAL AND METHODS

2.1. PLANT MATERIAL

Flowers from five different genotypes of Alstroemeria (Figure 2.1) at eight different stages of floral development (Figures 2.2, 2.3 and 2.4) were used in order to evaluate their floral scent through different techniques, including GC-MS, semi q and qRT-PCR and sensorial analysis. The flowers of the cultivars 'Sweet Laura' and 'Samora' and the species *A. caryophyllaea*, were grown at the University Botanical and Research Garden of Cardiff University (Cardiff, UK) in a greenhouse with temperature set at a minimum of 14 °C. Humidity and light were not controlled. Cultivars 'Rebecca' and 'Ajax' were obtained as cut flowers from commercial sources.

The non scented genotypes comprise *A.* cv. 'Rebecca' belonging to Royal Van Zanten and *A.* cv. 'Samora' from Van Staaveren B.V. The scented genotypes include the species *A. caryophyllaea*, native to southern Brazil; and the cultivars 'Sweet Laura' which is derived from the cross between *A. aurea* X *A. caryophyllaea* produced by Professor Mark Bridgen at Cornell University, USA; and 'Ajax' bred by Könst in the Netherlands with a pedigree including *A. caryophyllaea* as the only contributor of scent, and another seven non scented ancestors (Meijles, 2008).

Stages of development for *A.* cv. 'Samora' and cv. 'Rebecca' were already defined in Wagstaff *et al.* (2001) and Breeze *et al.*, (2004) respectively. For the remaining genotypes, stages were assigned following the patterns described by

Wagstaff *et al.* (2001) and Breeze *et al.*, (2004) where S0= coloured bud; S1= first tepal opening; S2= full flower opening; S3= three dehiscent anthers; S4= six dehiscent anthers; S5= stigma opening with stigmatic liquid on its surface; S6= loss of colour and start of wilting in tepals and S7= falling of wilted tepals (Figures 2.2, 2.3 and 2.4).

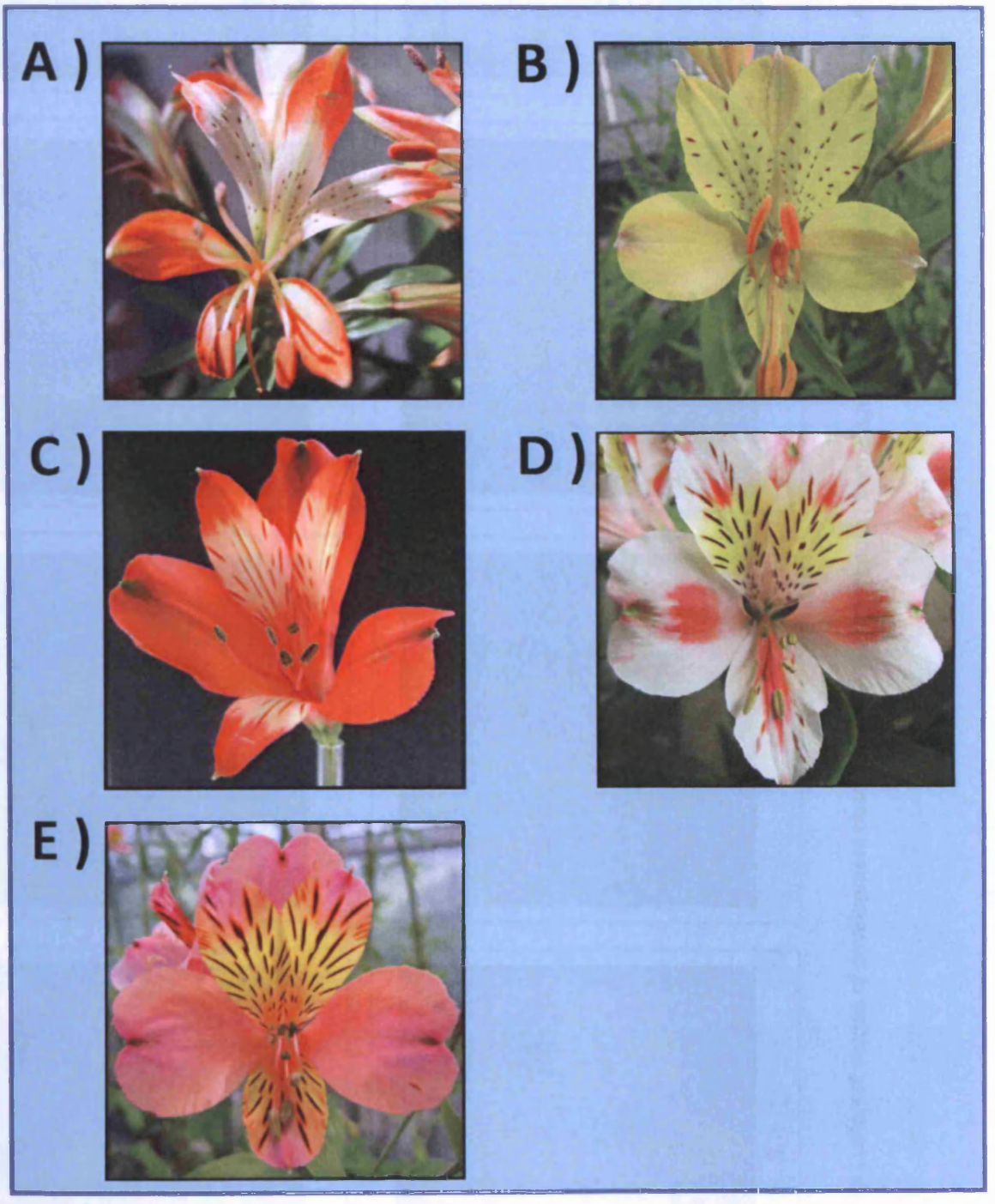


Figure 2.1. Alstroemerias used for the evaluation of floral scent through GC-MS, RT-PCR and sensorial analysis. Scented genotypes: A) *A. caryophyllaea,* B) *A.* cv. 'Sweet Laura', C) *A.* cv. 'Ajax' and non scented genotypes: D) *A.* cv. 'Rebecca' and E) *A.* cv. 'Samora'.

Figure 2.2. Eight different stages of development observed in *A.* cv.'Sweet Laura', from stage 0 (S0) to 7 (S7) according to Wagstaff *et al*, 2001.

Figure 2.3. Eight different stages of development observed in *A.* cv. 'Ajax', from stage 0 (S0) to 7 (S7) according to Wagstaff *et al*, 2001.

Figure 2.4. Eight different stages of development observed in *A.* caryophyllaea, from stage 0 (S0) to 7 (S7) according to Wagstaff *et al*, 2001

In addition, different organs of the flowers were used and analyzed for both the evaluation of the gene expression of a putative Alstroemeria TPS (Chapter V) and for the morphological description of the new lines of scented Alstroemerias (Chapter VII). Thus the reproductive organs studied were outer and inner tepals, pistil and stamens (Figure 2.5).

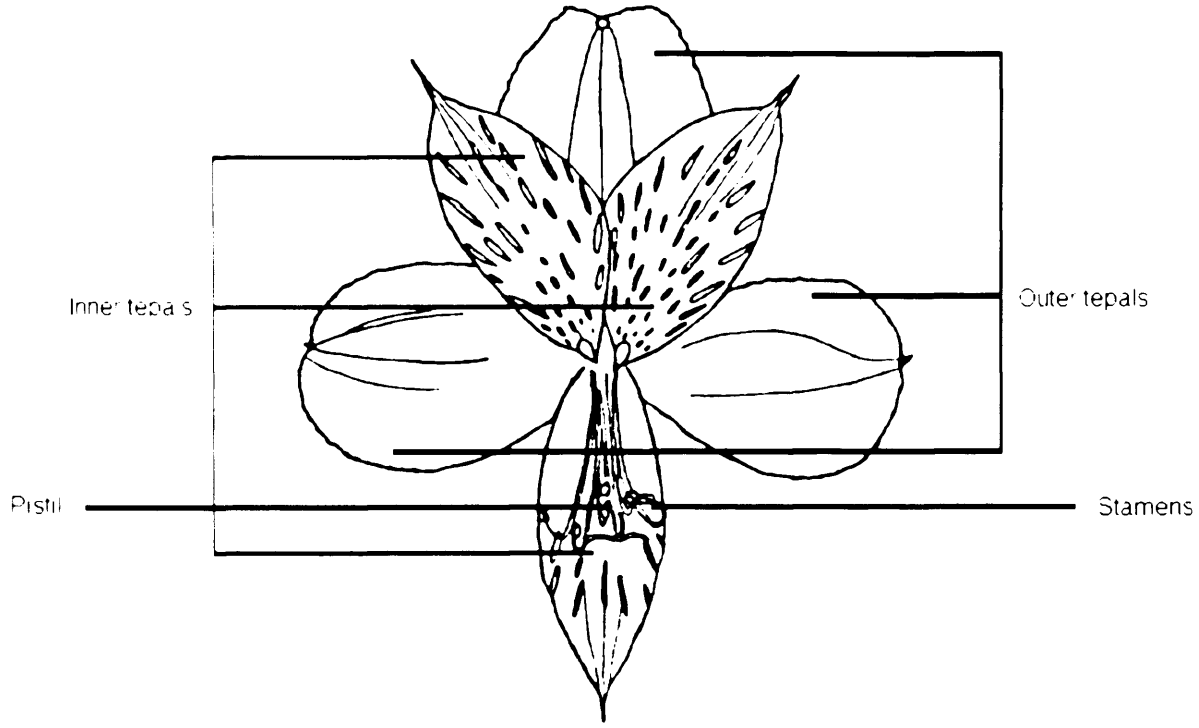


Figure 2.5. Alstroemeria flower scheme showing reproductive organs used for RNA extraction and morphological evaluation.

2.2. MOLECULAR BIOLOGY PROCEDURES

2.2.1. RNA extraction

RNA from flowers and leaves of Alstroemeria was extracted using approximately 200 mg of plant material collected from at least three independent samples (flowers or leaves). The extract was placed into sterile mortars and ground in liquid nitrogen with a pestle to a fine dust. Thereafter 2 ml of TRI Reagent® (Sigma) were added to form a homogeneous paste. Equal amounts of paste were transferred into two 1.5 ml Eppendorf tubes. Tubes were vortexed and left at room temperature for five minutes to permit the complete dissociation of nucleoprotein complexes. Next, the tubes were centrifuged using an Allegra 21R centrifuge (Beckman Coulter Inc., Germany) at 16,420 x g for 15 min at 4 °C. Aqueous top layers were transferred to fresh 1.5 ml Eppendorf tubes and 0.2 ml of chloroform was added to each tube. Tubes were vortexed and centrifuged at 16,420 x g for 15 min at 4 °C. Following centrifugation, the top layer was transferred again to fresh 1.5 ml Eppendorf tubes and 0.5 ml of isopropanol was added. Following mixing by inversion, tubes were kept at room temperature for 10 min and then centrifuged at 16,420 x g for 10 min at 4 °C. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. Tubes were centrifuged at 16,420 x g for 10 min at 4 °C. The supernatant was removed and the pellet was air-dried for 10 to 30 min. The pellet was resuspended in 50 µl of sterile water and contents of the tubes were combined.

In order to assess RNA in the samples, 10 µl were used for electrophoresis on an ethidium bromide stained agarose gel 1% at 80 V for 30 min (See section 2.2.5). The tank, comb and tray used for electrophoresis were treated with NaOH (0.1 M) and

washed copiously with distilled water prior to use. Concentration of RNA (ng · μl^{-1}) was measured by spectrophotometry using a Genequant instrument (Pharmacia).

2.2.2. DNAse treatment

In order to degrade any DNA contamination in the RNA samples, a DNAse digestion reaction was set up as follows: 2 µg of RNA, 2 µl of RQ1 DNAse 10X buffer (Promega, Madison, WI, USA), 2 µl of RQ1 DNAse (Promega, Madison, WI, USA) and water up to a total volume of 20 µl. The samples were incubated at 37 °C for 30 min. Following the incubation, 2 µl of RQ1 DNAse stop solution (Promega, Madison, WI, USA) were added to terminate the reaction. Finally, samples were exposed to 65 °C for 10 min to inactivate the DNAse.

2.2.3. cDNA synthesis

For cDNA synthesis, 19 µl of RNA (1-2 µg) and 1 µl of Oligo (dt) 15 (500 µg μl^{-1}) (Promega, Madison, WI, USA) were incubated at 70 °C for 10 min and then cooled at 4 °C for 10 min. The samples were mixed with 6 µl of M-MLV RT buffer (Promega, Madison, WI, USA), 2 µl of 0.1 M DTT (Dithiothreitol) and 1 µl of 10 mM dNTPs (Promega, Madison, WI, USA), and then incubated at 42 °C for 2 min. Finally, 1 µl of M-MLV enzyme (reverse transcriptase) (Promega, Madison, WI, USA) was added and the reaction incubated at 42 °C for 50 min followed by inactivation at 70 °C for 15 min.

2.2.4. PCR

PCR amplifications were carried out in a 25 µl volume containing 2.5 µl of 10X PCR buffer (500 mM KCl, 100 mM Tris HCl - pH 9 at 25 °C), 0.2 mM of each dNTP

(Promega, Madison, WI, USA), 0.4 µM of each primer (Sigma, Genosys), 1.0 µl of cDNA and 0.125 µl Taq Polymerase (~5u/µl) (Purified in house at Cadiff Univerisity, School of Biosciences). As a negative control, a PCR reaction containing a volume of water replacing the cDNA was included for each set of PCR reactions to ensure there was no contamination in any of the PCR reagents.

Thermal cycling was performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA) and the amplification was conducted following the thermal profile: Initial denaturation at 95 °C for 1 min; cycles of 1 min at 95 °C, 1 min at a Tm temperature according to the primers used and 1 min at 72 °C; and a final extension at 72 °C for 15 min. The number of cycles was specific for each experiment.

2.2.5. Agarose gel electrophoresis

For a 1% agarose gel, 1g of agarose (Bioline) was mixed into a flask with 100 ml of 1x TAE (0.484 g Tris base, 0.114 ml of glacial acetic acid, 0.2 ml of 0.5 M EDTA pH 8.0). The solution was heated in a microwave oven until agarose was completely dissolved. Afterwards, the solution was allowed to cool at room temperature until it reached 50 – 55 °C and then 3 µl of ethidium bromide (10 mg/mL) were added and gently mixed. The solution was poured into a gel tray and an appropriate number of combs inserted. The gel was allowed to set for 15 - 30 min at room temperature and then placed in an electrophoresis chamber covered with 1x TAE.

Each sample was mixed with 3 µl of 10 X loading buffer (50 mM Tris HCl pH 7.6, 60% glycerol and bromophenol blue) and then loaded in the wells of the gel. Electrophoresis was carried out at 100 V for 30 min or until an optimum separation of the bands was observed. DNA ladder 1 Kb (Invitrogen) 250 - 500 ng was used as marker for all the electrophoresis.

2.2.6. PCR clean up using QIAquick PCR Purification Kit Protocol

For the cleaning of PCR products and other enzymatic reactions, 5 volumes of Buffer PB (QIAGEN) were added to 1 volume of the PCR sample and mixed. The mix was placed in a QIAquick spin column with a 2 ml collection tube, and centrifuged at room temperature for 1 min at 12,100 x g using a microcentrifuge (Minispin plus, Eppendorf). The flow-through was discarded and in order to wash the samples, 0.75 ml Buffer PE (QIAGEN) was added to the QIAquick columns and then centrifuged at 12,100 x g for 1 min. The flow-through was discarded again and the samples were centrifuged for an additional 1 min at 12,100 x g to remove residual wash buffer. The DNA was eluted by placing the QIAquick columns into a clean 1.5 ml Eppendorf tube and 40 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the column. Columns were centrifuged for 1 min at 12,100 x g.

2.2.7. Excision of a band from agarose gel using QIAquick Gel Extraction Kit

Bands were excised from agarose gels using a clean scalpel and then weighed. 3 volumes of Buffer QG (QIAGEN) was added to 1 volume of gel (100 mg ~ 100 µl) and the samples were incubated at 50 °C for 10 min or until the gel slice was completely dissolved. One volume of isopropanol was added and the samples were placed into QIAquick spin columns with a 2 ml collection tube, and then centrifuged at room temperature for 1 min at 12,100 x g using a microcentrifuge (Minispin plus, Eppendorf). The flow-thourgh was discarded and to wash, 0.75 ml of Buffer PE were added to the QIAquick (QIAGEN) column followed by a centrifugation for 1 min at 12,100 x g. The flow–through was discarded and the columns were centrifuged for an

additional 1 min at 12,100 x g to remove residual wash buffer. Finally, the QIAquick columns were placed into a clean 1.5 ml Eppendorf tube and DNA was eluted in 40 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) by centrifuging the column for 1 min at 12,100 x g.

2.2.8. Ligation using pGEM-T easy vector

For DNA ligation, 7 µl of insert DNA, 1 µl of 50 $ng \cdot \mu l^{-1}$ pGEM-T easy vector (Promega, Madison, WI, USA), 1 µl of 10X rapid ligation buffer (Promega, Madison, WI, USA) and 1 µl of ligase (Promega, Madison, WI, USA) were mixed and incubated at 4 °C overnight.

2.2.9. Transformation of *E. coli* competent cells

Two different competent *E. coli* strains, DH5α and BL 21 (Purified in house at Cardiff University, School of Biosciences), were used for general cloning and protein expression respectively. The same transformation protocol was used for both strains.

Competent *E. coli* cells stored at -80 °C were thawed on ice and 70 µl were mixed with 2 µl of the DNA ligation in pre-cooled Eppendorf tubes. The tubes were kept on ice for 20 min, then exposed to 42 °C for 45 s in a water bath and transferred back to ice for 2 min. Liquid LB medium (900 µl) (Lysogeny broth; 10 g/L NaCl; 10 g/L tryptone and 5 g/L yeast extract, pH 7.0) were added and the mix was incubated at 37 °C for 1 h shaking at 100 rpm. The cells (100 µl) were plated onto solid LB medium (10 g/L NaCl; 10 g/L tryptone; 5 g/L yeast extract and 10 g/L agar, pH 7.0) with antibiotic (Ampicillin 100 $\mu g \cdot ml^{-1}$). As a positive control, 50 µl of a transformation with intact plasmid DNA was plated under same conditions. Plates were incubated at 37 °C overnight and then transferred to 4 °C.

2.2.10. Colony PCR

Each single colony was inoculated individually in 1.5 ml Eppendorf tubes containing 200 µl of liquid LB medium with antibiotic (Ampicillin 100 $\mu g \cdot ml^{-1}$). Tubes were incubated at 37 °C for 4-5 h shaking at 100 rpm. In order to confirm bacterial growth and successful transformation, the clones were analyzed by PCR with M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-AACAGCTATGACCATG-3') primers, using 1 µl of bacterial cultures and following the same PCR conditions described previously (2.2.4). PCR amplification products were separated on an ethidium bromide stained agarose gel (1 %) and clones were selected for sequencing based on size and amount of the product.

2.2.11. Plasmid DNA purification using QIAprep Spin Miniprep Kit

Cultures of selected clones (50 µl) were used to inoculate 3 ml of liquid LB medium with antibiotic (Ampicillin 100 $\mu g \cdot ml^{-1}$) and incubated at 37 °C overnight shaking at 200 rpm. From the overnight cultures, 0.5 ml were used to make glycerol stocks: Cells were centrifuged for 2 min at 7,224 x g using a microcentrifuge (Minispin plus, Eppendorf), the supernatant was removed and the pellet was resuspended in 400 µl of liquid LB medium and 100 µl of sterile glycerol. Glycerol stocks were stored at -80 °C. The rest of the culture was split in two samples of 1.25 ml each and and transferred to two 1.5 ml Eppendorf tubes for plasmid DNA extraction. Samples were centrifuged for 3 min at 7,224 x g using a microcentrifuge (Minispin plus, Eppendorf). The supernatant was removed and the two pellets obtained were mixed.

The pellets were resuspended in 250 µl Buffer P1 (QIAGEN) and then 250 µl of Buffer P2 (QIAGEN) was added. The samples were mixed thoroughly by inverting the tube 4–6 times and 350 µl Buffer N3 (QIAGEN) were added. The samples were centrifuged at room temperature for 1 min at 12,100 x g using a microcentrifuge (Minispin plus, Eppendorf). The supernatants were applied to QIAprep spin columns by decanting or pipetting and the samples were centrifuged at 13,000 for 1 min using a microcentrifuge (Minispin plus, Eppendorf). The flow-through was discarded and 0.75 ml Buffer PE (QIAGEN) were added followed by a centrifugation for 1 min at 12,100 x g, to wash the samples. This step was repeated and the flow-through was discarded again and the samples were centrifuged for an additional 1 min at 12,100 x g to remove residual wash buffer. Buffer EB (40 µl of 10 mM Tris·Cl, pH 8.5) (QIAGEN) at ~60 °C were added to the columns and the samples were centrifuged for 1 min at 12,100 x g to elute the DNA.

Purified DNA (3 µl) was analyzed on an ethidium bromide stained agarose gel (1.5 %) and DNA concentration (ng · μl^{-1}) of the samples was quantified using a spectrophotometer (GeneQuant, Pharmacia).

2.2.12. Sequencing

All the sequencing was carried out at Cardiff University, School of Biosciences, using an ABI Prism 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-AACAGCTATGACCATG-3') primers.

The sequences were viewed using the CHROMAS software (ver. 2.2.3, Technelysium, Qld, Australia) to check the quality of the sequence and exported into

the DNAstar package (DNAstar, Madison, WI) to edit the sequence and remove the areas of sequence corresponding to the plasmid vector.

Sequences obtained were analyzed using BLAST (Basic Local Alignment Search Tool), available on line in the National Center for Biotechnology Information webpage (www.ncbi.nih.gov/BLAST/), in order to find related sequences.

2.2.13. SDS-PAGE

SDS-PAGE 12% gels used for protein separation were made by mixing 3.3 mL 40% acrylamide/*bis*-acrylamide (37.5:1) (Melford Laboratories), 4.4 mL 2.5X separation buffer (1.875 M Tris-HCl, pH 7.5, 0.25% SDS), 3.3 mL dH_2O, 100 ul 10 % ammonium persulfate (APS), 10 µl N,N,N',N'-tetramethyl-ethylenediamine (TEMED) (Sigma) and cast between two glass plates. Stacking gel contained 0.56 mL 40 % acrylamide/*bis*-acrylamide (17.5:1), 0.66 mL 5X stacking buffer (0.3 M Tris-HCl, pH 6.7, 0.5% SDS), 2 mL dH_2O, 30 uL 10 % APS, 5 ul TEMED and cast on top of separation gel. Once this had set, samples were mixed with the appropriate amount of 5X loading buffer (250 mM Tris-HCl pH 6.8, 10 % SDS, 30 % glycerol, 0.5 M DTT, 0.02 % bromophenol blue) and boiled for 5 min before loading. The gel was run in 1X Laemmli buffer (10X: 10 g/L SDS, 30.3 g/L Tris base, 144.1 g/L glycine) at 120 V in a Biorad gel system. Finally, the proteins were detected by staining the gel with Coomassie Brilliant Blue stain (2.5 g Coomassie Brilliant Blue R-250) (Sigma), 450 mL ethanol, 100 mL acetic acid and 450 mL dH_2O).

CHAPTER III.

CHARACTERIZATION OF FLORAL SCENT OF ALSTROEMERIA THROUGH GC-MS ANALYSIS

III. CHARACTERIZATION OF FLORAL SCENT OF ALSTROEMERIA THROUGH GC-MS ANALYSIS

3.1. INTRODUCTION

Floral scent is mainly considered as a mechanism of attraction for pollinators and its composition is based on a combination of diverse volatile compounds, including aromatics, terpenoids and fatty acid derivatives (Knudsen *et al.*, 1993). The wide range of volatile compounds and their relative amounts in the final bouquet determines a high specificity of floral scent in different species of pollinator.

Considering both the important role of floral scent in plant reproduction and its aesthetic value, from a human point of view, many investigations have focused on characterizing the composition of floral scent in rose (Dobson *et al.*, 1990), snapdragon (Dudareva *et al.*, 2000), carnation (Jürgens *et al.*, 2003), petunia (Kondo *et al.*, 2006) and clarkia (Pichersky *et al.*, 1994) among other species.

Several methods have been used for the analysis of fragrance in flowers including sensorial analysis (Morinaka *et al.*, 2001; this study), e-nose (Fukai and Abe, 2002) and GC-MS (Dudareva *et al.*, 2000; Jürgens *et al.*, 2003; Kondo *et al.*, 2006; Pichersky *et al.*, 1994). Considering that sensorial analysis depends on human perception, therefore quantitative and objective evaluation is difficult, and the e-nose is a novel method that is also mostly qualitative, GC-MS has been the most commonly

used analytical method for floral scent evaluation because of its high-separation capacity of volatile mixtures (Yeon Oh *et al.*, 2008).

The GC-MS instrument is made up of two parts, the gas chromatography (GC) portion which separates the chemical mixture into pulses of pure chemicals and the mass spectrometer (MS) which identifies and quantifies the chemicals. Samples are injected into the GC and then carried by an inert (non-reactive) gas, usually helium, through the instrument. The chemicals in the mixture are separated based by partitioning between gas and column coating (stationary phase), as a rule of the thumb, small molecules travel more quickly than larger molecules. The MS is used to identify chemicals based on their structure. The column effluent is exposed to an electron beam, which ionises the molecules and, in most cases, cause fragmentation of the molecule into smaller moieties. The ions are focussed, accelerated and travel through an electromagnetic field that filters the ions based on mass to charge ratio. The ions are detected and recorded as mass spectrum of a compound (Hübschmann, 2008).

Different methods of collection or sampling methods can be performed before the GC-MS analysis and they can be divided in two main groups: Static headspace collection and dynamic headspace collection or 'Purge and trap' method. One of the most used static head space collection methods is solid-phase microextraction (SPME) because of its low cost, easy manipulation and solvent-free extraction procedure, involving the immersion of a phase-coated fused silica fibre into the liquid sample or the headspace above the liquid or solid sample, to adsorb/absorb the volatile compounds (Arthur and Pawliszyn, 1990; Wercinski, 1999).

In this study, the GC-MS technique using SPME has been utilized to characterize the fragrance of Alstroemeria flowers by identifying the volatile compounds emitted. In addition, some evaluations were performed at different times during the day in order to determine a possible circadian effect on the floral scent emission.

3.2. MATERIAL AND METHODS

Four single flowers of Alstroemeria collected at stage of development 4 or 5 (as described in chapter II) were used and 3 replicates were performed for each evaluation in order to identify the volatile compounds released by the flowers.

The flowers were enclosed in 300-ml flasks with 30 ml of distilled water for 1 hour (Figure 3.1). SPME was performed using two fibres, red and white, in order to capture a wider spectrum of volatile compounds as these fibres have different specificity (Table 3.1).

Table 3.1. Characteristics of the two fibres used for GC-MS analysis (Sigma-Aldrych, San Luis, MO, USA).

Fibre	Thickness	Material	Coated	Recommended for
Red	100 µm	Fused silica	Polydimethylsiloxane (PDMS)	Volatiles (MW 30-225)
White	85 µm	Fused silica	Polyacrilate	Polar semi-volatiles (MW 80-300)

The fibre (red or white) was exposed into the flasks for one hour to the headspace over the flowers. After this, the fibre was desorbed for 2 min at 220 °C in splitless mode in the injection port of the GC machine model Fisons GC 8000 (Fisons Instruments, Manchester, UK). Gas chromatography was performed on a 30 m 0.25 mm I.D. capillary column 0.25 µm VF-23 ms (Varian). Helium was used as a carrier gas (40 cm · s^{-1}). Chromatograms were recorded using the following temperature programme: start at 40 °C for 5 min, linear gradient of 5 °C · min^{-1} to 250 °C, final temperature 250 °C for 5 min (total of 52 min).

Before each set of samples, the fibre was conditioned at 200 °C for 1 h in the oven of the GC-MS, a blank sample (fibre non exposed) was also performed. Furthermore, an alkane standard solution C_8-C_{20} (Sigma-Aldrich) was analyzed regularly in the GC-MS in order to ensure that the conditions of the instrument were consistent in time and to allow calculation of retention indices. Quantitative standards were not performed and relative quantities derived from normalisation of peak areas to the largest signal observed.

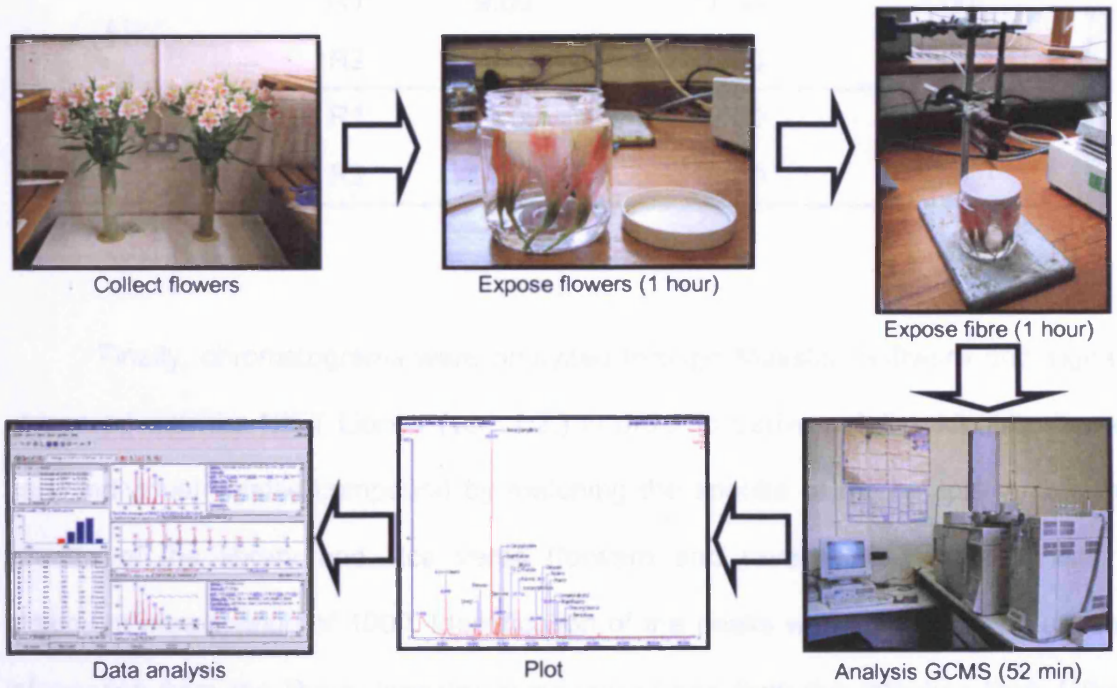


Figure 3.1. Scheme of the methodology performed during the analysis of flowers of Alstroemeria through GC-MS.

Mass spectrometry analysis was carried out in a Finnigan MD 800 (Fisons Instruments, Manchester, UK). Data was recorded at two scan per sec with an m/z 35.00 to 500.00 scanning range, using positive electron impact mode (EI+).

All the evaluations aimed at detecting the volatile compounds in each sample were carried out around midday-afternoon (11 am - 15 pm). In addition, some evaluations aimed at identifying whether a circadian rhythm affects the floral scent

emission were performed. For this purpose, *A.* cv. 'Ajax' was evaluated every 8 hours and cv. 'Sweet Laura' was evaluated in the morning (9 am – 11 am) and afternoon (17 pm – 18 pm), performing two replicates in each case (Table 3.2).

Table 3.2. Summary of the evaluations performed using the GC-MS in Alstroemeria cvs. 'Sweet Laura' and 'Ajax'. R1 and R2 stands for replicates performed.

		Morning	Afternoon	Night
'Ajax'	R1	9:00	17:00	1:00
	R2	10:00	18:00	2:00
'Sweet Laura'	R1	10:00	17:00	-
	R2	11:00	18:00	-

Finally, chromatograms were analyzed through Masslab Software and signals compared with the NIST Library (ver. 1.2.) in order to derive putative identification of each individual volatile compound by matching the spectra of the sample versus the spectra of the library and vice versa (forward and reverse respectively), with a maximum match value of 1000. Identification of the peaks was achieved by matching information from the library (spectra comparison) and from the retention time. When identification was not possible, volatile compounds were named according to the group they belong to (monoterpene, sesquiterpene, aromatic) and numbered in order of appearance.

3.3. RESULTS

3.3.1. Volatile compound identification

3.3.1.1. *Alstroemeria* cv. 'Rebecca'

A. cv. 'Rebecca' is a non scented cultivar so as expected, no consistent signal of any volatile compound was observed in the chromatograms obtained from the analysis through GC-MS using both red (Figure 3.2) and white fibre (Figure 3.3). This finding supports the suggestion of a good correlation between human perception and GC-MS results.

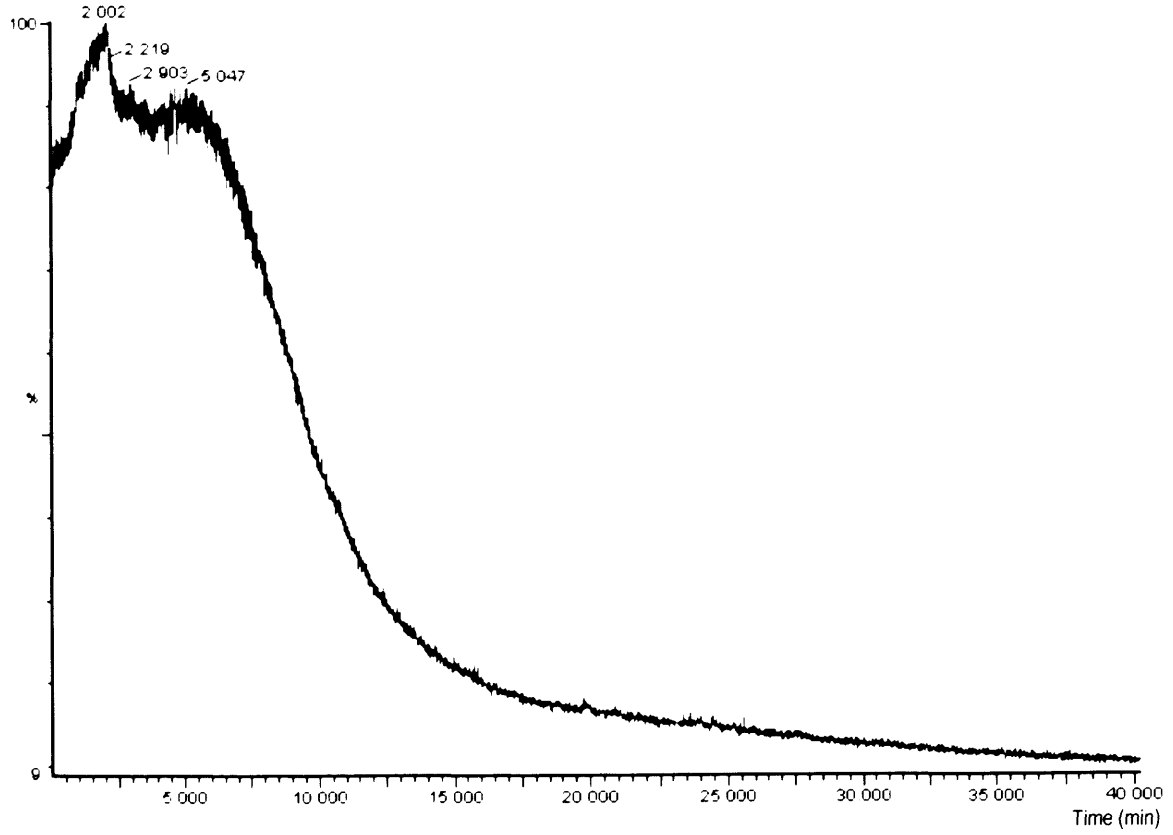


Figure 3.2. Chromatogram obtained from the analysis through GC-MS assayed in *A.* cv. 'Rebecca' flowers (Stage 4-5) using the red fibre.

Only an alcohol species was detected at 17 min using the white fibre, probably due to a contamination during the analysis. This compound is a common alcohol and could be present in the flasks used during the headspace collection, but is definitely not a component of floral scent since this compound is an industrial solvent also known under trade names Carbitol, Carbitol cellosolve, Transcutol.

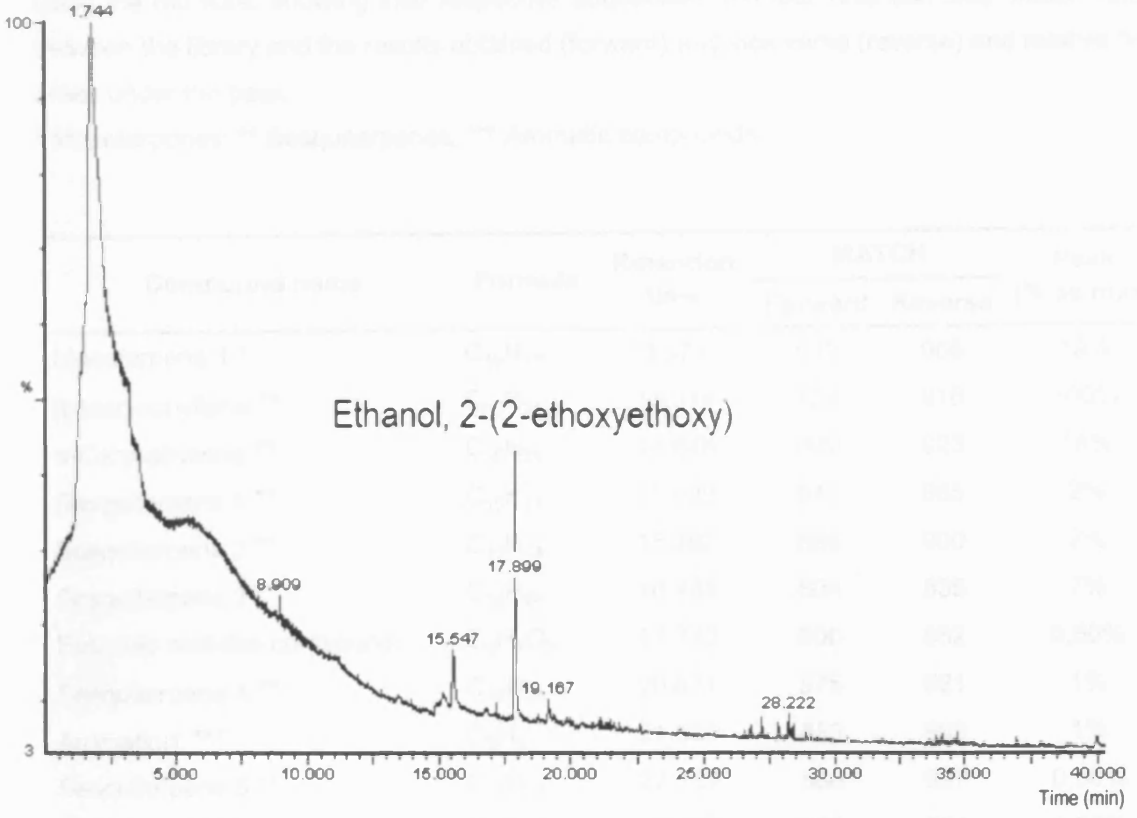


Figure 3.3. Chromatogram obtained from the analysis through GC-MS assayed in *A.* cv. 'Rebecca' flowers (Stage 4-5) using the white fibre.

Since no volatile compounds were identified in non scented 'Rebecca', this result can act as a reference or negative control and is useful as a comparison for the analysis using GC-MS of the scented genotypes (*A.* cv. 'Sweet Laura', *A. caryophyllaea* and *A.* cv 'Ajax').

3.3.1.2. *Alstroemeria* cv. 'Sweet Laura'

The analysis of 'Sweet Laura' flowers through GC-MS using red and white fibres showed 14 and 17 different compounds respectively, released between min 5 and 26 (Tables 3.3 and 3.4; Figures 3.4 and 3.5).

Table 3.3. List of compounds detected in *A.* cv. 'Sweet Laura' flowers through GC-MS analysis using the red fibre, showing their respective abbreviated formula, retention time, match values between the library and the results obtained (forward) and vice versa (reverse) and relative % of areas under the peak.

* Monoterpenes; ** Sesquiterpenes; *** Aromatic compounds

Compound name	Formula	Retention time	MATCH		Peak (% as max)
			Forward	Reverse	
Monoterpene 1 *	$C_{10}H_{16}$	5.573	942	965	19%
Isocaryophyllene **	$C_{15}H_{24}$	13.314	834	916	100%
α-Caryophyllene **	$C_{15}H_{24}$	14.648	899	923	18%
Sesquiterpene 1 **	$C_{15}H_{24}$	15.023	943	955	2%
Sesquiterpene 2 **	$C_{15}H_{24}$	15.282	885	900	2%
Sesquiterpene 3 **	$C_{15}H_{24}$	16.458	804	835	7%
Butanoic acid-like compound	$C_4H_8O_2$	17.743	906	962	0,50%
Sesquiterpene 4 **	$C_{15}H_{24}$	20.671	875	921	1%
Aromatic 1 ***	C_6H_6	21.639	853	899	1%
Sesquiterpene 5 **	$C_{15}H_{24}$	22.189	860	937	0,50%
Sesquiterpene 6 **	$C_{15}H_{24}$	22.472	735	864	0,50%
Aromatic 2 ***	C_6H_6O	22.931	936	959	1%
Aromatic 3 ***	$C_9H_{10}O$	23.732	886	911	0,50%
Aromatic 4 ***	$C_{10}H_8$	24.174	864	947	0,50%

Sesquiterpenes were observed as the main group of compounds found in all the experiments assayed using both red and white fibre. A monoterpene possibly identified as ocimene, and some aromatic compounds were also common using both fibres (Tables 3.4 and 3.5).

Table 3.4. List of compounds detected in *A.* cv. 'Sweet Laura' flowers through GC-MS analysis using the white fibre, showing their respective abbreviated formula, retention time, match values between the library and the results obtained (forward) and vice versa (reverse) and relative % of areas under the peak.

* Monoterpenes; ** Sesquiterpenes; *** Aromatic compounds

Compound name	Formula	Retention time	MATCH		Peak (% as max)
			Forward	Reverse	
Monoterpene 1	$C_{10}H_{16}$	5.546	959	981	23%
Linalyl/Anthranilic acid-like compound	$C_7H_7NO_2$	11.309	846	928	4%
Sesquiterpene 1 **	$C_{15}H_{24}$	12.459	830	925	3%
Isocaryophyllene **	$C_{15}H_{24}$	13.210	944	965	100%
Aromatic 1 ***	C_6H_6	14.527	924	940	11%
α-Caryophyllene **	$C_{15}H_{24}$	14.619	895	921	11%
Aromatic 2 ***	$C_{10}H_8$	15.011	902	947	2%
Sesquiterpene 2 **	$C_{15}H_{24}$	16.428	748	775	10%
Butanoic acid-like compound	$C_4H_8O_2$	17.712	923	961	20%
Sesquiterpene 3 **	$C_{15}H_{24}$	20.647	874	922	8%
Aromatic 3 ***	C_6H_6	21.623	851	896	7%
Sesquiterpene 4 **	$C_{15}H_{24}$	21.165	796	900	1%
Aromatic 4 ***	C_6H_6O	22.915	919	957	18%
Aromatic 5 ***	$C_9H_{10}O$	23.699	912	930	5%
Aromatic 6 ***	$C_{10}H_8$	24.149	865	942	1%
Aromatic 7 ***	$C_9H_{10}O$	25.266	828	945	1%
Aromatic 8 ***	C_8H_6O	26.175	915	932	1%

A cluster of sesquiterpenoid compounds was found after the monoterpene and includes isocaryophyllene as the major volatile compound. The rest of the compounds belonging to this group were α-caryophyllene and other 4 (using the white fibre) and 6 (using the red fibre) none identified sesquiterpenoids. Sesquiterpene compounds have a heavier molecular weight because of their C_{15} structure, so it is reasonable to expect a longer retention time than monoterpenes.

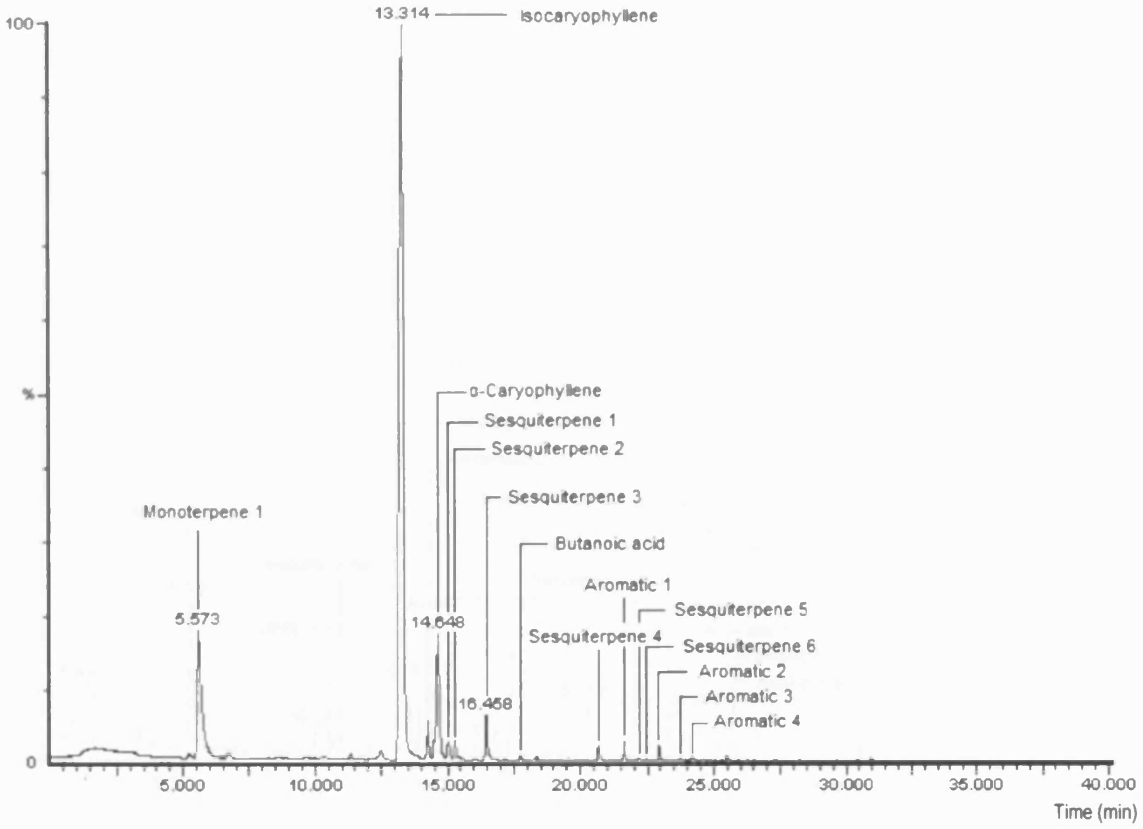


Figure 3.4. Chromatogram obtained from the analysis through GC-MS assayed in *A.* cv. 'Sweet Laura' flowers (Stage 4-5) using the red fibre.

The aromatic compound group was identified after the sesquiterpene group and comprises 8 (using the white fibre) and 4 (using the red fibre) none identified aromatic compounds. Isocaryophyllene was the highest peak (100%) detected at min 13.31 and 13.21 using the red and white fibres respectively (Figure 3.3 and 3.4). This result compares well with the retention time of 14.09 min reported by Hochmuth (2006).

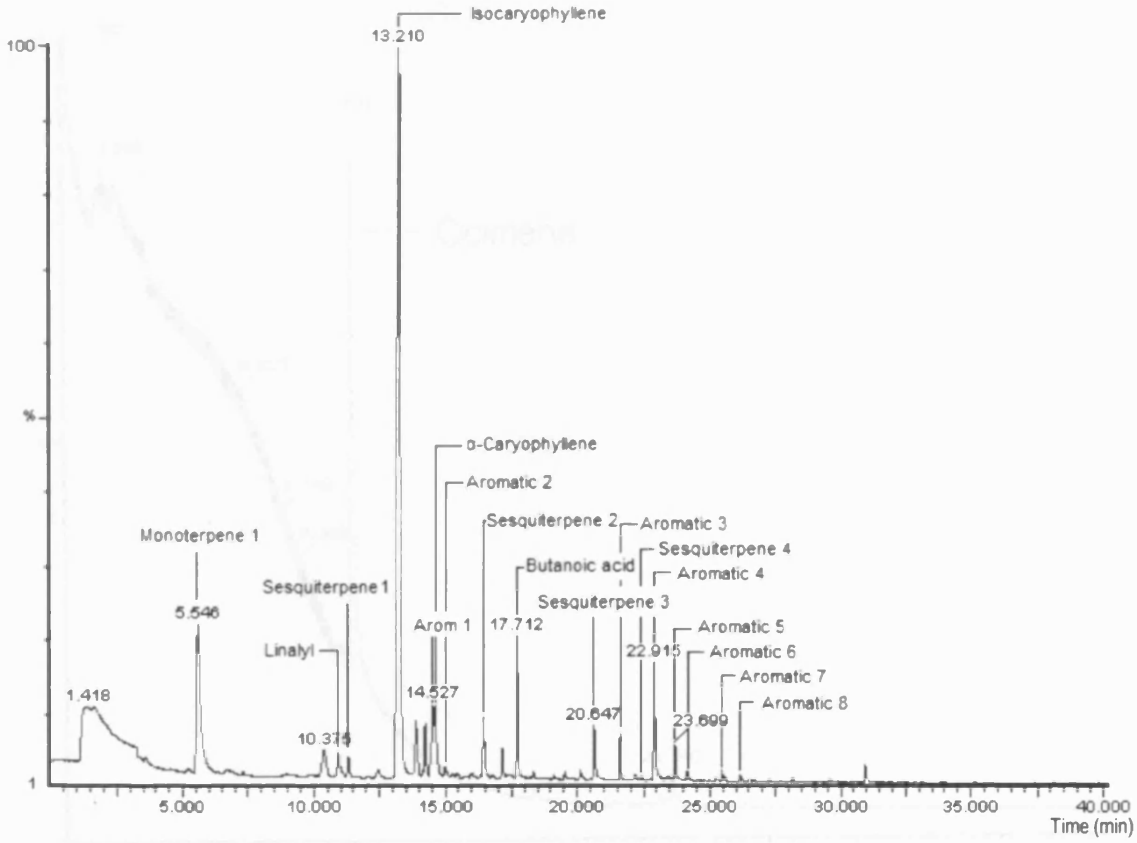


Figure 3.5. Chromatogram obtained from the analysis through GC-MS assayed in *A.* cv. 'Sweet Laura' flowers (Stage 4-5) using the white fibre.

3.3.1.3. *Alstroemeria caryophyllaea*

A monoterpene, putatively identified as ocimene, was the only volatile compound found through the GC-MS analysis with the red fibre (Figure 3.6). No signals were observed using the white fibre (Figure 3.7).

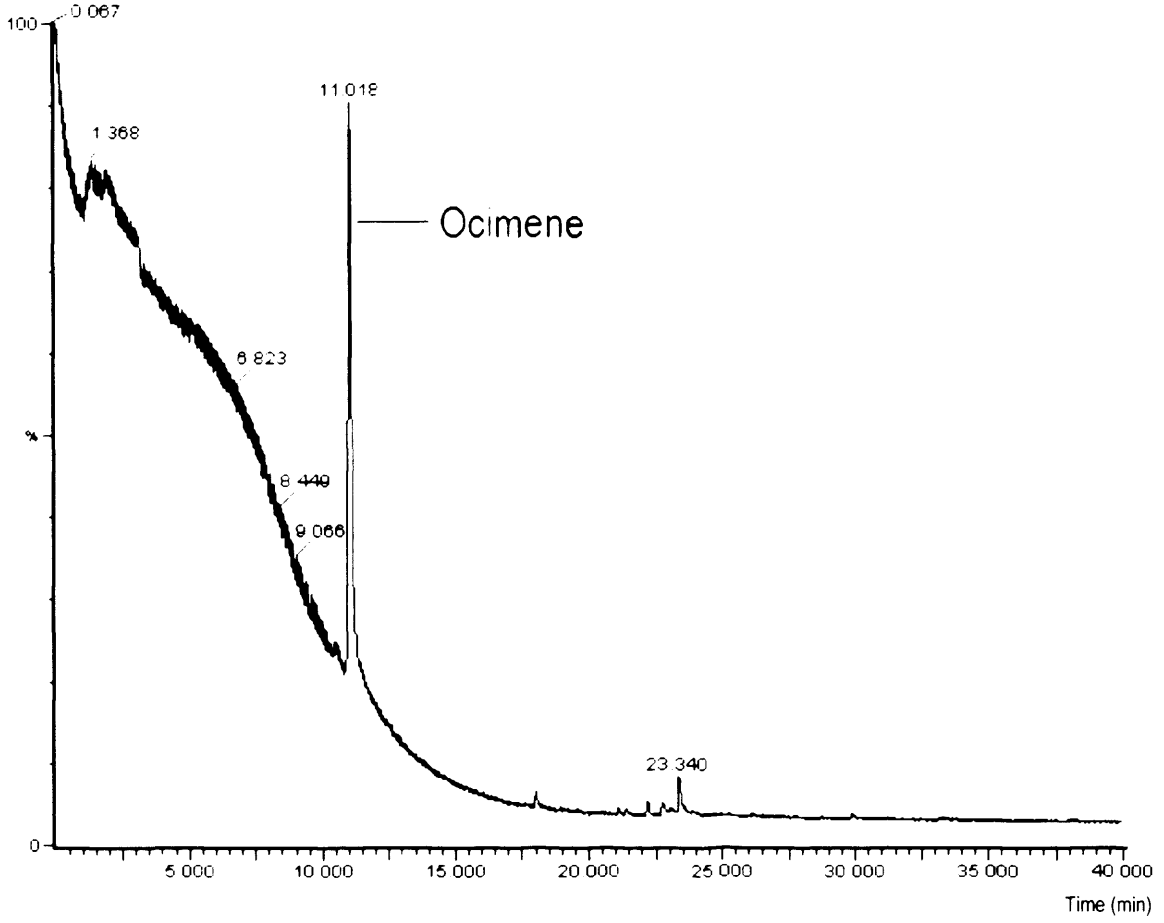


Figure 3.6. Chromatogram obtained from the analysis through GC-MS assayed in *A. caryophyllaea* flowers (Stage 4-5) using the red fibre.

The retention time of 11 min of the ocimene in the scented *A. caryophyllaea* compared well to 10.29 and 10.41 min reported for ocimene and some of its isomers (Hochmuth, 2004).

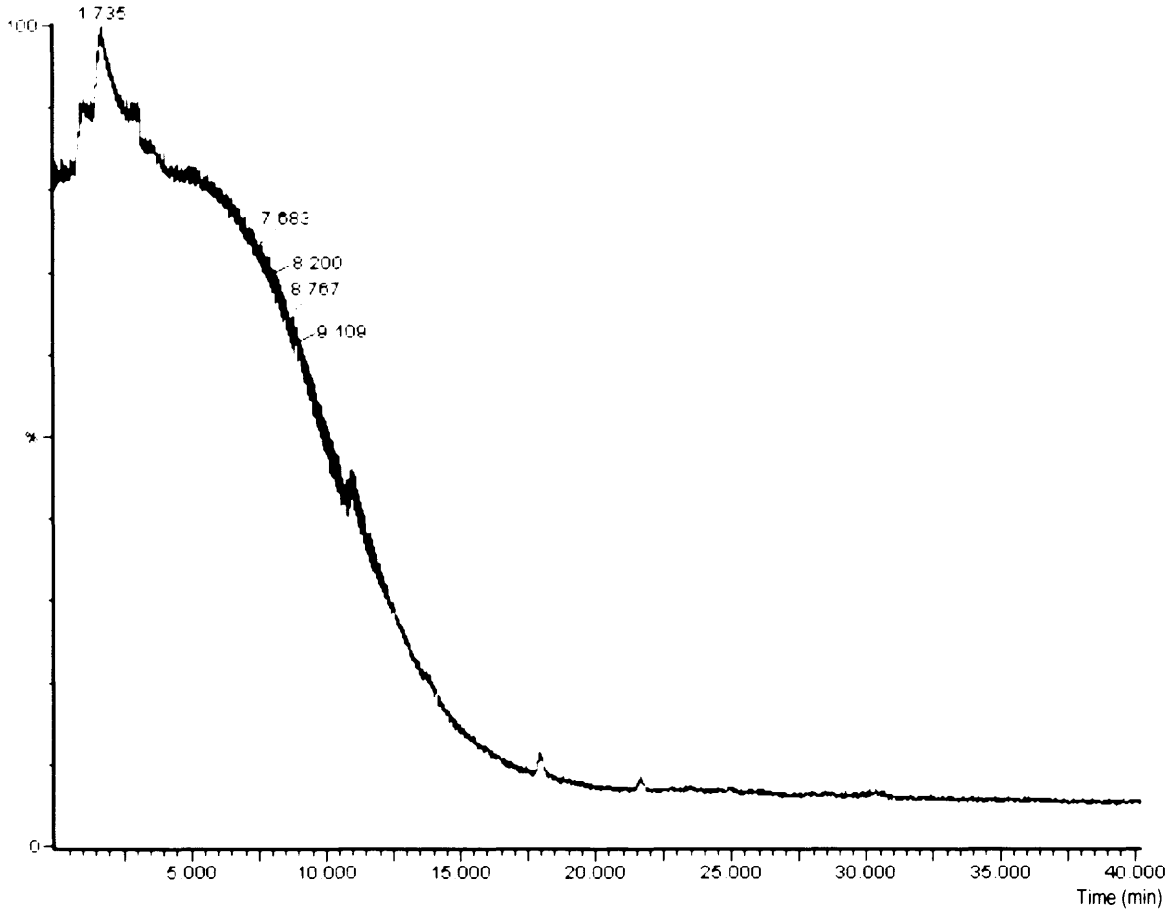


Figure 3.7. Chromatogram obtained from the analysis through GC-MS assayed in *A. caryophyllaea* flowers (Stage 4-5) using white fibre.

3.3.1.4. *Alstroemeria* cv. 'Ajax'

A single peak corresponding to a sesquiterpene was detected through the GC-MS analysis using the red fibre (Figure 3.8) at 17 min. No signals were observed using white fibre.

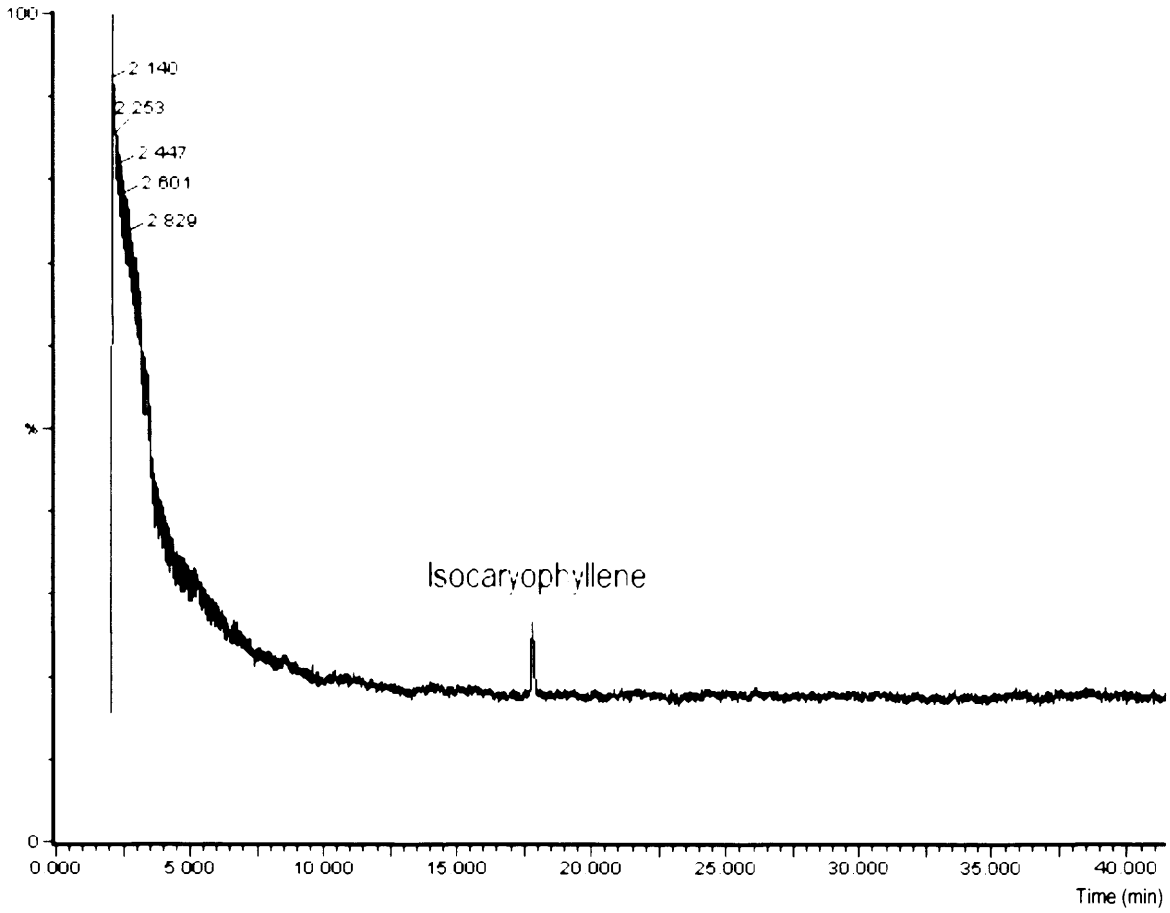


Figure 3.8. Chromatogram obtained from the analysis through GC-MS assayed in *A.* cv. 'Ajax' flowers (Stage 4-5) using red fibre.

3.3.2. Analysis of the volatile compounds emitted by different genotypes using different fibres

A comparison between the scented Alstroemerias cvs. 'Sweet Laura' and 'Ajax' and *A. caryophyllaea*, detected substantial differences in the major monoterpene and sesquiterpene found (Figure 3.9). In general, higher peaks were observed in *A.* cv. 'Sweet Laura', particularly using the red fibre by which the highest peak was detected (isocaryophyllene 100 %). On the other hand the isocaryophyllene peak present in *A.* cv. 'Ajax' was undetectable because of its insignificant size compared to the *A.* cv. 'Sweet Laura' peak.

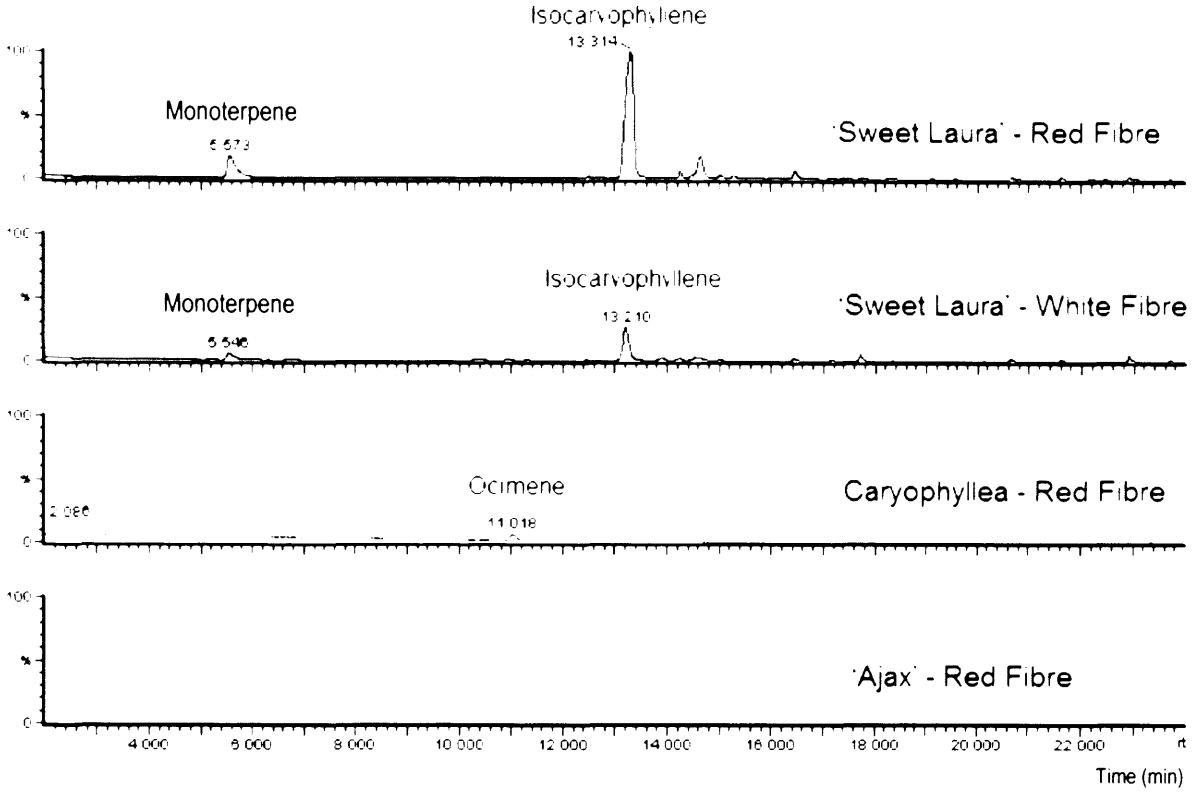


Figure 3.9. Chromatograms obtained from the analysis through GC-MS assayed in *A.* cv. 'Sweet Laura', *A. caryophyllaea* and A. cv. 'Ajax' flowers (Stage 4-5) using the white and red fibre.

Significant differences between the fibres were found also in *A.* cv. 'Sweet Laura' as the percentage of isocaryophyllene detected by the red fibre (100 %) was about five times higher than the white fibre (19.5 %), and the percentage of ocimene - monoterpene was about three times higher when using the red (18.9 %) compared to the white fibre (5.4 %) (Table 3.5). Looking at the descriptions of the fibres made by the manufacturer (Table 3.1), the higher selectivity of the red fibre in the analysis of floral scent was expected, since this fibre is recommended for non-polar volatiles with MW around 30-225.

Table 3.5. Relative % of areas under the peak of ocimene isomers and isocaryophyllene detected in *A.* cv. 'Sweet Laura', *A. caryophyllaea* and A. cv. 'Ajax' flowers using both red and white fibre.

Sample	% monoterpene	% isocaryophyllene
A. cv 'Sweet Laura' Red fibre	18.9	100
A. cv 'Sweet Laura' White fibre	5.4	19.5
A. caryophyllaea Red fibre	4.6	N/A
A. cv. 'Ajax' Red fibre	N/A	~ 0

Monoterpenes showed similar peak area values in *A. caryophyllaea* (4.6 %) and *A.* 'Sweet Laura' (5.4 %) using red and white fibres respectively. However if we compared the monoterpene detection using the same fibre, 'Sweet Laura' (18.9 %) showed values of more than three times the value of this compound detected in *A. caryophyllaea* (4.6 %) (Table 3.5; Appendix A, Tables A.1 and A.2).

3.3.3. Circadian effect on floral scent emission

3.3.3.1. *Alstroemeria* cv. 'Sweet Laura'

A. cv. 'Sweet Laura' flowers showed higher emission of both monoterpene and isocaryophyllene during the afternoon as shown in Figure 3.10.

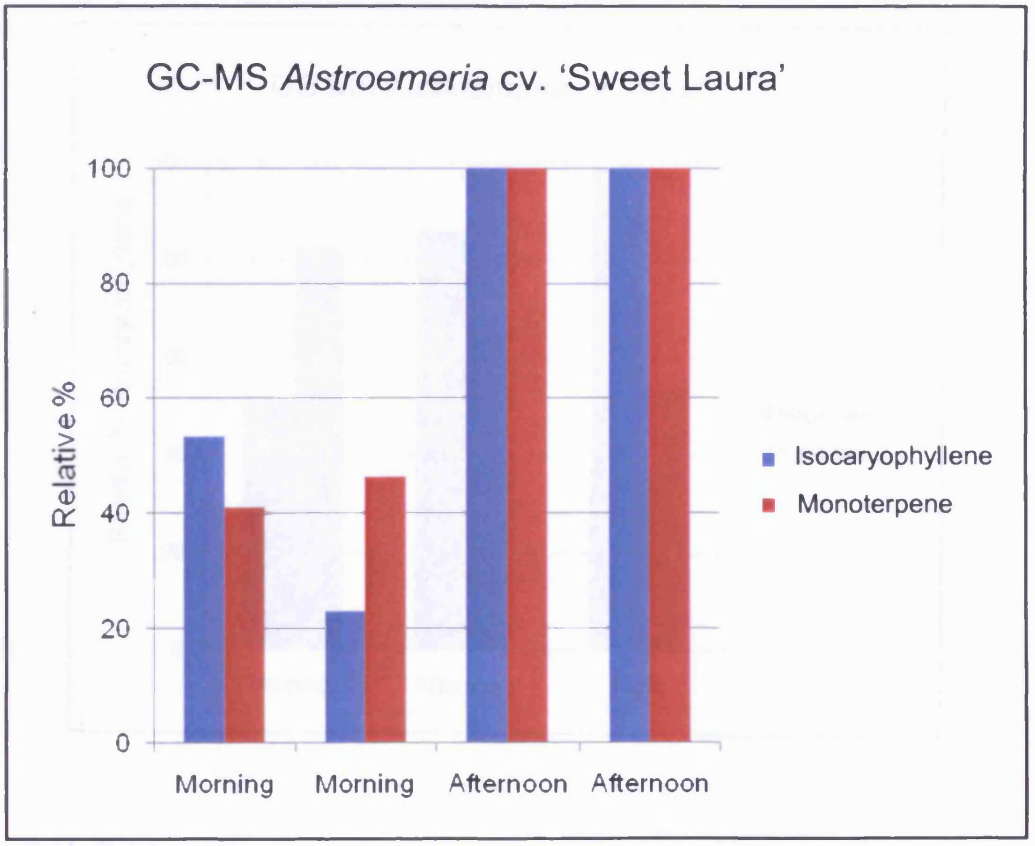


Figure 3.10. Graph showing the relative percentage of areas under the peaks of two replicates of isocaryophyllene and ocimene detected in *A.* cv. 'Sweet Laura' flowers through GC-MS.

3.3.3.2. *Alstroemeria* cv. 'Ajax'

The emission of floral scent by flowers of *A.* cv. 'Ajax' did not follow any pattern when evaluations at different times during the day were performed (Figure 3.11). The

isocaryophyllene peak was detected at irregular values during the evaluations at different times throughout the day.

As shown in the Figure 3.11, the results were not consistent between the replicates. Moreover the differences observed among the evaluations performed in the morning, afternoon and night did not respond to any pattern or circadian rhythm expected.

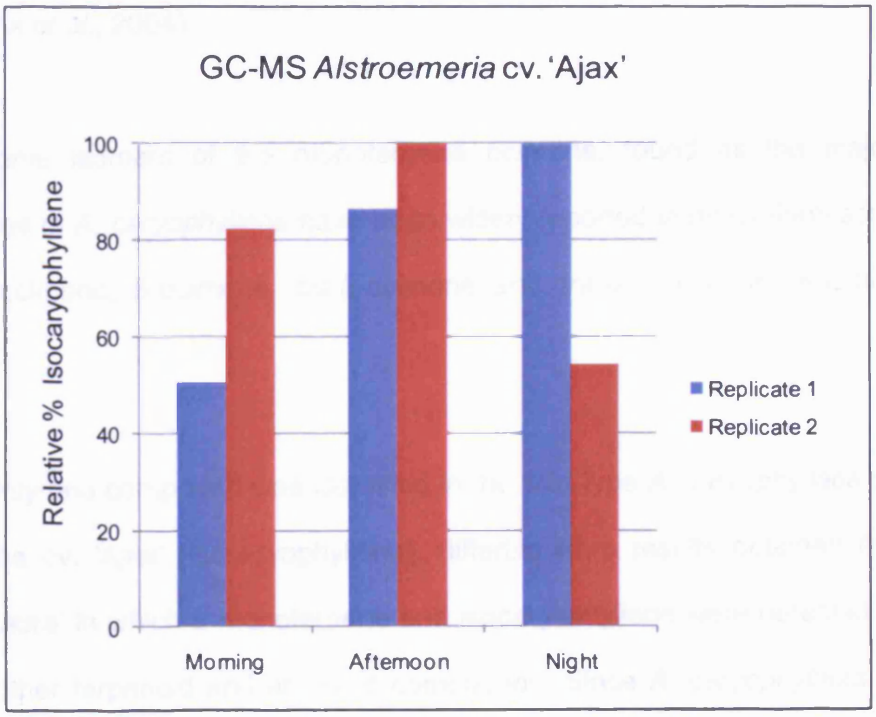


Figure 3.11. Relative percentage of areas under the peak of two replicates of isocaryophyllene detected in *A.* cv. 'Ajax' flowers through GC-MS at different times throughout the day.

3.4. DISCUSSION

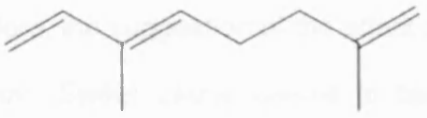
Isocaryophyllene, the major volatile compound observed in *A.* cv. 'Sweet Laura' and *A.* cv. 'Ajax', has rarely been found in common floral scents, although some previous investigations have observed this compound as a component in the floral fragrance of rose (Knudsen *et al.*, 1993), *Haplopapus berterii* (Urzúa *et al.*, 2000), *Maxillaria tenuifolia* (Perraudin *et al.*, 2006) and also in leaves and glands of sweet basil (Iijima *et al.*, 2004).

Some isomers of the monoterpene ocimene, found as the major volatile compounds in *A. caryophyllaea* have been widely reported in many floral scents in the forms α-ocimene, β-ocimene, *cis*-β-ocimene and *trans*-β-ocimene (Knudsen *et al.*, 1993).

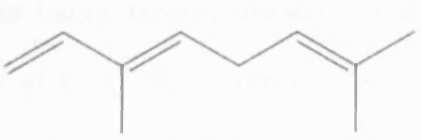
Only one compound was identified in the wild type *A. caryophyllaea* (ocimene), and in the cv. 'Ajax' (isocaryophyllene), differing from results obtained from *A.* cv. 'Sweet Laura' in which a monoterpene and isocaryophyllene were detected as well as several other terpenoid and aromatic compounds. Since *A. caryophyllaea* is a direct ancestor of *A.* cv. 'Sweet Laura' (*A. aurea* x *A. caryophyllaea*) (Pounders *et al.*, 2003) and *A.* cv. 'Ajax' (Meijles, 2008), it was reasonable to expect a similar composition of their floral fragrance. The other ancestor of *A.* cv. 'Sweet laura' is a non scented species (*A. aurea*), thus none of these ancestors showed any emission of the majority of the volatile compounds detected in *A.* cv. 'Sweet Laura'. The origin of these novel products in terms of heredity therefore remains unclear. One possibility is that the genetic information related to biosynthesis of volatile compounds found in this cultivar, is present but not expressed in the parental species (*A. caryophyllaea* and *A. aurea*). The same hypothesis could be suggested by analyzing the *A.* cv. 'Ajax' pedigree,

which includes *A. caryophyllaea* as the only contributor of scent, as the other seven ancestors are non scented Alstroemeria (Meijles, 2008). Heredity of floral scent has been previously described as a very complex process since huge differences in this character are found in closely related species. Furthermore, floral scent has been described before as an easily acquired or lost character through evolution (Dudareva *et al.*, 1996).

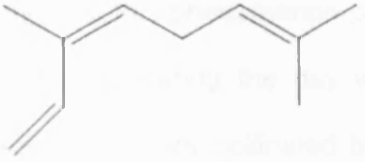
The monotepene identified as ocimene had a retention time around 11 min in *A. caryophyllaea*, however this result differs from the ocimene-like compound found in the analysis of *A.* cv. 'Sweet Laura' in which this compound was also identified through the library, but at around minute 5 using both red (Figure 3.4) and white fibres (Figure 3.5). Ocimene and its isomers β-cis-ocimene and β-trans-ocimene are monoterpenes with a molecular weight of 136 g and differ in the position of one of the three double bonds present in this molecule (Figure 3.12).



Ocimene
1,3,7-octatriene,3,7-dimethyl



β-trans-ocimene
1,3,6-octatriene,3,7-dimethyl-,(E)-



β-cis-ocimene
1,3,6-octatriene,3,7-dimethyl-,(Z)-

Figure 3.12. Molecule of ocimene and two of its isomers β-trans-ocimene and β-cis-ocimene, detected by the analysis through GC-MS in *A. caryophyllaea* and *A.* cv. 'Sweet Laura'.

However, the slight differences between two isomers are not enough to result in a discrepancy of more than 5 min in the retention time. Thus combining the information of the library (comparison of spectra) and the fact that ocimene has been described with a retention time around minute 10.29 and 10.41 min (Hochmuth, 2004), ocimene seems to be a volatile compound present only in *A. caryophyllaea*. Further analysis using specific standards should be performed in order to clarify the identification of the monoterpene found in *A.* cv. 'Sweet Laura'.

Concerning the circadian effect on the floral scent emission, the results obtained from *A.* cv. 'Sweet Laura' flowers suggest a correlation between floral scent emission and circadian rhythm, since different levels of abundance of compounds were detected throughout the day. Besides the circadian rhythm, some other variables have been described affecting the floral scent emission. However, stage of floral development suggested by Dudareva and Pichersky (2000) and temperature suggested by Jakobsen and Olsen (1994) as factors provoking changes in floral scent emission, could be eliminated as both these parameters were maintained constant during the experiment. Therefore, the suggestion of the effect of circadian rhythm in the floral scent emission of *A.* cv. 'Sweet Laura' seems to be consistent and reliable according to the results obtained. Thus both isocaryophyllene and monoterpene were clearly produced more abundantly during the afternoon compared to the morning in A. cv. 'Sweet Laura' flowers. An ecological explanation of this phenomenon proposed by Loughrin *et al.* (1990) suggests that flowers pollinated during the day would show higher scent output at this time and the opposite for flowers pollinated by nocturnal insects such as moths. In Alstroemeria, the more frequent floral visitors are diurnal Hymenopteran insects (Aizen and Basilio, 1998; Cavieres *et al.* 1998; Botto-Mahan and Ojeda-Camacho 2000). However, the circadian rhythm and its interaction with the pollinators could not be demonstrated in *A.* cv. 'Ajax' as no clear differences were found between evaluations performed during the day and night. Thus perhaps a GC-

MS analysis performed during the night in *A.* cv. 'Sweet Laura' flowers would be interesting as lower levels of emission at this time would be expected. Furthermore, other species responding to circadian rhythm in their floral scent emission have been studied in order to find correlations with the expression of genes involved in the biosynthesis their volatile compounds throughout the day (Lu *et al.*, 2002; Dudareva *et al.*, 2005). These types of studies, could explain whether these changes in emission during the day are directly regulated by an intrinsic circadian clock or by some intermediates in the pathway of the biosynthesis of the volatile compounds. They can also form the basis for the isolation of scent-related genes.

Finally, results obtained from the analysis of *A.* cv. 'Ajax' did not show conclusive results about whether a circadian rhythm is affecting the emission of floral scent. Despite the fact that 'Ajax' and 'Sweet Laura' are two cultivars of the same species with a common ancestor (*A. caryophyllaea*), they showed different scent emission behaviour. This fact supports the opinion of many researchers about how complex the scent character is, showing remarkable differences even between plants that are closely related taxonomically (Dudareva *et al.*, 1996; Dudareva and Pichersky, 2000) such as those in this study, that are three closely related genotypes of the same genus. Furthermore, the problem of obtaining consistent data on circadian patterns of scent emission, previously noted by some Alstroemeria breeders (Meijles, 2008; Kuiper, 2008 and Bridgen, 2008), was supported in this study when trying to find a clear pattern of floral scent emission for *A.* cv. 'Ajax'.

CHAPTER IV.

CHARACTERIZATION OF A PUTATIVE ALSTROEMERIA TERPENE SYNTHASE

IV. CHARACTERIZATION OF A PUTATIVE ALSTROEMERIA TERPENE SYNTHASE

4.1. INTRODUCTION

Terpenoids comprise a large number of primary and mostly secondary metabolites with a wide variety of structural types including monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}) and triterpenes (C_{30}) (Dudareva and Pichersky, 2000; Degenhardt *et al.* 2009). The biosynthesis of these products is controlled by a group of enzymes called terpene synthases (TPS) and many efforts have been made to characterize both their amino acid sequences (Bohlmann *et al.*, 1998; Dudareva *et al.*, 2003) and their nucleotide sequences to examine evolutionary relationships within the gene family (Cseke *et al.*, 1998; Trapp and Croteau, 2001; Lee and Chappell, 2008). Thus a total of 91 mono TPS and 113 sesqui TPS has been isolated and described to date (Degenhardt *et al.* 2009).

In a previous project studying petal senescence and responses to stress (Breeze et al., 2004; Wagstaff et al., 2010), a number of Alstroemeria petal cDNA libraries were constructed from Alstroemeria cv. 'Rebecca' and a total of 1849 ESTs were sequenced. Amongst these were four ESTs which showed homology to TPS based on BLAST comparisons to the databases. All four ESTs were derived from small cDNA clones representing only a small portion of the ORF. These TPS have been studied because of their putative role in scent production, although they were isolated

from petals of *A.* cv. 'Rebecca' which is unscented. Moreover, due to the complexity and diversity of TPS gene families (Dudareva and Pichersky, 2000), it is not easy to isolate genes for TPS from degenerate primers as observed in Appendix B, where no consistent results were found for the identification of sesquiterpene and diterpene synthases in Alstroemeria.

A full length of the ORF is necessary for a full characterization of phylogeny and function, and an effective method known as rapid amplification of cDNA ends (RACE), which is a technique based on PCR, allows the cloning of full-length cDNA 5'- and 3'-ends starting from a partial cDNA sequence obtained previously by other methods (Schaefer, 1995). Thus the first aim of the work described in this chapter was to obtain a full length sequence for a novel Alstroemeria TPS focusing on the *A.* cv. 'Rebecca' TPS clones, but using *A.* cv. 'Sweet Laura' material as template since this is a scented variety. Another aim of the work described in this chapter was to investigate whether sequence data could provide any clues on the sub-cellular compartmentalization of the putative Alstroemeria TPS. Douglas *et al.* (1995) proposed that mono and diterpenes are synthesized in the plastids while sesqui and triterpenes in the cytoplasm, hence identifying the target compartment would provide useful information on the possible enzymatic activity of this putative Alstroemeria TPS. One way to study evolutionary relationships within gene families is to examine intron/exon structure (Brown *et al.*, 1995; Wattler *et al.*, 1998). Genomic organization of TPS, that is the position and size of introns and exons, has been widely studied in many species including Magnolia (Lee and Chappell, 2008), Arabidopsis (Bohlmann *et al.*, 2000) and tobacco (Trapp and Croteau, 2001), among others. This characterization was therefore included in this investigation in order to find out more about the evolution and putative classification of a novel Alstroemeria TPS into one of the TPS classes proposed by previous investigations (Cseke *et al.*, 1998; Trapp and Croteau, 2001; Lee and Chappell, 2008).

4.2. METHODOLOGY

4.2.1. Primer design

Four DNA sequences from an *Alstroemeria* cv. 'Rebecca' petal S2-S0 subtracted library (Breeze *et al.*, 2004), CF569327, CF569368, CF588473 and CF588421 were analyzed using BLAST and a *Quercus ilex* mRNA for putative chloroplast terpene synthase (Fischbach *et al.*, 2001) was the nearest match.

In order to amplify a sequence to be used as starting point for the 5' and the 3'-RACEs, primers were designed flanking the whole sequence of the clones *CF 327* and *CF421*. The primers designed were: *NCF327F* (5'-TAGAAAATGCAAGGAATTCGG-3') and *NCF327R* (5'-TAGCCTTGCGGTCTTTGTTC-3') for *CF327* and *NCF421F* (5'-CAGGCTCGAGAAGCTGAA-3') and *NCF421*R (5'-ACGTCGTAGATGTCATCGAT-3') for *CF 421.*Combinations of these primers were also tested aimed to check the relative position of these clones and find the longest sequence as a starting point for the RACE.

4.2.2. RACE

4.2.2.1. Ready cDNA synthesis

Starting with RNA from *A.* cv. 'Sweet Laura' at stage 4, extracted as described in section 2.2, 5'- and 3'-RACE-Ready cDNA was synthesized using a Clontech kit (BD SMART™ RACE cDNA Amplification Kit) as follows: ~1 µg RNA, 1 µl 5'-CDS primer or 3'-CDS primer (12 µM), 1 µl BD SMART II A oligo and 2 µl sterile water were incubated at 70°C for 2 min and then cooled on ice for 2 min. Afterwards 2 µl 5X First-Strand

Buffer, 1 µl DTT (20 mM), 1 µl dNTP Mix (10 mM) and 1 µl BD PowerScript Reverse Transcriptase (Clontech) were added to a final volume of 10 µl. Samples were incubated at 42°C for 1.5 h and then diluted in 100 µl Tricine-EDTA Buffer (10 mM Tricine-KOH pH 8.5 and1.0 mM EDTA). Finally the samples were incubated at 72 °C for 7 min.

4.2.2.2. Gene specific primer (GSPs) design

Two GSPs were designed, GSP1 for the 5'-RACE and GSP2 for the 3'-RACE, applying the following conditions as recommended in the Clontech kit (BD SMART™ RACE cDNA Amplification Kit):

- GC content of 50–70%
- Tm of at least 65°C
- Length of 23 to 28 bp, as primers longer than 30 bp do not show any advantage.

The primers were designed based on the previous amplification and sequencing of the *CF 327* clone. The selected primers were: ALSTGSP1 (5'-CACCTCTCTCTCCTTCTGCCTCTGAAGTTG-3') and ALSTGSP2 (5'-TGCATGAAACGGATGCTTCAGAGGTGATGG-3').

Two more GSP primers were design for extra RACEs aimed at reaching the N-terminus of the gene. These primers were: *ALSTGSP3* (5'-CAGCTTCTCGAGCCTGGCAGCACATTCC-3') and *ALSTGSP4* (5'-GTTTGCAAATAGTTGTTGTTCCAGACGG-3').

4.2.2.3. Rapid Amplification of cDNA Ends (5' and 3'-RACE)

The RACE was carried out in a 50 µl total mix containing 34.5 µl sterile water, 5 µl 10X BD Advantage 2 PCR Buffer, 1 µl dNTP Mix (10 mM), 1 µl 50X BD Advantage 2 Polymerase Mix, 1 µl GSP1 or GSP2, 5 µl UPM (10X) and 2.5 µl 5'- or 3'-RACE-Ready cDNA (obtained experimentally during 5'- or 3'-RACE-Ready cDNA synthesis, section 2.1.3.1).

Thermal cycling was performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA) and the amplification was conducted according to the following thermal profile: 5 cycles of 94°C for 30 sec and 72°C for 3 min; 5 cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 3 min; 25 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 3 min.

4.2.2.4. Analysis of the amplification and sequencing

Purification, ligation, transformation, cloning and sequencing of the amplified fragment after both the 5' and the 3'-RACE were carried out following the protocols described in the 'General materials and methods' chapter.

4.2.2.5. Construction of a consensus with sequences obtained as result of the RACE

All the cDNA sequences obtained as result of the 5' and the 3'-RACE were assembled into a finished contig using the programs EDITSEQ and SEQMAN (DNASTAR, Madison, WI). When discrepancies were found among overlapping

sequences, chromatograms of the sequencing were analyzed and a final consensus was built.

The final consensus of the ORF associated to the putative Alstroemeria monoterpene synthase was analyzed by alignments against other known TPS using BioEdit (v. 7.0.5.3) (Hall, 1999).

4.2.3. Phylogenetic analysis

In order to characterize the putative Alstroemeria TPS obtained, its amino acid sequence consensus was compared with other 44 plant terpenoid synthases previously described, including hemiterpene (C_5), monoterpene (C_{10}), sesquiterpene (C_{15}) and diterpene (C_{20}) synthases found in both gymnosperm and angiosperm species. A fungal diterpene synthase was also included (*G. fujikuroi* - copalyl diphosphate synthase) as an out-group to enrich the analysis. The list of TPS analyzed comprises proteins involved in both primary metabolism (copalyl diphosphate and kaurene synthase) and secondary metabolism (all the others). All the TPS were selected based on previous phylogenetic analysis, adding some others interesting for this study, and their amino acid sequences were obtained from a public database (http://www.ncbi.nlm.nih.gov/) (Table 4.1).

An unrooted phylogenetic tree based on amino acid alignments of 45 terpene synthases was constructed by the neighbour-joining method using MEGA 4.0.2 (Tamura *et al.*, 2007).

Table 4.1. List of 45 terpenoid synthases genes used for comparison analyses with the putative Alstroemeria TPS characterized in this study. N/I stands for no information. Highlighted is the Alstroemeria TPS

Gene bank accession no	Designation in tree and alignments	Terpene Synthase	aa	Type	Species	References
AAC24192	Ag bisabolene	(E)-α-bisabolene synthase	817	C_{15}	Abies grandis	Bohlmann et al., 1998
AAB70707	Ag camphene	(-)-Camphene synthase	618	C_{10}	Abies grandis	Bohlmann et al., 1997
AAB71084	Ag myrcene	Myrcene synthase	627	C_{10}	Abies grandis	Bohlmann et al., 1997
AAB71085	Ag pinene	Pinene synthase	628	C_{10}	Abies grandis	Bohlmann et al., 1997
O64404	Ag d-selinene	δ-selinene synthase	581	C_{15}	Abies grandis	Steele et al., 1998
N/I	Alstroemeria TPS	Alstroemeria TPS	567	N/I	Alstroemeria sp.	N/I
AAO42614	Am b-ocimene	(E)-β-ocimene synthase	579	C_{10}	Antirrhinum majus	Dudareva et al., 2003
AAO41727	Am myrcene	Myrcene synthase	584	C_{10}	Antirrhinum majus	Dudareva et al., 2003
Q38802	At copalyl diphos	Copalyl diphosphate synthase	802	C_{20}	Arabidopsis thaliana	Sun and Kamiya, 1994
NP_567511	At b-ocim/myrc TPS03	(E)-β-ocimene/myrcene synthase	565	C_{10}	Arabidopsis thaliana	Bohlmann et al., 2000
NP_179998	At b-ocim/myrc TPS10	(E)-β-ocimene/myrcene synthase	591	C_{10}	Arabidopsis thaliana	Bohlmann et al., 2000
AAL79181	Aa b-caryophyllene	β-caryophyllene synthase	548	C_{15}	Artemisia annua	Cai et al., 2002
AAK58723	Aa b-pinene	(-)-β-pinene synthase	582	C_{10}	Artemisia annua	Lu et al., 2000
CAC08805	Aa 8-epicedrol	Epi-cedrol synthase	547	C_{15}	Artemisia annua	Mercke et al., 1999
ABI21838	Cas a-pinene	(+)-α-pinene synthase	615	C_{10}	Cannabis sativa	Guennewich et al., 2006
AAQ04608	Cis valencene	Valencene synthase	548	C_{15}	Citrus sinensis	Sharon-Asa et al., 2003
BAD27260	Ciu b-pinene	β-pinene synthase	602	C_{10}	Citrus unshiu	Shimada et al., 2005
Q96376	Cb linalool	Linalool synthase	870	C_{10}	Clarkia breweri	Dudareva et al., 1996
AAU05951	Cus a-farnesene	(E,E)-α-farnesene synthase	561	C_{15}	Cucumis sativus	Mercke et al., 2004
CAA75244	Gf copalyl diphos	Copalyl diphosphate synthase	959	C_{20}	Gibberella fujikuroi	Tudzynski et al., 1998
AAF74977	Gh d-cadinene	(+)-δ-cadinene synthase	551	C_{15}	Gossypium hirsutum	Townsend et al., 2005
AAA86340	Hm vetispiradiene	Vetispiradiene synthase	555	C_{15}	Hyoscyamus muticus	Back et al., 1995
AAM11626	Ls germacrene A	Germacrene A synthase	559	C_{15}	Lactuca sativa	Bennett et al., 2002
ABB73045	La linalool	Linalool synthase	564	C_{10}	Lavandula angustifolia	Landmann et al., 2007
AAT86042	Lc b-ocimene	(E)-β-ocimene synthase	595	C_{10}	Lotus corniculatus	Arimura et al., 2004
ACC66282	Mg a-terpineol	α-terpineol synthase	592	C_{10}	Magnolia grandiflora	Lee and Chappell, 2008
ACC66281	Mg b-cubebene	β-cubebene synthase	550	C_{15}	Magnolia grandiflora	Lee and Chappell, 2008
AAL99381	Ma linalool	Linalool synthase	606	C_{10}	Mentha aquatica	Crowell et al., 2002
CAH10288	Mxp muuroladiene	Cis-muuroladiene synthase	551	C_{15}	Mentha x piperita	Prosser et al., 2006
Q40577	Nt Aristolochene	Aristolochene synthase	548	C_{15}	Nicotiana tabacum	Facchini and Chappell, 1992
AAK06663	Pf limonene	Limonene synthase	604	C_{10}	Perilla frutescens	Takeya et al., 2000
AAS47689	Pa a-bisabolene	(E)-α-bisabolene synthase	807	C_{15}	Picea abies	Martin et al., 2004
AAS47693	Pa linalool	(-)-Linalool synthase	623	C_{10}	Picea abies	Martin et al., 2004
AAS47695	Pa longifolene	Longifolene synthase	578	C_{15}	Picea abies	Martin et al., 2004
AAS47696	Pa myrcene	Myrcene synthase	633	C_{10}	Picea abies	Martin et al., 2004
CAC35696	PaxPt isoprene	Isoprene synthase	595	C_5	Populus alba x P. tremula	Miller et al., 2001
CAC41012	Qi myrcene	Myrcene synthase	597	C_{10}	Quercus ilex	Fischbach et al., 2001
CAK55186	Qi pinene	Pinene synthase	597	C_{10}	Quercus ilex	Schnitzler, 2006
AAC26018	So sabinene	(+)-Sabinene synthase	590	C_{10}	Salvia officinalis	Wise et al., 1998
BAA84918	Sl copalyl diphos	Copalyl diphosphate synthase	800	C_{20}	Solanum lycopersicum	Imai, 1998
CAE47440	Sc germacrene D	Germacrene D synthase	551	C_{15}	Solidago canadensis	Prosser et al., 2004
AAD34295	Sr kaurene	Kaurene synthase	784	C_{20}	Stevia rebaudiana	Richman et al., 1999
AAS66358	Vv valencene	(+)-Valencene synthase	556	C_{15}	Vitis vinifera	Lucker et al., 2004
AAS66357	Vv germacrene	(-)-Germacrene D synthase	557	C_{15}	Vitis vinifera	Lucker et al., 2004
ABY79206	Zm b-caryophyllene	(E)-β-caryophyllene synthase	547	C_{15}	Zea mays	Kollner et al., 2008
BAG12020	Zz a-humulene	α-humulene synthase	548	C_{15}	Zingiber zerumbet	Yu et al., 2008

4.2.4. Signal peptide analysis

Three programs were used to predict the compartment target (cytoplasm or plastids) of the putative Alstroemeria TPS signal peptide. These were "Predotar v. 1.03", "ChloroP 1.1 Server" (Emanuelsson *et al.*, 1999) and "WoLF PSORT Protein Subcellular Localization Prediction" (Horton et al., 2007). Their results were compared and matched for a more reliable prediction.

4.2.5. Analysis of the genomic organization

4.2.5.1. Genomic DNA extraction

Genomic DNA was extracted from young leaves of Alstroemeria cv. 'Sweet Laura', according to Dempster *et al.* (1999). Approximately 200 mg of plant material were placed into sterile 1.5 ml Eppendorf tubes and ground in liquid nitrogen with an Eppendorf pestle to a fine dust. Then 700 µL of 2% CTAB buffer (100 mM Tris-HCL, 1.4 M NaCL, 20 mM EDTA, 2% CTAB, 1% PVP and 0.3% de β-mercaptoetanol) were added and the samples were incubated for 30 min at 65°C, mixing gently every 10 minutes.

One volume (700 µL) of chloroform- isoamyl alcohol (24:1) was added and the tubes were vortexed and then centrifuged at room temperature for 10 minutes at 16,420 x g using an Allegra 21R centrifuge (Beckman Coulter Inc., Germany). The aqueous top phase was transferred to fresh 1.5 ml Eppendorf tubes and again 700 µL of chloroform- isoamyl alcohol were added repeating the same procedure.

The top phase recovered from the last step was transferred into a new 1.5 mL tube containing 700 µL cold isopropanol. The tubes were stored at 4°C for 30 minutes

or until pellet precipitation. The pellet was washed with 200 µL 70% ethanol by vortexing and then centrifuged at room temperature for 5 min at at 16,420 x g. The supernatant was decanted and the tubes were left to air-dry for 30 minutes at room temperature. The pellet was resuspended in 50 µl of TE and 0.5 µg of RNAse (Invitrogen) were added. The samples were incubated at 37°C for 30 minutes to degrade contaminating RNA.

DNA was detected by electrophoresis on an ethidium bromide stained 1% agarose gel loading 10 µl of the samples. Concentration of DNA ($ng \cdot \mu l^{-1}$) was determined by spectrophotometry using a Nanodrop instrument (Thermo Scientific).

4.2.5.2. Design of primers

Using the Alstroemeria TPS consensus obtained in this chapter in Section 4.3.3, several primers were designed to cover the whole genomic sequence related to the ORF of this TPS.

All the primers were designed using IDT Scitools, Oligo Analyzer 3.1 (http://eu.idtdna.com) and were designed to divide the target sequence in segments that overlapped each other by about 100 nucleotides. Sequence length and Tm s were similar for all the primers, thus several combinations of primers could be tested at the same time.

A temperature of 55 °C was successfully used as Tm for all the primer combinations, although the optimal Tm calculated for each primer ranged from 50.1 (ALSTER R) to 55.8 °C (INTRON1 F). Furthermore primer sequences ranged from 18 (NCF421 F) to 25 bp length (INTRON2 R) (Table 4.2).

Table 4.2. List of the primers used for the amplification of the gDNA showing their sequence (5' - 3'), length (bp) and Tm (°C).

Primer	Sequence (5' - 3')	Length (bp)	Tm (°C)
INTRON1 F	CGCCGCTCGGCAAATTATA	19	55.8
INTRON1 R	TAGCAATATAGGTTCCACCAACA	23	53.7
INTRON2 F	CCTGGAGATGCAGACAAGTATT	22	54.5
INTRON2 R	GCGTTCTACAGATTCTACAGATACA	25	54.0
INTRON3 F	CCCGTGACATTAAGGGATTATTG	23	53.6
INTRON3 R	GCTCTAAAGCATGTAAGGCTC	21	53.5
INTRON4 R	CCTCCTCAAAGTGATACGCC	20	55.3
INTRON5 F	GGCGTATACAAGACAGAAATCAG	23	53.4
NCF421 F	CAGGCTCGAGAAGCTGAA	18	54.5
NCF421 R	ACGTCGTAGATGTCATCGAT	20	53.1
ALSTER F	GGGGGACATCTATTACTCACT	21	53.2
ALSTER R	GTTTCATGCATGTAGCATTG	20	50.1

4.2.5.3. Amplification

PCR was carried out following the protocol described in section 2.5 of 'General materials and methods'. Thermal cycling was conducted following the thermal profile: Initial denaturation at 95 °C for 1 min; 40 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 15 min.

Purification, ligation, transformation, cloning and sequencing of the amplified products was carried out following the protocols described in the 'General materials and methods' chapter.

4.2.5.4. Identification of introns and exons

The genomic sequences obtained were assembled into a finished contig using the programs EDITSEQ and SEQMAN (DNASTAR, Madison, WI), and then the contig was aligned against the ORF of the Alstroemeria TPS consensus using BioEdit (v. 7.0.5.3) (Hall, 1999) to identify intron positions. Introns were numbered according to their location, starting with "Intron 1" as the closest to the 5' terminus.

4.3. RESULTS

4.3.1. Alstroemeria TPS identification

Results from a BLAST search performed using the Alstroemeria sequences (*CF327*, *CF368*, *CF473 and CF421*), cDNA clones derived from petal libraries (Breeze *et al*, 2004; Wagstaff *et al.*, 2010), indicated that *Quercus ilex* mRNA for a putative chloroplast terpene synthase (Fischbach *et al.*, 2001) was the nearest match (Figure 4.1).

Clone	Code from Genebank	Putative function	Size
CF327	CF569327	Terpene synthase	309
CF368	CF569368	Limonene cyclase	309
CF473	CF588473	Terpene synthase	310
CF421	CF588421	Sesquiterpene cyclase	495

Quercus ilex mRNA for putative chloroplast terpene synthase

MALKLLTSLPMYNFSRVPVSSKDPILLVTSRTRNGYLARPVQCMVANKVSTSPDILRRSANYQPSIWNHD
YIESLRIEYVGETCTRQINVLKEQVRMMLHKVVNPLEQLELIEILQRLGLSYHFEEEIKRILDGVYNNDH
GGDTWKAENLYATALKFRLLRQHGYSVSQEVFNSFKDERGSFKACLCEDTKGMLSLYEASFFLIEGENIL
EEARDFSTKHLEEYVKQNKEKNLATLVNHSLEFPLHWRMPRLEARWFINIYRHNQDVNPILLEFAELDFN
IVQAAHQADLKQVSTWWKSTGLVENLSFARDRPVENFFWTVGLIFQPQFGYCRRMFTKVFALITTIDDVY
DVYGTLDELELFTDVVERWDINAMDQLPDYMKICFLTLHNSVNEMALDTMKEQRFHIIKYLKKAWVDLCR
YYLVEAKWYSNKYRPSLQEYIENAWISIGAPTILVHAYFFVTNPITKEALDCLEEYPNIIRWSSIIARLA
DDLGTSTDELKRGDVPKAIQCYMNETGASEEGAREYIKYLISATWKKMNKDRAASSPFSHIFIEIALNLA
RMAQCLYQHGDGHGLGNRETKDRILSLLIQPIPLNKD

Figure 4.1. Summary of the four nucleotide sequences of Alstroemeria including clones *CF327* (CF569327), *CF368* (CF569368), *CF473* (CF588473) and *CF 421* (CF588421) (gi:35511103, gi:35200653, gi:35200694 and gi:35510770 respectively) petal S2-S0 subtracted *Alstroemeria peruviana* cDNA and their relative position in the *Quercus ilex* putative chloroplast terpene synthase (Fischbach *et al.*, 2001).

The length of the predicted open reading frame (ORF) for the chloroplast TPS found in *Quercus ilex* is 597 amino acids and three of the sequences (*CF327*, *CF368* and *CF473*) were homologous to a sequence beginning at amino acid 452. The other Alstroemeria sequence (*CF421*) was homologous to a different sequence of 146 amino acids still within the *Quercus ilex* mRNA but starting at amino acid 84 (Figure 4.1).

Since these two cloned fragments represented by the cDNA library clones are homologous to different regions of the *Q. ilex* chloroplast terpene synthase they may either represent a different portion of the same Alstroemeria gene or alternatively another different Alstroemeria gene which also shows homology to terpene synthase. Thus the two sequences were treated separately.

For this work only the sequences represented by the three clones (*CF327*, *CF368* and *CF473*) were used in RACE to obtain a full length TPS clone. *Alstroemeria* cv. Sweet Laura was used as the cDNA template with the aim of obtaining a full length sequence of this putative TPS from a scented variety of Alstroemeria.

4.3.2. Results of the RACE

4.3.2.1. 5'-RACE

The synthesis of RACE-Ready cDNA starting with RNA from A. cv. 'Sweet Laura' was successfully performed. 5' RACE was carried out and a consistent product of about 1300 bp was obtained as result of the amplification (Figure 4.2).This is the expected size for the 5'-RACE since the clone *CF327* from which the ALSTGSP1 primer was designed, starts at amino acid 452. Therefore there are 1352 bp from the beginning of this clone to the N terminus of the TPS found in *Quercus ilex*.

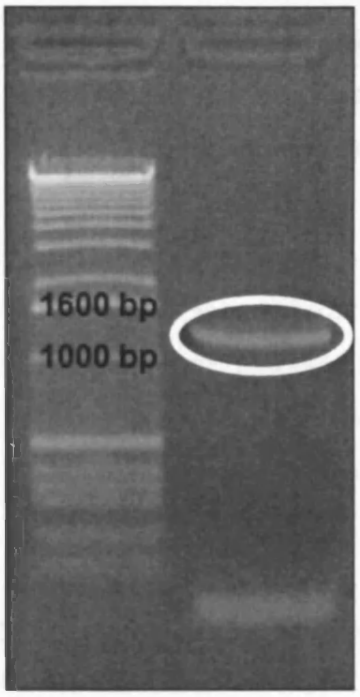


Figure 4.2. Amplification product of the 5'-RACE on agarose gel (1%) electrophoresis.

Three clones of the 1300bp fragment obtained from 5' RACE were sequenced and found homologous to a *Quercus ilex* TPS sequence located between amino acids 57 and 286 (Figure 4.3). However, the sequence did not reach the N-terminus of the *Q. ilex* sequence so an extra round of 5' RACE was performed. Two new GSPs were designed (ALSTGSP3 and ALSTGSP4) and a total of twelve clones were obtained as result of the four repetitions of the 5' RACE using the two new primers (RACE1_08, RACE2_09, RACE3 09 and RACE4_09). All twelve clones were sequenced and aligned (Figure 4.4).

The major differences found between the twelve sequences were the alternative 'RR/CR' at the beginning of the $R(R)X_8W$ motif and the presence of an extra 'PR' sequence. The three clones containing this 'PR' sequence (A1, B6 and G34) also contained an 'RR' instead of a 'CR' at the $R(R)X_8W$ motif (Figure 4.4). Hence all the clones were divided in two main groups, the 'RR' group and the 'CR' group, and these differences are analyzed in the discussion section.

```
Score =  223 bits (569), Expect = 3e-57, Identities = 105/232 (45%)
 Positives = 160/232 (68%), Frame = +2

C1-F  : 59  RRSANYTPTVWNNNYLQTLESEFIGMECAARLEKLKSEAKSLIAGTTSLVEKLELVDTLR 238
          RRSANY P++WN++Y+++L  E++G  C  ++  LK + + ++    + +E+LEL++ L+
Qi.TPS: 57  RRSANYQPSIWNHDYIESLRIEYVGETCTRQINVLKEQVRMMLHKVVNPLEQLELIEILQ 116

C1-F  : 239 QLGLAYHFEEEIMDVLAAILQSADLDSVARQLDGLHATALLFRLLREHGFEISQDILRWF 418
          +LGL+YHFEEEI  +L  +  + D      + + L+ATAL FRLLR+HG+ +SQ++   F
Qi.TPS: 117 RLGLSYHFEEEIKRILDGVYNN-DHGGDTWKAENLYATALKFRLLRQHGYSVSQEVFNSF 175

C1-F  : 419 HDETTGGFKACITRDIKGLLSFYEASYVAIEEENIMDDAREFTTKHLKDFIENSTEPWLR 598
           DE  G FKAC+  D KG+LS YEAS+  IE ENI+++AR+F+TKHL+++++ + E  L
Qi.TPS: 176 KDER-GSFKACLCEDTKGMLSLYEASFFLIEGENILEEARDFSTKHLEEYVKQNKEKNLA 234

C1-F  : 599 ERALHALELPLNWRFQRLHSRWFIDMYERGTDTNLCLLELAKLDFNIVQGVY 754
             H+LE PL+WR  RL +RWFI++Y    D N  LLE A+LDFNIVQ  +
Qi.TPS: 235 TLVNHSLEFPLHWRMPRLEARWFINIYRHNQDVNPILLEFAELDFNIVQAAH 286
```

Figure 4.3. BLASTX performed to compare a *Quercus ilex* TPS (Qi.TPS, GenBank: AJ304839.1) with a 'Sweet Laura' clone (C1-F) obtained through 5'-RACE and sequenced with M13R primers.

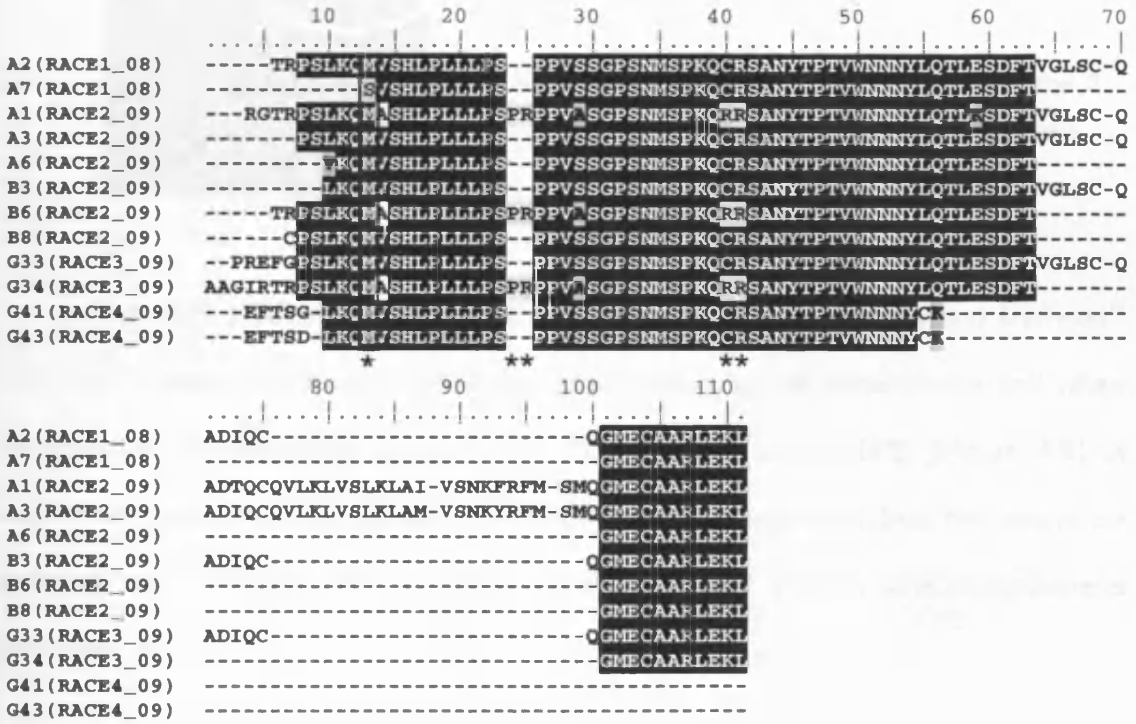


Figure 4.4. Alignment of the 12 clones obtained as result of the 5'-RACEs performed using three different GSPs. (*) The putative starting 'M'; (**) Highlighted are the 'PR' extra amino acids in three of the clones, and the 'RR/CR' presence at the beginning of the R(R)X_8W motif.

4.3.2.2. 3'-RACE

The 3'-RACE was carried out successfully obtaining an amplified fragment of about 350 bp, which is of the expected size (Figure 4.5).

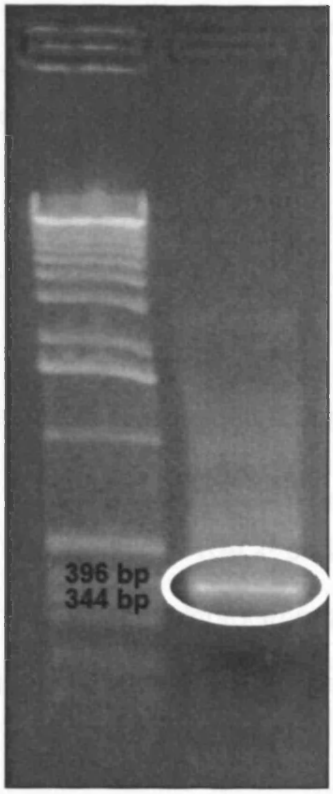


Figure 4.5. Amplification products of the 3'-RACE on agarose gel (1%) electrophoresis.

The PCR product was cloned (See chapter 'General Materials and Methods') and three clones were sequenced. The clones were identical in sequence and when compared to the databases using BLAST identified the *Q. Ilex* TPS. (Figure 4.6). A stop codon was found at the end of the sequence, suggesting that the sequence includes the C-terminus of the protein of this putative Alstroemeria monoterpene synthase

```
Score = 68.6 bits (166), Expect = 2e-10, Identities = 35/79 (44%)
Positives = 49/79 (62%), Gaps = 0/79 (0%), Frame = -3

A'1-F     311  TDASEVMAYMFMRDMIADKWKILNKDRKASTKNEKYFKSVAINTLQLTQWTYQHGDGFGE  132
               T ASE  A  +++ +I+  WK +NKDR AS+     F  +A+N  ++ Q  YQHGDG G
Q.i TPS   516  TGASEEGAREYIKYLISATWKKMNKDRAASSPFSHIFIEIALNLARMAQCLYQHGDGHGL  575

A'1-F     131  PQHRTKDTILALLVEPILL  75
                  TKD IL+LL++PI L
Q.i TPS   576  GNRETKDRILSLLIQPIPL  594
```

Figure 4.6. BLASTX performed to compare a *Quercus ilex* TPS (Qi.TPS, GenBank: AJ304839.1) with a 'Sweet Laura' clone (A'1-F) obtained through 3'-RACE and sequenced with M13F primers.

4.3.3. Final consensus

The sequences obtained as result of the first and extras 5'-RACEs and the 3'-RACE were assembled based on the information of the *Quercus ilex* TPS amino acid sequence. From the first 5'-RACE and the 3'-RACE all the clones were included for the alignment, while only the clones from the 'RR' group were considered from the extra 5'-RACEs (Figure 4.7) as the 'CR' sequences are unlikely to belong to a terpene synthase.

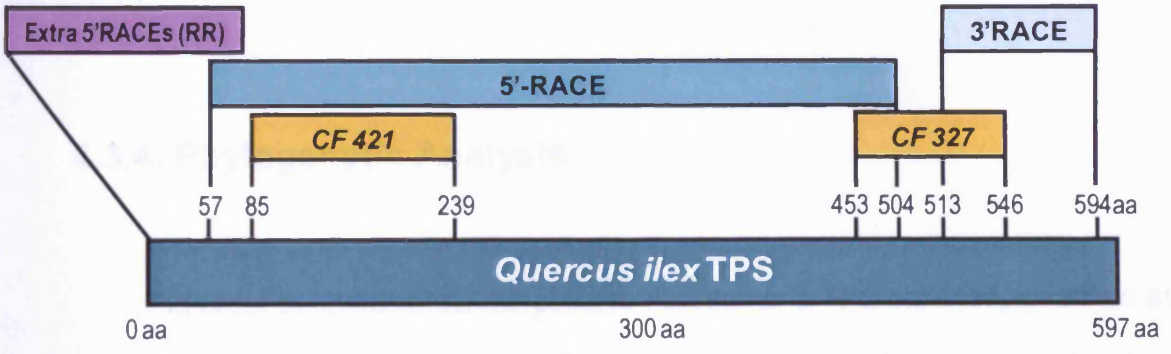


Figure 4.7. Scheme of the relative position in comparison with *Quercus ilex* TPS of the deduced amino acids obtained through the first 5'-RACE forward and reverse; the 3'-RACE and the extra 5'-RACEs. Previously described clones CF 421 and CF327 are also included.

A consensus of 1701 bp starting from the presumed starting 'M' was established for this putative Alstroemeria monoterpene synthase and following analyses described in this chapter, alignments, phylogeny, and genomic organization, were based on this amino acid sequence (Figure 4.8).

```
         10        20        30        40        50        60        70        80
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MASHLPLLLPSPRPPVASGPSNMSPKQRRSANYTPTVWNNNYLQTLESEFIGMECAARLEKLKSEAKSLIAGTTSLVEKL

         90       100       110       120       130       140       150       160
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ELVDTLRQLGLAYHFEEEIMDVLAAILQSADLDSVARQLDGLHATALLFRLLREHGFEISQDILRWFHDETTGGFKACIT

         170       180       190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RDIKGLLSFYEASYVAIEEENIMDDAREFTTKHLKDFIENSTEPWLRERALHALELPLNWRFQRLHSRWFIDMYERGTDT

         250       260       270       280       290       300       310       320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NLCLLELAKLDFNIVQGVYKTEIRQLSKWWADLDLIGDKLSFARDRLLECYLWAAGGSPEPESWRCRQVFTKCICLATII

         330       340       350       360       370       380       390       400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DDIYDVYGTLEELELFTKAVERWEVSAIEQLPDYMKICVLALFNTFNEIAYKTLKEKGLDIIPFLRKAWSDLCNAYLVEA

         410       420       430       440       450       460       470       480
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
KWYYKGHSPPFGEYLENAGISIGGHLLLTLAFFVNDYVSVESVERFKAYQSLMCWSGIIVRLYDDLATSEAEGERGDVSK

         490       500       510       520       530       540       550       560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AIQCYMHETDASEVMAYMFIRDMIADKWKILNKDRKASTKNEKYFKSVAINTLQLTQWTYQHGDGFGEPQHRTKDTILAL

....|..
LVEPILL
```

Figure 4.8. Amino acid sequence of the consensus established for a putative Alstroemeria monoterpene synthase. Highlighted are the R(R)X_8W and DDXXD motifs.

4.3.4. Phylogenetic Analysis

In order to characterize the putative Alstroemeria TPS obtained, its amino acid sequence consensus was compared with other 45 terpenoid synthases previously described (Table 4.1) and a phylogenetic tree was built (Figure 4.9).

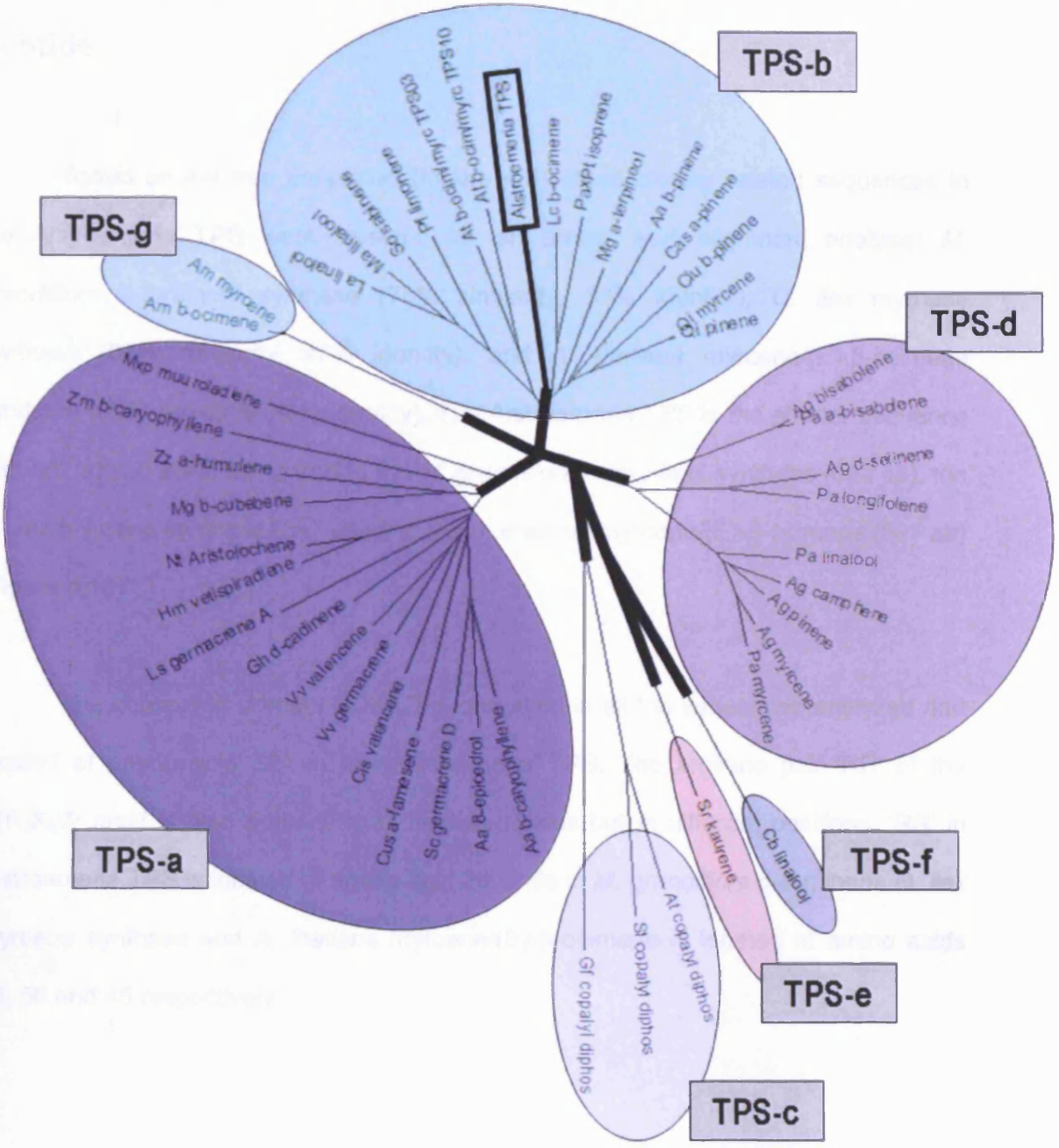


Figure 4.9. Phylogenetic tree of 46 plant terpene synthase genes based on estimation of pair-wise distances at amino acid level and analyzed by the neighbour-joining method using MEGA 4.0.2 (Tamura *et al.*, 2007). Circles cluster TPS grouped in each of the 6 subfamilies proposed by Bohlmann *et al.* (1998) (TPS-a, b, c, d, e and f) and the additional subfamily TPS-g suggested by Dudareva *et al.* (2003). The Alstroemeria TPS is showed boxed in black. All the other sequences were obtained from a public data base and their details are shown in table 4.1.

4.3.5. Analysis of conserved domains and putative signal peptide

Based on the tree presented (Figure 4.9), three closely related sequences to the Alstroemeria TPS were selected for an amino acid alignment analysis: *M. grandiflora* α-terpineol synthase (70% similarity, 50% identity), *Q. ilex* myrcene synthase (67% similarity, 47% identity), and *A. thaliana* myrcene/(E)-β-ocimene synthase (62% similarity, 40% identity). The Alstroemeria TPS is the shorter sequence with 567 amino acids compared to the *M. grandiflora* α-terpineol synthase (592 aa), the *Q. ilex* myrcene synthase (597 aa) and the *A. thaliana* myrcene/(E)-β-ocimene (591 aa) (Figure 4.10).

The conserved domain DDXXD is observed in all the sequences analyzed and located at amino acid 321 in the Alstroemeria TPS. The arginine pair 'RR' of the R(R)X_8W motif is also present in all the sequences but at different positions. 'RR' in Alstroemeria TPS is located at amino acid 28 while in *M. grandiflora* α-terpineol, *Q. ilex* myrcene synthase and *A. thaliana* myrcene/(E)-β-ocimene is located at amino acids 53, 56 and 45 respectively.

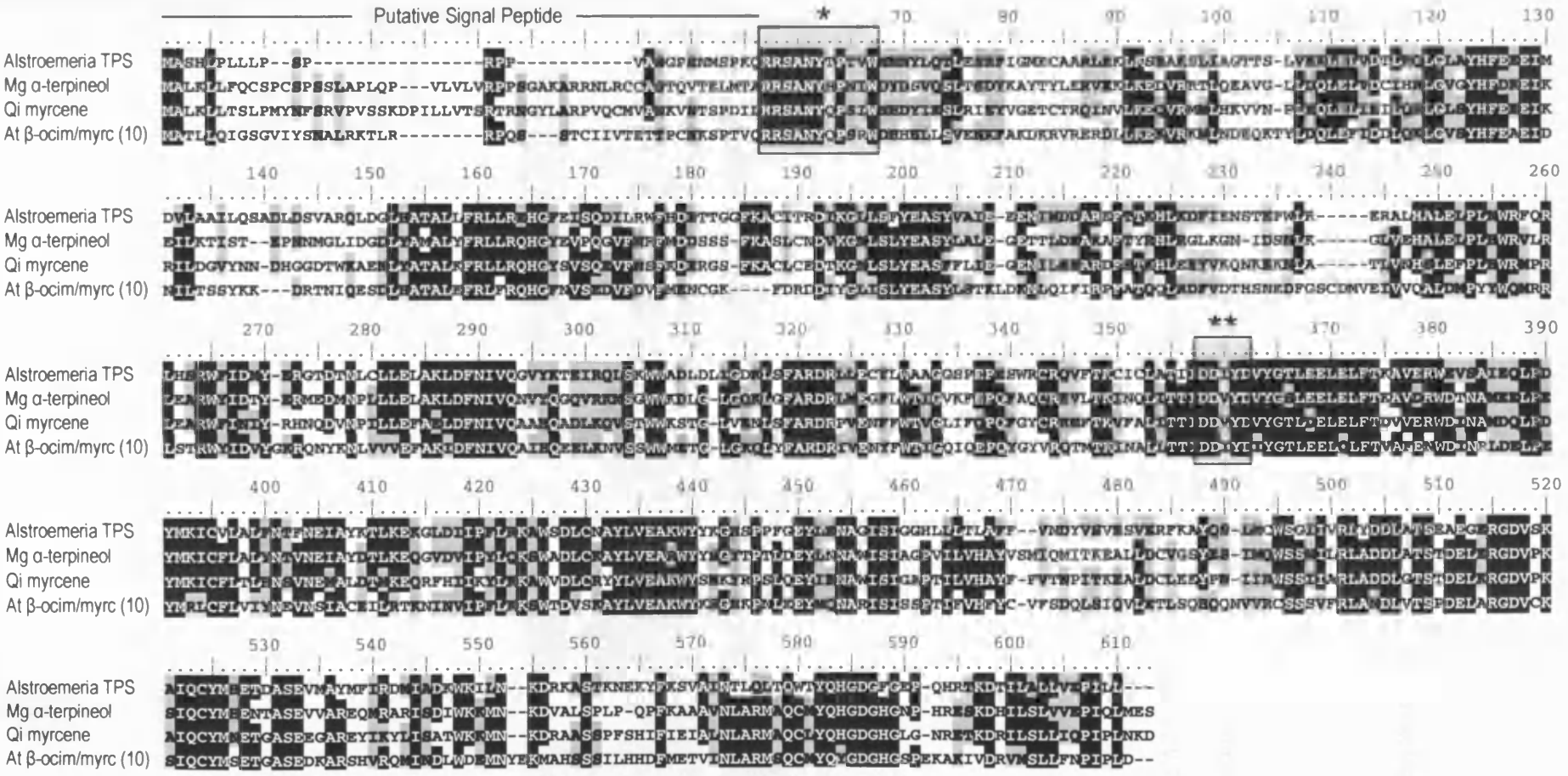


Figure 4.10. Alignment of deduced amino acid sequences of the Alstroemeria TPS and other three closely related sequences M.g. α-terpineol synthase, Q.i. myrcene synthase and A.t. (E)-β-ocimene/myrcene synthase (TPS10).
* R(R)X_8W motif
** DDXXD motif

4.3.6. Genomic Organization

4.3.6.1. Amplification of the genomic sequencing covering the putative Alstroemeria TPS ORF

After several attempts, a total of 12 primers were sufficient to amplify 9 sequences (AA2, AC1, AD5, AE2, AF3, AG1, C33, C02 and D11) by PCR that covered a total genomic region of 2323 bp including adequate overlap (>100 bp) across them (Figure 4.11 and Figure 4.12).

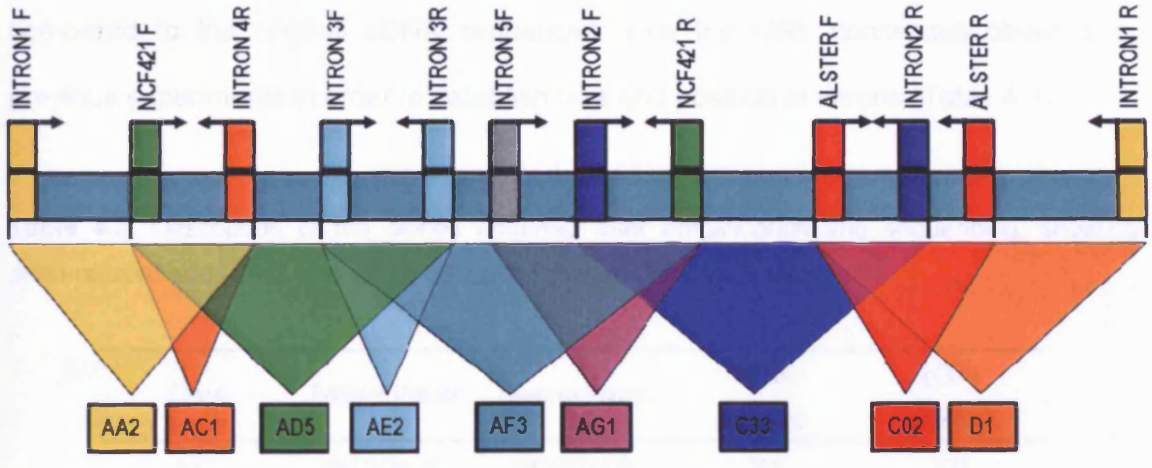


Figure 4.11. Scheme of the primers used showing their respective position in the ORF of the Alstroemeria TPS and the clone (boxed codes) that they amplified.

In order to enhance the amplification and obtain clear bands for further excision and cloning of the PCR products, it was necessary to increase both concentration of primers (from 0.4 to 0.8 µM) and the concentration of $MgCl_2$ (from 1.5 to 2.5 mM) in the PCR mix (Figure 4.12)

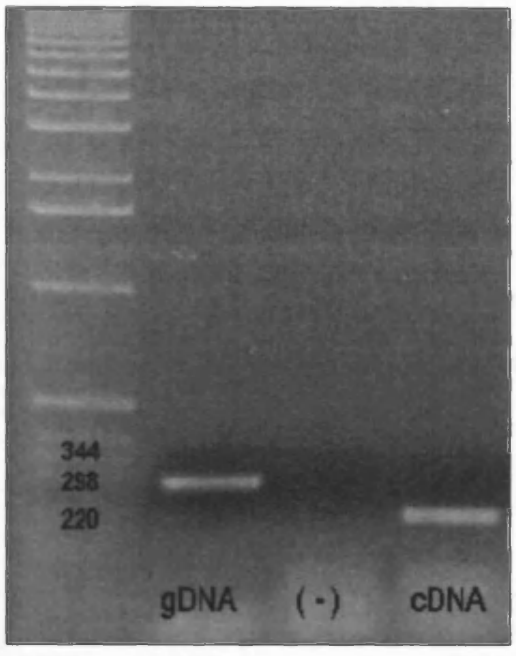


Figure 4.12. Amplification of *Alstroemeria* cv. 'Sweet Laura' (SL) cDNA and gDNA by the primer combination (INTRON5 F + NCF421 R). The band observed corresponds to the clone AG1 with a sequenced size of 209 and 311 bp for cDNA and gDNA respectively. Hence the difference found (102 bp) was assigned as an intron.

After the amplification and sequencing of the nine clones, sequences were compared to the original cDNA sequences from the ORF consensus obtained in previous experiments in order to establish size and position of introns (Table 4.3).

Table 4.3. Description of the clones obtained after amplification and sequencing, showing primers used and gDNA and cDNA sequence size (bp) for each clone.

Clone	Forward Primer	Reverse Primer	cDNA size (bp)	gDNA size (bp)
AA2	INTRON1 F	INTRON4 R	211	322
AC1	NCF421 F	INTRON4 R	122	122
AD5	NCF421 F	INTRON3 R	476	698
AE2	INTRON3 F	INTRON3 R	168	168
AF3	INTRON3 F	NCF421 R	499	604
AG1	INTRON5 F	NCF421 R	209	311
C33	INTRON2 F	INTRON2 R	428	538
C02	ALSTER F	ALSTER R	200	273
D1	ALSTER F	INTRON1 R	435	508

4.3.6.2. Identification of exons and introns

After the alignment of the genomic contig with the ORF of the putative Alstroemeria TPS, five introns were identified along the original cDNA sequence. Introns 1 to 5 were inserted at amino acids 52, 141, 269, 388, 470 of the putative Alstroemeria TPS respectively (Figure 4.13).

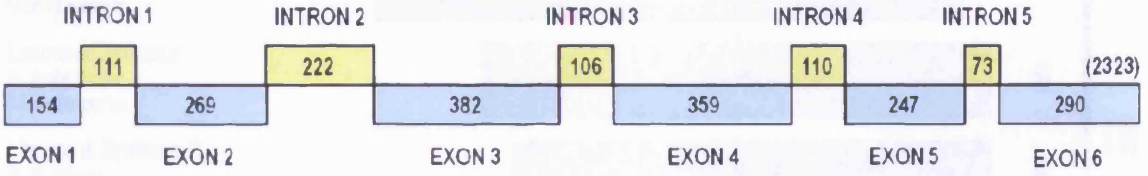


Figure 4.13. Organization exon-intron of the Alstroemeria TPS. Numbers represent the total number of bp of each exon and intron.

4.3.6.3. Classification of Alstroemeria TPS based on its genomic organization

The exon-intron distribution and size of introns observed in Alstroemeria TPS were compared with genomic organizations previously described for other terpene synthases. As observed in the Figure 4.14, the Alstroemeria TPS shows a unique genomic organization with 5 introns and 6 exons.

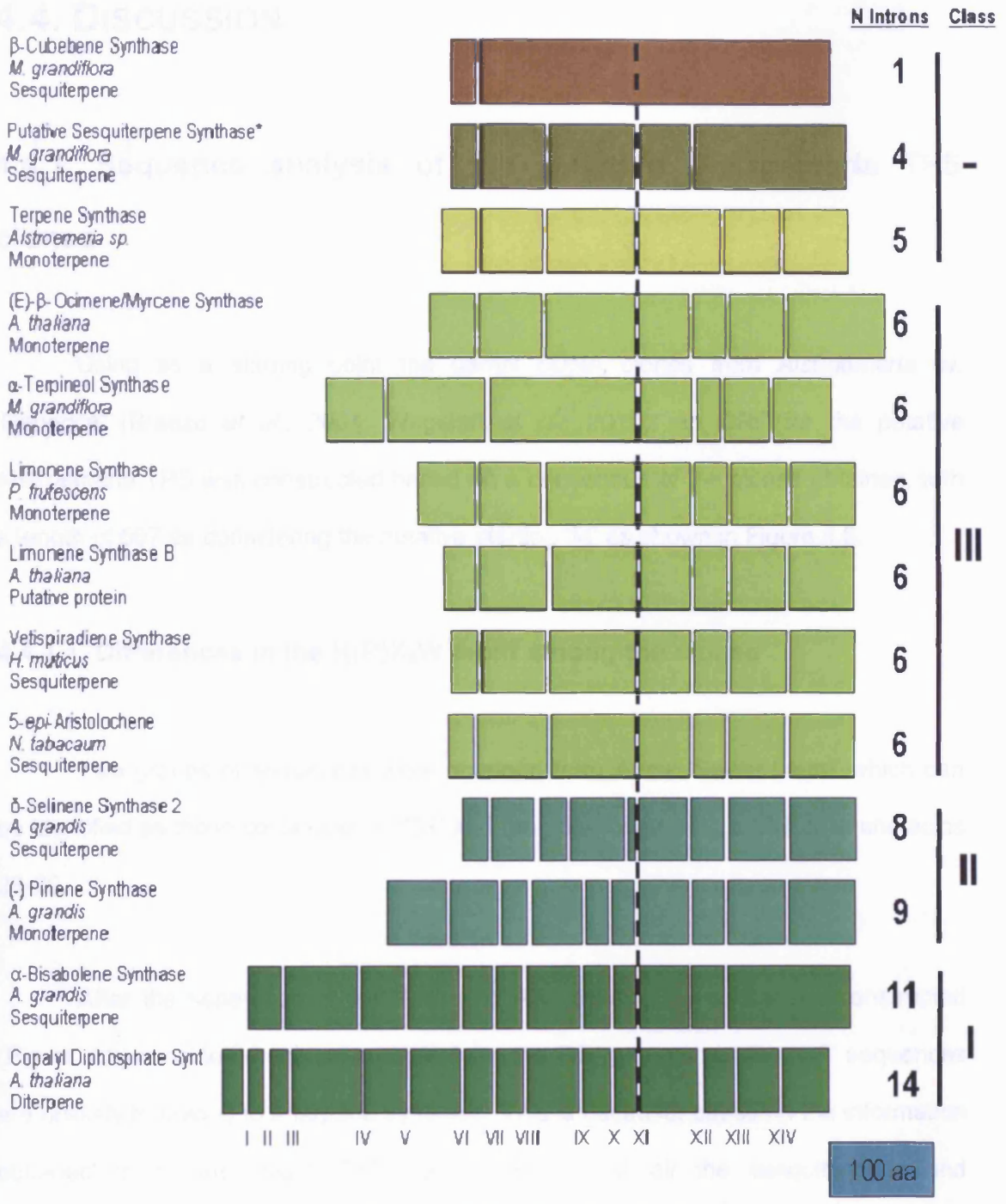


Figure 4.14. Genomic organization of plant TPS genes. Coloured boxes show exons and are drawn to scale. Gaps between exons correspond to introns and are numbered in the bottom of the figure with Roman numerals. All the sequences have been aligned to the intron XI (dashed line) of copalyl diphosphate synthase (*A.thaliana*) as the following exon contains the highly conserved aspartate rich domain (DDXXD), common for all the plant TPS. On the right, number of introns and the class (according to Trapp and Croteau, 2001) which each TPS belongs to are shown. Classes (I, II and III) are also illustrated in different colours and (-) was assigned to identify TPS with unique exon intron organization. (*) Described by Lee and Chappell (2008) as member of a putative sub Class III-a.

4.4. DISCUSSION

4.4.1. Sequence analysis of the putative Alstroemeria TPS clones

Using as a starting point the partial cDNA clones from *Alstroemeria* cv. 'Rebecca' (Breeze *et al.*, 2004; Wagstaff *et al.*, 2010), an ORF for the putative Alstroemeria TPS was constructed based on a consensus of the clones obtained, with a length of 567 aa considering the putative starting 'M' as shown in Figure 4.8.

4.4.1.1. Differences in the R(R)X_8W motif among the clones

Two groups of sequences were obtained from *A.* cv. 'Sweet Laura' which can be identified as those containing an 'RR' and the other containing a 'CR' at amino acids 28-29.

After the separation of the 'CR' and 'RR' groups, the consensus constructed (Figure 4.8) considered only sequences from the 'RR' group since the 'CR' sequences are unlikely to belong to a terpene synthase. This is because, based on the information obtained from other plant TPS sequences, almost all the sesquiterpene and monoterpene synthases observed in angiosperms contain the R(R)X_8W motif but none of them contain a 'CR' nor even a 'XR' (Table 4.4).

Table 4.4. Comparison of the R(R)X_8W motif present in the Alstroemeria clones (groups 'RR' and 'CR') and 12 TPS from other species including hemiterpenes (C_5), monoterpenes (C_{10}) and sesquiterpenes (C_{15}).
(*) Assigned to 'RR' sequences.

TPS	Type	Sequence
Aa β-pinene*	(C_{10})	RRSANYAPSL
Aa 8-epicedrol	(C_{15})	RPNANFPSEI
Cus α-farnesene*	(C_{15})	RRSAQFQASV
Cas α-pinene*	(C_{10})	RRSANYDPPI
Ciu β-pinene*	(C_{10})	RRSADYGPTI
Lc β-ocimene	(C_{10})	RKSANYQPNI
Ls germacrene A	(C_{15})	RPLANFPPSV
Mg β-cubebene	(C_{15})	RAFVNYHPSI
Nt Aristolochene	(C_{15})	RPVADFSPSL
PaxPt isoprene*	(C_5)	RRSANYEPNS
Qi myrcene*	(C_{10})	RRSANYQPSI
Vv valencene	(C_{15})	RPVANFHPNI
Alst TPS 'RR'*	(?)	RRSANYTPTV
Alst TPS 'CR'	(?)	CRSANYTPTV

The first R of this motif is conserved in all the angiosperm TPS and the 'CR' observed in some of the 5' RACE clones may reflect a mutation which would result in this protein being non-functional as a TPS. This suggestion is supported by the hypothesis of Williams *et al.* (1998) who described this arginine-rich motif as involved in the isomerisation of GPP to a cyclizable intermediate. Empirically they support this hypothesis by an experiment where they prove that a limonene synthase without the RR motif was unable to accept GGP as substrate.

4.4.1.2. Putative signal sequence

The arginine pair 'RR' of the R(R)X_8W motif in Alstroemeria TPS is located at amino acid 28, and this position is of particular importance since this site has been suggested as the starting point of the functional protein or, in other words, the N terminus of the mature protein (Bohlmann *et al.*, 1998). Therefore the amino acid sequence going from the initial 'M' up to this point presumably corresponds to the

signal or transit peptide (Dudareva *et al.*, 2003; Shimada *et al.*, 2004; Shimada *et al.*, 2005; Lee and Chapell, 2008). Supporting this suggestion, the alignment presented before (Figure 4.10) shows that the sequence upstream of the R(R)X_8W motif is less conserved, again an indication that it is not part of the functional enzyme. Furthermore, some studies have demonstrated that truncations of monoterpene synthases upstream of the R(R)X_8W motif have resulted in fully active enzymes (Bohlmann *et al.*, 1999; Williams *et al.*, 1998).

Considering this information and looking at the alignment (Figure 4.10), one conclusion is that the Alstroemeria TPS is shorter than the other sequences because its putative signal peptide (27 aa) is shorter. Turner *et al.* (1999) suggested that sesquiterpene synthases (550–580 amino acids) are normally shorter than monoterpene synthases (600–650 amino acids) due to differences in the signal peptide, which is longer in monoterpene synthases because they target the initial translation product towards the plastids. However, the assumption that the Alstroemeria TPS has a sesquiterpene-like signal peptide seems to be contradictory with the analysis of the phylogenetic tree, where it was clustered in the subfamily-b which includes only monoterpene synthases (Figure 4.9). However, the members of this subfamily have been described with signal peptides of approximately 50 to 60 amino acids (Bohlmann *et al.*, 1998; Bohlmann *et al.*, 2000).

The average size of the putative signal peptides of each member of the TPS subfamilies a and b, was determined by considering the amino acid sequence from the initial M up to the arginine pair. The sesquiterpene synthases included in the TPS-a subfamily showed an average signal peptide size of about 14 amino acids. An average of about 49 amino acids was found in the subfamily TPS-b. The Alstroemeria TPS included in this group with a signal peptide of 27 amino acids, is significantly below the average. However, another two members of this subfamily had signal peptides of very

similar length, to the Alstroemeria TPS: the Arabidopsis (E)-β-ocimene/myrcene synthase (TPS03) and the *Lavandula angustifolia* linalool synthase with signal peptides of 25 and 26 amino acids respectively (Table 4.5)

Table 4.5. Comparison of the putative signal peptide sizes of member of TPS subfamilies 'a' and 'b' as observed in Figure 4.9, including sesquiterpene and monoterpenes respectively. Averages, standard deviations (STEDV) and standard errors (SE) are shown.
(*) TPS without a clear $R(R)X_8W$ motif, thus the no identification of the signal peptide.

Subfamily TPS-a Sesquiterpene synthases	Putative signal peptide size (aa)	Subfamily TPS-b Monoterpene synthases	Putative signal peptide size (aa)
Aa b-caryophyllene	9	At b-ocim/myrc TPS03	25
Aa 8-epicedrol	9	At b-ocim/myrc TPS10	45
Cus a-farnesene	15	Aa b-pinene	47
Cis valencene	7	Alstroemeria TPS	27
Gh d-cadinene	18	Cas a-pinene	70
Hm vetispiradiene	16	Ciu b-pinene	55
Ls germacrene A	21	La linalool	26
Mg b-cubebene	14	Lc b-ocimene	55
Mxp muuroladiene	14	Ma linalool	68
Nt Aristolochene	14	Mg a-terpineol	53
Sc germacrene D	9	PaxPt isoprene	52
Vv germacrene	21	Pf limonene	60
Vv valencene	18	Qi myrcene	56
Zm b-caryophyllene	*	Qi pinene	56
Zz a-humulene	16	So sabinene	51
Average	14,36	Average	49,73
STEDV	4,48	STEDV	13,95
± SE (n=14)	3,17	± SE (n=15)	9,86

Hence, although the size of the Alstroemeria TPS signal peptide seems to be unusually short to belong to the TPS-b subfamily, the similarity in this aspect with another two TPS members of this group support the classification of the Alstroemeria TPS as a monoterpene synthase clustered in the TPS subfamily-b.

All three programs used to predict the targeting function of the putative Alstroemeria TPS signal peptide, predicted it as a plastid targeting sequence. Thus since GPP and FPP seem to be located only in plastids and cytoplasm respectively, the plastidial targeting sequence of this putative Alstroemeria TPS would suggest its activity only with GPP, the main precursor of monoterpenes.

4.4.2. Phylogenetic Analysis

The six subfamilies suggested by Bohlmann *et al.* (1998) and the additional subfamily suggested later by Dudareva *et al.* (2003), were clearly distinguished in the unrooted phylogenetic tree (Figure 4.9), based on amino acid alignments of the putative Alstroemeria terpene synthase with the other 45 terpene synthases previously described (Table 4.1).

Subfamily TPS-a comprises 15 sesquiterpene synthases observed in a wide range of angiosperm families including *Asteraceae, Solanaceae* and *Vitaceae* among others (Appendix C) (Figure 4.9). Although only sesquiterpenes were clustered in TPS-a in this study, the tree constructed by Bohlmann *et al.* (1998) included also an angiosperm diterpene synthase in this subfamily.

Fifteen monoterpene synthases and one hemiterpene synthase (isoprene synthase) were clustered in the subfamily TPS-b, including the Alstroemeria TPS described in this study (Appendix C). Although this subfamily was initially described only with species of the *Lamiaceae* family (Bohlmann *et al.*, 1998), an Arabidopsis (*Brassicaceae*) myrcene/(E)-β-ocimene synthase was also included later (Bohlmann *et al.*, 2000). Subsequently, some more families were included in this group by Dudareva

et al. (2003) (*Asteraceae*, *Fagaceae* and *Rutaceae*) and Lee and Chappell (2008) (*Fabaceae*, *Magnoliaceae* and *Salicaceae*). Finally, the tree presented in this study comprises also *Cannabaceae* and *Alstroemeriaceae* mono TPS in this subfamily, revealing that as for the TPS-a subfamily, this subfamily includes a wide range of taxonomic families too (Figure 4.9).

Only a single diterpene synthase present in three different organisms is observed in the subfamily TPS-c, copalyl diphosphate synthase which is a TPS of primary metabolism (Appendix C). Despite the considerable taxonomic distance between the fungal *G. fujikuroi* compared to the two plants, they still were clustered together in this subfamily (Figure 4.9). This fact seems to contradict the hypothesis proposed by Bohlmann *et al.*, (1998) who suggested that despite the high similarity among amino acid sequences of sesquiterpene and monoterpene synthases, similarity is based more on taxonomic affinities of the plant species from which the gene was isolated rather than the type of products formed. Hence the tree shown in the present study clustered three TPS in the same subfamily TPS-c because of their enzymatic activity but not because of their taxonomic relationship. However more studies, including a wider range of species, should be done in order to provide a more reliable answer to this question.

Subfamily TPS-d includes nine gymnosperm monoterpene and sesquiterpene synthases present in *A. grandis and P. Abies* (Appendix C). Two sub-branches are observed clustering together all the monoterpene synthases (Pinene, myrcene, (-)-linalool and (-)-camphene synthase) on one side and all the sesquiterpene synthases (Longifolene, (E)-α-bisabolene and δ-selinene synthase) on the other (Figure 4.9).

A diterpene synthase involved in secondary metabolism from *Stevia rebaudiana*, kaurene synthase, is the single member of the subfamily TPS-e (Appendix

C). Subfamily TPS-f also has a single member, a linalool synthase discovered in *Clarkia breweri* (Appendix C) (Figure 4.9).

The additional subfamily TPS-g suggested by Dudareva *et al.* (2003) includes two monoterpene synthases (myrcene and (E)-β-ocimene synthase) from *A. majus* (Appendix C) (Figure 4.9). Although in a later publication (Lee and Chapel, 2008) these TPS were clustered in the subfamily TPS-b with other monoterpene synthases, the tree presented in the present study shows clearly how they clustered in a completely different branch. Moreover, the amino acid sequences related to these two TPS are characterized by a lack of the well conserved arginine-rich region R(R)X_8W, hence an important difference to other monoterpene synthases.

Summarizing the information, this Alstroemeria TPS characterized here could be classified as a monoterpene synthase with a particularly short plastidial signal peptide. Nevertheless this suggestion is based on phylogenetic relationships and signal peptide analysis. Only the actual catalytic activity of the enzyme can determine with more certainty whether it as mono or sesquiterpene, by performing a functional analysis and thus experimentally obtaining the final product.

4.4.3. Genomic organization

According to the classification proposed by Trapp and Croteau (2001), plant terpene synthases are clustered into three different classes based on intron/exon pattern: 12–14 introns (class I), 8-9 introns (class II), and 6 introns (class III). Class I includes conifer diterpene and sesquiterpene synthases (i.e. α-bisabolene synthase/*A. grandis*) and angiosperm terpene synthases only involved in primary metabolism (i.e. copalyl diphosphate synthase/*A. thaliana*). TPS belonging to this class contain the

conifer diterpene internal sequence (CDIS) domain, not present in classes II and III. Moreover, this class has been identified as an ancestral class throughout the evolution of TPS in the plant kingdom. Hence it is proposed that classes II and III have evolved from Class I by loss of introns; this hypothesis was proposed by Trapp and Croteau (2001) and Lee and Chappell (2008) who contradicted the evolution through gain of introns in plant TPS (Rogers, 1985; Coghlan and Wolfe, 2004). Moreover, they suggested that plant and fungal TPS genes might have had different evolutionary origins, since the trichodiene synthase gene found in the fungus *Myrothecium roridum* contains a single intron (Gallo *et al.*, 2004), and no equivalent TPS gene in the plant kingdom had been described so far (Trapp et al., 1998).

Class II comprises only conifer monoterpene (i.e. (-) pinene synthase/*A. grandis*) and sesquiterpene synthases (i.e. δ-selinene synthase/*A. grandis*) and class III includes only angiosperm monoterpene (i.e. limonene synthase/*P. frutescens*), sesquiterpene (i.e. 5-*epi*-aristolochene/*N. tabacaum*) and diterpene synthases involved in secondary metabolism, all of them with 7 exons and 6 introns. Introns III, VIII and XI-XIV are conserved in this class and, according to Trapp and Croteau (2001), among all the plant terpene synthases. Nevertheless, a later publication (Lee and Chappell, 2008) described a putative Magnolia sesquiterpene synthase without intron XIV and a very unusual Magnolia β-cubebene synthase with only one intron (Figure 4.14).

The Alstroemeria TPS characterized in this study could be clustered into class III although intron XII is lost, resulting in only five introns: III, VIII, XI, XIII and XIV. Although position of introns is conserved, intron size is not. Hence, even the most conserved introns found in all the TPS included in Figure 5.4 showed a high diversity in size, ranging from 76 to 698 bp for intron III, from 82 to 826 bp for intron VIII and 55 to 1134 bp for intron XI (Table 4.5).

Table 4.5. Comparison of the size and position of the introns of Alstroemeria TPS (A.sp.Terpene synthase) with other terpene synthase previously described. Roman numerals correspond to intron numbers. Dashes and ND symbolize absence of intron and intron not determined respectively.

([1]) Information provided by J.Chappel (personal communication).

([2]) Sequence obtained from a public database (http://www.ncbi.nlm.nih.gov/).

([3]) Data adapted from Trapp and Croteau (2001).

Terpene synthase	Intron Size (bp)														TOTAL Introns
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	
M.g.β-Cubebene Synthase[1]	-	-	687	-	-	-	-		-	-	-	-	-	-	1
M.g.Putative Sesq Synthase[1]	-	-	677	-	-	-	-	801	-	-	1134	266	-	-	4
Alstroemeria TPS	-	-	111	-	-	-	-	222	-	-	106	-	110	73	5
A.t.(E)-β-Oc/Myrc Synthase[2]	-	-	100	-	-	-	-	151	-	-	387	145	279	234	6
M.g.α-Terpineol Synthase[1]	-	-	76	-	-	-	-	97	-	-	158	84	114	480	6
P.f.Limonene Synthase[3]	-	-	698	-	-	-	-	826	-	-	125	326	91	119	6
A.t.Limonene Synthase B[3]	-	-	100	-	-	-	-	212	-	-	437	89	308	178	6
H.m.Vetispirad Synthase[3]	-	-	ND	-	-	-	-	ND	-	-	ND	93	ND	ND	6
N.t.5-*epi*-Aristolochene[3]	-	-	127	-	-	-	-	87	-	-	76	131	155	113	6
A.g.δ-Selinene Synthase2[3]	-	-	122	-	-	-	84	147	101	-	55	142	361	76	8
A.g.(-) Pinene Synthase[3]	-	-	107	-	-	-	85	146	92	537	99	94	106	120	9
A.g.α-Bisabolene Synthase[3]	-	-	205	164	428	546	156	134	108	96	116	141	103	-	11
A.t.Copalyl Diph Synthase[3]	822	115	283	253	198	121	79	82	212	357	263	927	494	270	14

Analysis of exon size reveals that exon 4 of the Alstroemeria TPS is almost exactly the same size as the addition of exon 4 plus exon 5 of the TPS included in class III. Therefore is reasonable to suggest exon 5 of the Alstroemeria TPS may have resulted from the fusion of exons 4 and 5 observed in class III TPS (Table 4.6).

Table 4.6. Comparison of the size of exons 4 and 5 of the Alstroemeria terpene synthase and other six terpenoid synthases grouped in the class III. Numbers indicate number of amino acids for each exon and for the total (exon 4 + exon 5).

	Alstroemeria TPS	A.t.(E)-β-Oc/Myrc Synt	M.g.α-Terp Synthase	P.f.Limonene Synthase	A.t.Limonene Synthase B	H.m.Vetispirad Synthase	N.t.5-*epi*-Aristolochene
Exon 4	119	73	73	73	73	73	73
Exon 5	-	46	46	47	47	47	46
Total	119	119	119	120	120	120	119

The rest of the exons of the Alstroemeria TPS show very similar size compared to the other class III TPS, excepting the first exon. This exon has been described as longer in monoterpene and diterpene synthases compared to sesquiterpene synthases, because of the presence of a plastidial transit peptide (Vogel *et al.*, 1996 and Bohlmann *et al.*, 1997). Furthermore the class III TPS in Figure 4.14, supports this pattern, with the first exon of *M. grandiflora*, *H. muticus* and *N. tabacum* sesquiterpene synthases being shorter than the *A. thaliana, M. grandiflora* and *P. frutescens* monoterpene synthases. Thus based on a genomic organization criterion, the Alstroemeria TPS described in this study seems to be a member of class III with a merged 4-5 exon and a sesquiterpene-like first exon.

CHAPTER V.

EVALUATION OF THE GENE EXPRESSION OF A PUTATIVE ALSTROEMERIA TPS

V. EVALUATION OF THE GENE EXPRESSION OF A PUTATIVE ALSTROEMERIA TPS

5.1. INTRODUCTION

The vast diversity of terpenoid compounds found in nature is possible due to a class of enzymes involved in the conversion of the acyclic prenyl diphosphates and squalene into a multitude of cyclic and acyclic forms (Degenhardt *et al.*, 2009). This class of enzymes comprises the well known terpenoid synthases which have been widely studied for different purposes and using different methods. Thus studies, including phylogenetic analysis (Bohlmann *et al.*, 1998), genomic organization (Trapp and Croteau, 2001), functional analysis (Dudareva *et al.*, 2003), transformation (Davidovich-Rikanati *et al.*, 2008) and gene expression (Tholl *et al.*, 2004), have been carried out aimed at characterizing plant terpenoid synthases.

The evaluation of gene expression has been widely used in studies related to TPS, focused on two main objectives: the analysis of temporal and spatial expression. Temporal expression of TPS comprises evaluations performed at different floral stages of development, for example in rose (Guterman *et al.*, 2002), iris (van Doorn *et al.*, 2003) and snapdragon (Tholl *et al.*, 2004); and at different hours throughout the day looking for a circadian regulation (Dudareva *et al.*, 2005). Spatial expression analyses have been focused on evaluations performed on different organs of the plant, including vegetative organs such as roots, leaves and stems (Cai *et al.*, 2002; Chen *et al.*, 2004)

and reproductive organs such as ovary, stamens, petals, sepals and fruits (Tholl *et al.*, 2004; Chen *et al.*, 2003b). Furthermore, some evaluations aimed at describing ecological functions of terpenoid compounds have been carried out, assessing gene expression of TPS in wounded tissues (Gomez *et al.*, 2005; Wang *et al*, 2009).

Different methodologies have been used for the evaluation of gene expression, from those developed for the analysis of single and specific genes (e.g., Northern, slot, and dot blotting; PCR-based techniques; and nuclease protection assays) to methodologies focused on identifying multiple genes with diverse expression between different experimental samples (e.g., subtractive hybridization, differential display, sequencing of expressed sequence tags, serial analysis of gene expression, and hybridization to microarrays) (Moody, 2001).

Although several studies of TPS gene expression have been performed using northern blots (Bohlmann *et al.*, 1997; Cseke *et al.*, 1998; Lu *et al.*, 2002; Schnee *et al.*, 2002), the PCR-based techniques such as semi quantitative RT-PCR (semi qRT-PCR) and quantitative RT-PCR (qRT-PCR) have shown some advantages over the northern blot analysis. With northern blot analysis there is the risk of mRNA degradation during electrophoresis, affecting the quality and quantity of expression (Streit *et al.*, 2008), whereas RT-PCR techniques avoid this sort of problem. Moreover, through qRT-PCR quantitative data can be obtained with a dynamic range of 7 to 8 log orders of magnitude (Wong and Medrano, 2005).

Even though recent studies aimed at evaluating gene expression prefer to use qRT-PCR because of its more accurate assessment, semi quantitative methods are still widely used and appropriate for many purposes (Marone *et al.*, 2001). Thus TPS expression has been successfully evaluated in plants through both real time qRT-PCR

(van Schie *et al.*, 2007; Cheng *et al.*, 2007; Wang *et al.*, 2008) and semi qRT-PCR (Wilderman *et al.*, 2004; Sasaki *et al.*, 2005; Wang *et al*, 2009).

In the present study, semi qRT-PCR and Real time qRT-PCR were performed in order to evaluate the temporal and spatial expression of a putative Alstroemeria TPS, in scented and non scented genotypes of Alstroemeria.

5.2. METHODOLOGY

5.2.1. RNA extraction

RNA was extracted from flowers of Alstroemeria at the 8 different stages of development previously described (Wagstaff *et al.*, 2001); from different organs of flowers at stage 4-5: inner tepals, outer tepals, pistil and stamens (Chapter II, figure 2.5) and from leaves, following the protocol described in section 2.2.1 of 'General Materials and Methods'.

5.2.2. DNAse treatment and cDNA synthesis

DNAse treatment and cDNA synthesis of the samples evaluated was carried out following the protocol described in sections 2.2.2 and 2.2.3 of 'General Materials and Methods', respectively.

5.2.3. Design of primers

From the clones previously characterized (*CF327*, *CF368*, *CF473*) in Chapter IV, forward and reverse primers were designed using Biotools (http://biotools.umassmed.edu/).

For the semi qRT-PCR, the primers designed for *ALSTER* amplification were the forward *ALSTERSF*: 5'-GGGGGACATCTATTACTCACT-3' and the reverse *ALSTERSR*: 5'-GTTTCATGCATGTAGCATTG-3', flanking a nucleotide sequence of 200 bp in size (Figure 5.1).

For the Real time qRT-PCR the primers selected were *RTALSTERSF*: 5'-ACTTTATGACGACTTGGGAACTTCA-3' and the reverse *RTALSTERSR*: 5'-CCTTATAAACATGCGAGCCATCA-3', flanking a nucleotide sequence of 121 bp in size (Figure 5.1).

Figure 5.1. Nucleotide sequence alignment of three Alstroemeria clones homologous to a *Quercus ilex* putative chloroplast terpene synthase. Highlighted are the primers used for the semi qRT-PCR (*) and the Real time qRT-PCR (**) for the amplification of *ALSTER*.

5.2.4. Semi qRT-PCR

5.2.4.1. Amplification

Semi quantitative PCR was carried out as described in section 2.5 of 'General Materials and Methods', using cDNA of the different organs evaluated. Three experimental replicates were performed for each set of PCR and the number of cycles was optimized for each genotype.

5.2.4.2. Cycle number optimization

To establish the optimal number of cycles, an equal amount of the cDNA from each sample (each developmental stage or each organ) was mixed and PCR reactions were performed with different number of cycles. Graphs plotting number of cycles v/s band intensity were performed in order to find the exponential phase and therefore the optimum number of cycles for semi qRT-PCR. Limiting the cycle number ensures that the reaction remains in exponential phase, and thus differences in band intensity are directly proportional to differences in the number of transcripts of the initial mRNA population. Details of cycle numbers used here are shown in Appendix D (Table D.1).

5.2.4.3. Normalization

Normalization controls were performed using the *PUV* primers (puv2: 5'-TTCCATGCTAATGTATTCAGAG-3'; puv4: 5'-ATGGTGGTGACGGGTGAC-3'), (Dempster *et al.*, 1999); which amplify a 488 bp fragment of 18S rRNA. PCR products obtained from the 18S target were used to normalise results of the other primer sets. Although oligo (dt) was used for the cDNA synthesis, rRNA contains sufficiently high AT rich regions for some of it to be retrotranscribed. This has already been used in previously published papers for normalization of RT-PCR (Price *et al.*, 2008; Orchard *et al.*, 2005; Wagstaff *et al*, 2005).

5.2.4.4. Electrophoresis and data analysis

The PCR products were analyzed on an ethidium bromide stained agarose gel (1 %) as described in section 2.6. of 'General Material Methods', and band intensities were quantified in arbitrary units using the Gene genius bioimaging system and

Genesnap software, both from Syngene (Synoptics Ltd., Cambridge, UK). The measurements were corrected by subtracting the intensity value of the negative control (sterile water). Values were expressed as percentage of maximum giving a value of 100% to the highest intensity found in each reaction set. Analysis of variance (ANOVA) for ALSTER expression was performed in each case using SPSS 17.0 for Windows, using Tukey's HSD (Honestly Significant Difference) test with a significance level of 0.05 (Appendix E; Tables E.1 to E.7).

5.2.5. Real time qRT-PCR

5.2.5.1. Amplification

The Real time qRT-PCR was conducted in a 6 µl volume containing 3 µl of SyBR Green Mix (Applied Biosystems), 0.25 µM of each primer (Sigma, Genosys) and approximately 20 ng of cDNA. Two biological replicates were used for the amplifications, that is cDNA synthesized from two separate RNA extractions. Furthermore all the reactions were carried out in triplicate for each experiment and two experimental replicates were performed for each set of PCR (Figure 5.2).

As a negative control, a PCR reaction containing a volume of water replacing cDNA was performed for each set of PCR reactions to ensure there was no contamination in any of the PCR reagents.

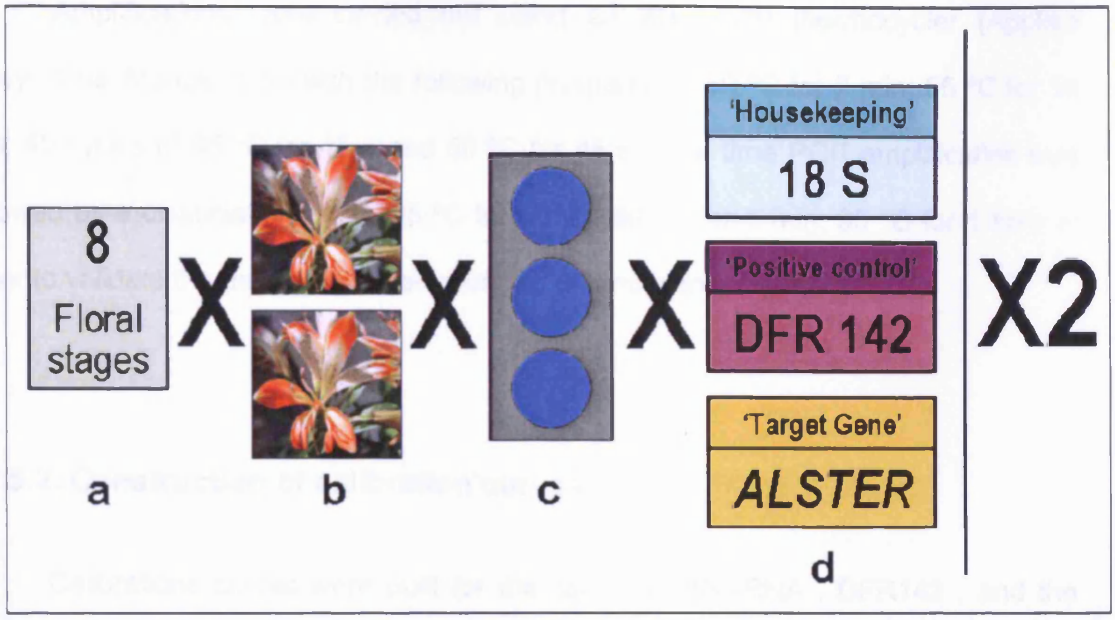


Figure 5.2. Scheme of the sampling method used for the Real time qPCR The amplification was performed using 8 floral stages of development (a) using RNA extractions from two biological replicates (b). Samples were amplified in triplicate for each reaction (c) including three genes (d). Finally, two experimental replicates were performed for each reaction.

A housekeeping gene and a positive control were selected in order to normalize and validate the results obtained for the *ALSTER* expression. The housekeeping gene amplified for the normalization was the 18S rRNA, using the primers Alstro18Sfor: 5'-CGAACACTTACCACGACGACTCT-3', and Alstro18Srev: 5'-CGTTCAAAGACTCGATGGTTCAC-3'), flanking a DNA sequence of 120 bp.

As a positive control to validate the results, DFR142 expression was evaluated. This gene has been described as a dihydroflavonol 4-reductase, playing an important role in anthocyanin accumulation (Helariutta *et al.*, 1993). The primers used for the amplification were AlstroDFR142for: 5'-GCAATTCGATCTTGCAACGAA-3' and AlstroDFR142rev: 5'- AATTTCTACCTCCCCTCCATTATCA-3', flanking a sequence of 110 bp.

Amplifications were carried out using an ABI 7900 thermocycler (Applied Biosystems, Monza, Italy) with the following programme: 50 °C for 2 min; 95 °C for 10 min; 45 cycles of 95 °C for 15 s and 58 °C for 45 s. Real time PCR amplification was followed by a dissociation cycle (95 °C for 5 min; 60 °C for 1 min; 95 °C for 1 min) in order to validate the denaturation temperature of amplicons.

5.2.5.2. Construction of calibration curves

Calibrations curves were built for the controls 18S rRNA , DFR142 , and the target gene *ALSTER*, in order to obtain the PCR amplification efficiencies for each set of primers. Serial cDNA dilutions starting from 10^{-1} [ng · μl^{-1}] were used to build the curves and a dilution factor was assigned for each concentration to facilitate the data analysis (Table 5.2) (Appendix D, Figure D.1).

Table 5.1. Serial dilutions used to build the calibration curves. cDNA concentrations [ng ·μl^{-1}] and their respective dilution factor assigned are shown.

Dilution Factor	cDNA concentration [ng • μl^{-1}]
6	10^{-1}
5	10^{-2}
4	10^{-3}
3	10^{-4}
2	10^{-5}
1	10^{-6}
0	10^{-7}
-1	10^{-8}
-2	10^{-9}
-3	10^{-10}

5.2.5.3. Efficiency and calibrated quantity calculations

Real time qRT-PCR amplification efficiencies (E) were calculated for each gene from the slope values obtained in the calibration curve, using the following formula:

$$E = 1 - (10^{-1/slope})$$

Efficiencies close to 100% are obtained with slope values of 3.3 according to the manufacturer (www.gene-quantification.de/mx4000-appnotes10.pdf).

Calibrated quantity was calculated from the calibration curves and the Ct (Cycle threshold) obtained for each gene, at each stage of development, using the following formula:

$$\text{Calibrated quantity} = 10^{(Ct - b)/slope}$$ where b= y intercept

Finally, all the values were normalized against the housekeeping gene, 18S rRNA. Results were expressed as 'Relative expression' where as the value '1' corresponds to the expression of 18S rRNA at each particular stage. Analysis of variance (ANOVA) for gene expression was performed in each case using SPSS 17.0 for Windows, using Tukey's HSD (Honestly Significant Difference) test with a significance level of 0.05 (Appendix E; Tables E.8 to E.15).

5.3. RESULTS

5.3.1. Semi qRT-PCR

5.3.1.1. *Alstroemeria* cv. 'Rebecca' – Stages of development

A remarkable high expression of *ALSTER* was observed in early stages of development of *A.* cv. 'Rebecca', starting with high levels of expression at stages 0 and 1 with no statistically significant differences between them. Thereafter expression fell sharply to stage 3, in which the lowest expression was found (~ 25 % of maximum). After stage 3, an irregular behaviour in the expression was observed until stage 7, with higher expression at stages 4 and 6 and lower expression at stages 5 and 7 (Figure 5.3).

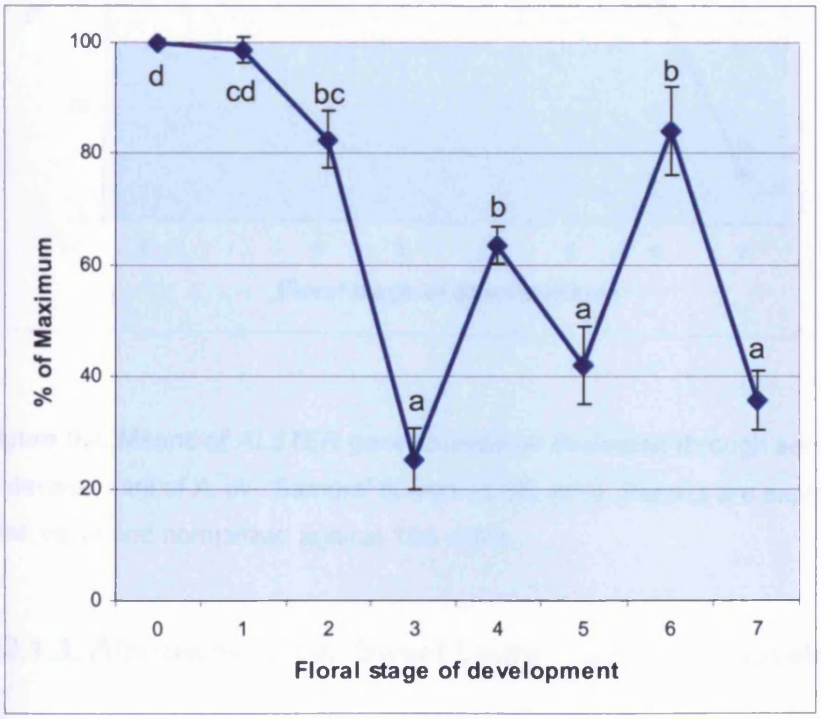


Figure 5.3. Means of *ALSTER* gene expression evaluated through semi qRT-PCR on 8 stages of development of *A.* cv. 'Rebecca' flowers (± SE, n=4). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.1.2. *Alstroemeria* cv. 'Samora' – Stages of development

Expression of *ALSTER* in *Alstroemeria* cv. 'Samora' remained high and fairly constant throughout development until stage 4, including the highest peak at stage 1, with no statistically significant difference among them. After stage 5 there was a continuous decrease finishing at stage 7 with the lowest expression (~ 9 % of maximum) (Figure 5.4).

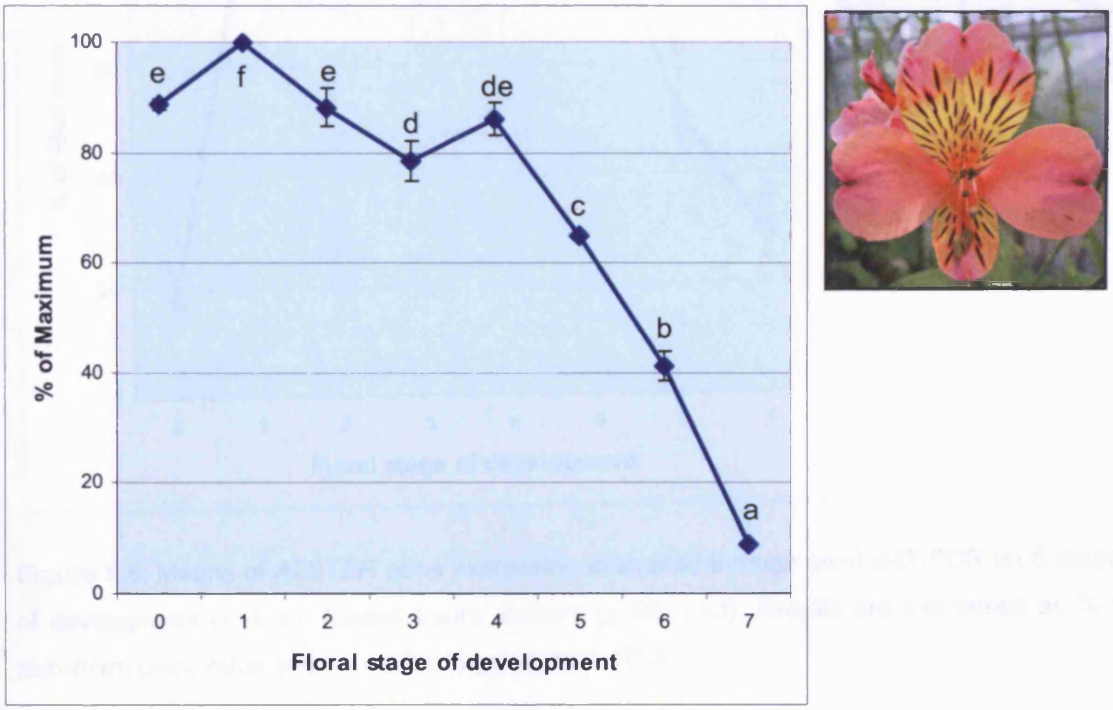
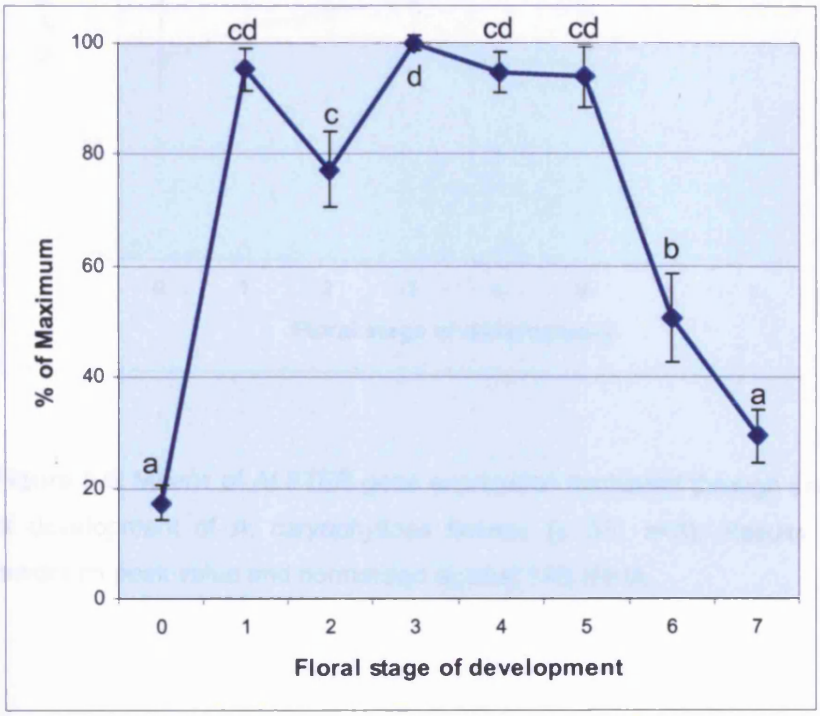


Figure 5.4. Means of *ALSTER* gene expression evaluated through semi qRT-PCR on 8 stages of development of *A.* cv. 'Samora' flowers (± SE, n=3). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.1.3. *Alstroemeria* cv. 'Sweet Laura' - Stages of development

The lowest expression of *ALSTER* was observed in the first stage of development, stage 0 (~ 17 % of the maximum level) which was not statistically

significant different from stage 7. Thereafter a strong and sustained up-regulation was observed from stage 1 to stage 5 with the highest peak at stage 3 which was not significantly different from stages 4, 5 and 1. Expression decreased in the last two stages of development (Figure 5.5).



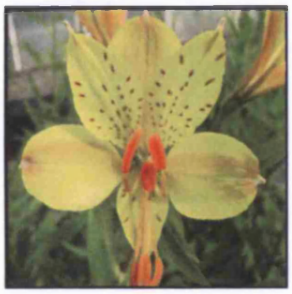


Figure 5.5. Means of *ALSTER* gene expression evaluated through semi qRT-PCR on 8 stages of development of *A.* cv. 'Sweet Laura' flowers (± SE, n=3). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.1.4. *Alstroemeria caryophyllaea* - Stages of development

A. caryophyllaea showed constant expression of *ALSTER* from stages 0 to 3 with values close to 40 % of the maximum and no statistically significant differences. The highest peak was observed at stage 4. From stages 5-7 expression levels fluctuated between 50-80% of maximum, but with no statistically significant changes. (Figure 5.6).

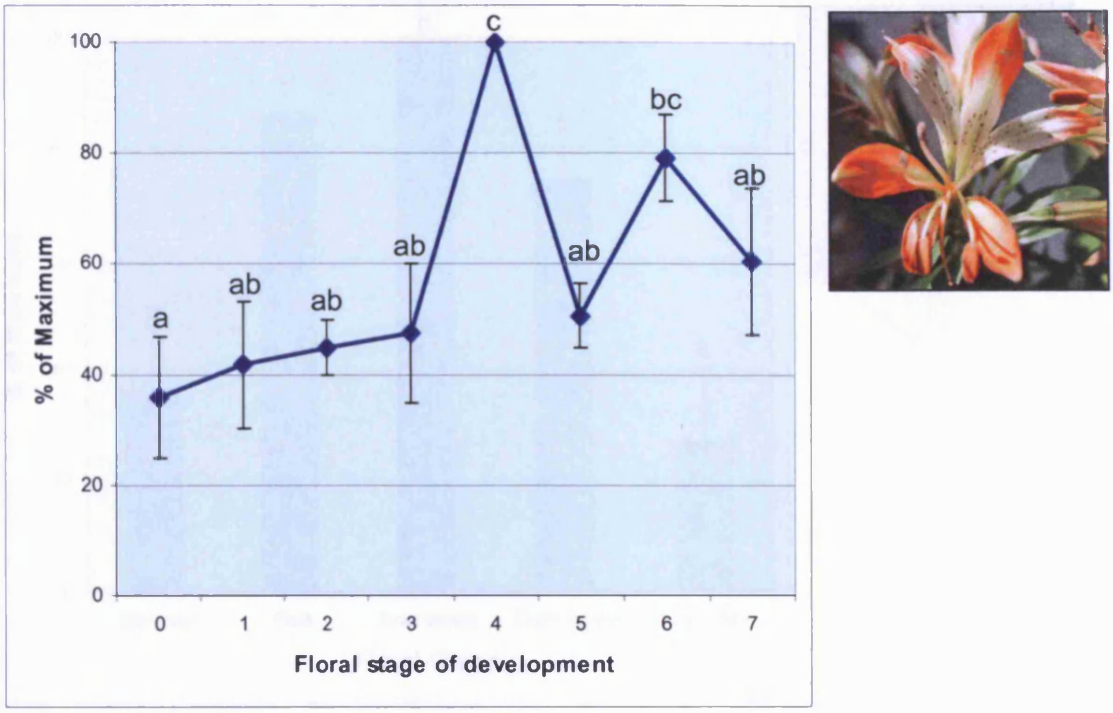


Figure 5.6. Means of *ALSTER* gene expression evaluated through semi qRT-PCR on 8 stages of development of *A. caryophyllaea* flowers (± SE, n=3). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.1.5. *Alstroemeria* cv. 'Sweet Laura' – Organs

From the evaluation of *ALSTER* expression in different organs of *A.* cv. 'Sweet Laura', the highest level of expression was found in inner tepals followed closely by pistil (~ 87 % of maximum) and then outer tepals (~ 74 % of maximum), with no statistically significant differences among them. Stamens and leaves were statistically similar showing the lowest expressions with 36 and 27 % of maximum respectively (Figure 5.7).

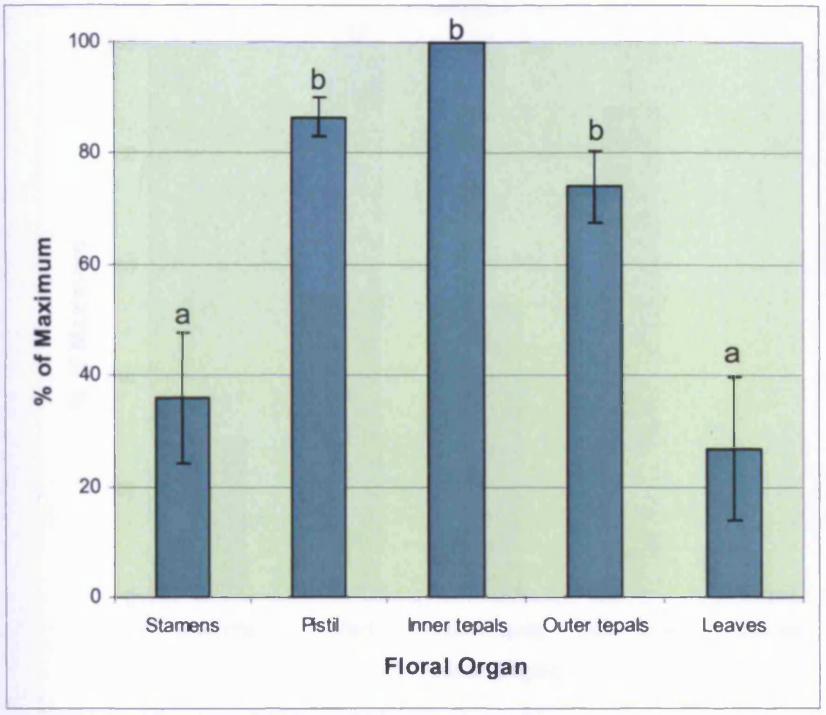


Figure 5.7. Means of *ALSTER* gene expression evaluated through semi qRT-PCR on 5 different organs of *A.* cv. 'Sweet Laura' flowers (± SE, n=3). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.1.6. *Alstroemeria caryophyllaea* – Organs

The evaluation of different organs of *A. caryophyllaea* showed pistil as having the highest and leaves as the lowest levels (~ 1 % of maximum) of *ALSTER* expression. A high expression was also found in outer tepals (89 % of maximum), statistically no different from pistil. Inner tepals showed about 54 % of maximum expression and were statistically different from all the other organs (Figure 5.8).

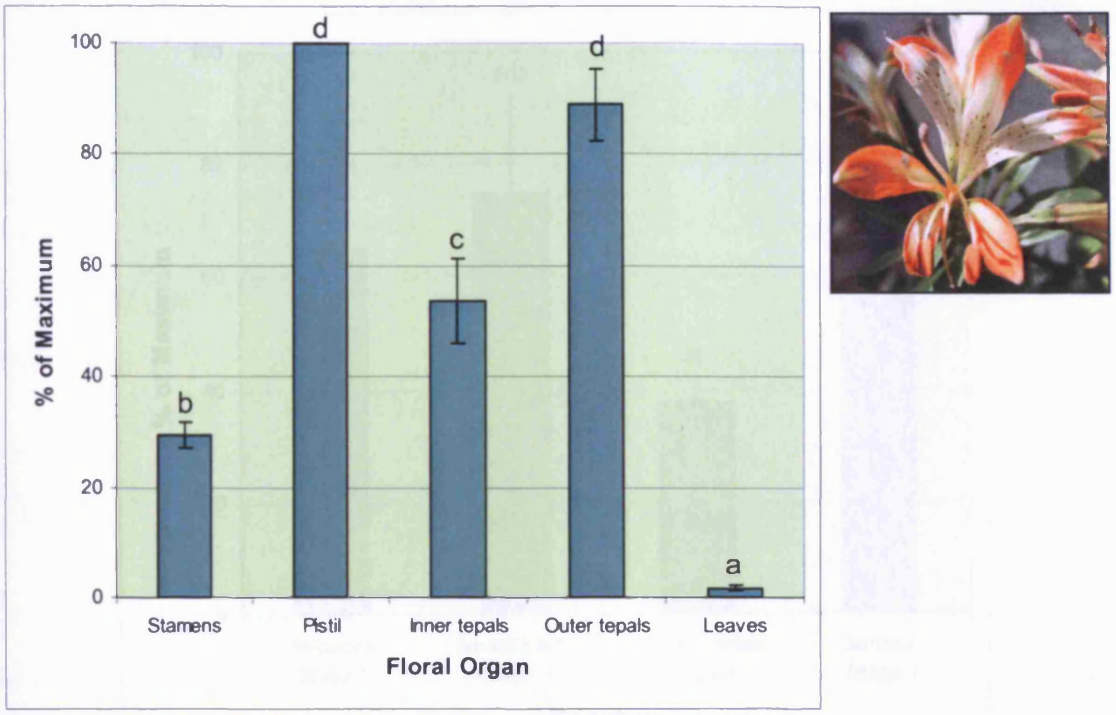


Figure 5.8. Means of *ALSTER* gene expression evaluated through semi qRT-PCR on 8 stages of development of *A. caryophyllaea* flowers (± SE, n=3). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.1.7. Comparison of the four genotypes

PCR was carried out using cDNAs from the stage of floral development in which maximal levels of *ALSTER* expression were observed in each genotype. The comparison was performed under the same PCR conditions and number of cycles. Expression was greatest in *A.* cv. 'Samora', although showing no statistical difference with *A.* cv. 'Rebecca' and 'Sweet Laura'. The lowest expression was found in *A. caryophyllaea*, which was statistically different from *A.* cv. 'Samora', but not from the others (Figure 5.9).

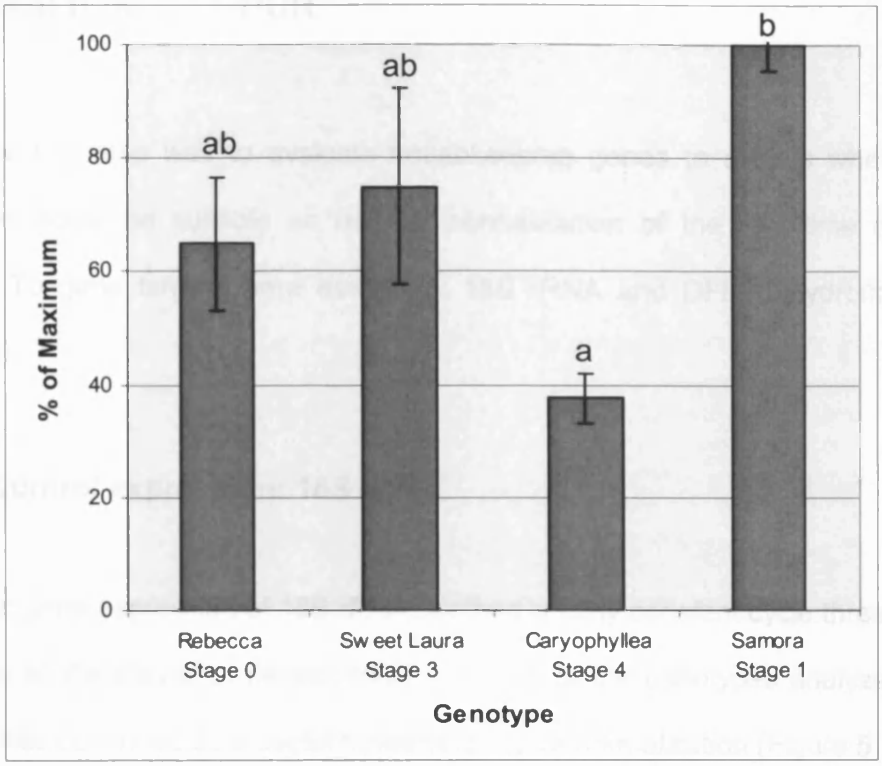


Figure 5.9. Means of *ALSTER* gene expression evaluated through semi qRT-PCR at the stage of maximum expression of 4 Alstroemeria genotype flowers (± SE, n=3). Results are expressed as % of maximum peak value and normalized against 18S rRNA.

5.3.2. Real time qRT-PCR

The first step was to evaluate housekeeping genes to assess whether their expression would be suitable as use for normalisation of the real time qPCR for *ALSTER*. To gene targets were assessed: 18S rRNA and DFR (dihydroflavonol 4-reductase).

5.3.2.1. Control expression: 18S rRNA

The gene expression of 18S rRNA reached a fairly constant cycle threshold (Ct) throughout all the stages of development, for each of the genotypes analyzed. Thus, this gene was confirmed as a useful housekeeping for normalization (Figure 5.10).

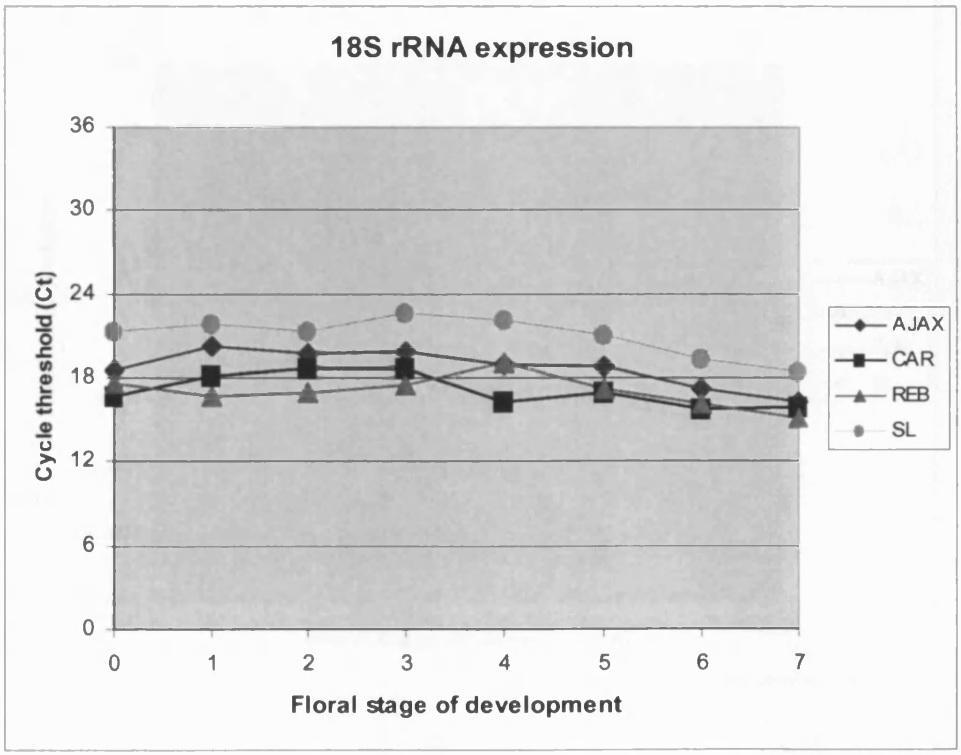


Figure 5.10. Cycle threshold of the 18S rRNA gene expression, found at each stage of development (0 to 7) for each genotype (*A.* cvs. 'Ajax', 'Sweet Laura', 'Rebecca' and *A. caryophyllaea* flowers) evaluated through Real time qPCR (± SE, n=12).

5.3.2.2. Control expression: DFR 142

Results obtained for DFR 142 expression ranged in *A.* cv. 'Ajax' from 4.1 (S 2) to 0.01 (S 7). Far below these levels of expression, *A.* cv. 'Sweet Laura' and *A. caryophyllaea* showed values in the order of 10^{-1} to 10^{-4}. The lowest expression was observed in *A.* cv. 'Rebecca' with values ranging in the order of 10^{-2} to 10^{-6}. Despite the differences in terms of quantity, the expression of this gene was always higher at early stages (S0 - S2 in *A.* cv. 'Rebecca' and S0 - S3 in the rest of the genotypes). After that, a constant decrease in the expression was observed in all the genotypes throughout development, reaching the lowest levels during senescence at stage 5 in *A.* cv. 'Rebecca' and stage 7 in the other genotypes (Figure 5.11).

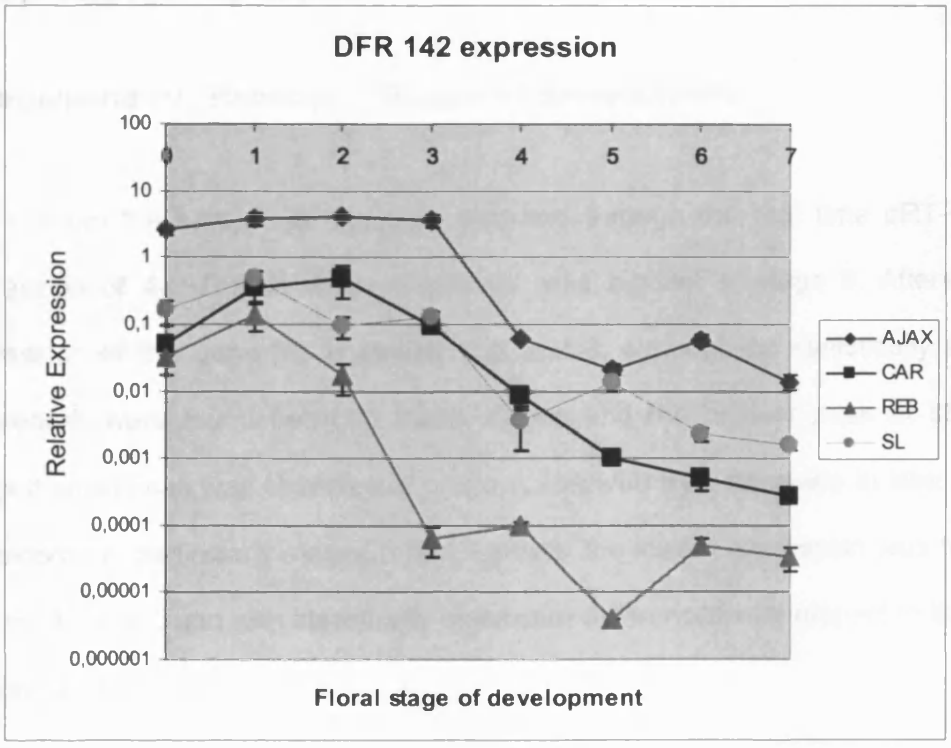


Figure 5.11. Means of DFR142 gene expression evaluated through real time qPCR on 8 stages of development of *A.* cvs. 'Ajax', 'Sweet Laura', 'Rebecca' and *A. caryophyllaea* flowers (± SE, n=12). Results are expressed as relative units, given by the ratio (transcript level of gene / transcript level of 18S rRNA). Scale has been adjusted to a logarithmic scale.

Thus, the expression of the dihydroflavonol 4-reductase (DFR 142) followed an expected pattern, as this gene is involved in anthocyanin accumulation (Helariutta *et al.*, 1993) the higher levels of expression were observed at early stages of development, when the flowers are just opening and still developing and gaining colour.

Furthermore, this anthocyanin-related gene showed the highest expression in *A.* cv. 'Ajax', which is characterized by its deep red tepals, while the mostly white *A.* cv. 'Rebecca' showed the lowest levels of expression. Hence this gene worked as a positive control, confirming the results concerning *ALSTER* expression.

5.3.2.3. *ALSTER* expression

Alstroemeria cv. 'Rebecca' – Stages of development

From the analysis of the data obtained through the real time qRT-PCR, the expression of *ALSTER* in *A.* cv. 'Rebecca' was highest at stage 0. Afterwards the expression of this gene fell in stages 1, 2 and 3, although no statistically significant differences were found between these stages and the highest peak at stage 0. A second small peak was observed at stage 4, followed by a decrease in later stages of development, particularly stages 6 and 7 where the lowest expression was found with values close to 0 and with statistically significant differences with respect to the stage 0 (Figure 5.12).

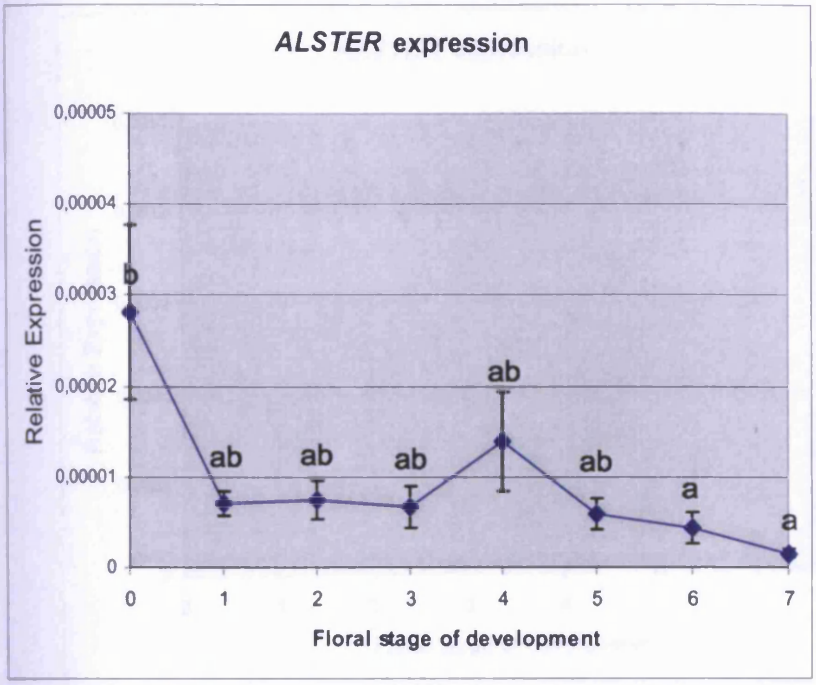


Figure 5.12. Means of *ALSTER* gene expression evaluated through real time qPCR on 8 stages of development of *A.* cv. 'Rebecca' flowers (± SE, n=12). Results are expressed as relative units, given by the ratio (transcript level of gene / transcript level of 18S rRNA).

Alstroemeria cv. 'Ajax' – Stages of development

The expression of *ALSTER* observed in cv. 'Ajax', was highest at stage 0, with statistically significant differences with respect to the rest of the stages. Then *ALSTER* expression fell and remained stable through floral stages 1, 2 and 3, with no significant differences among them. The expression fell again after anthesis, with values in the order of 10^{-6} to 10^{-5} relative expression at stages 4, 5, 6 and 7, which were statistically different to the rest of the stages but not to each other (Figure 5.13).

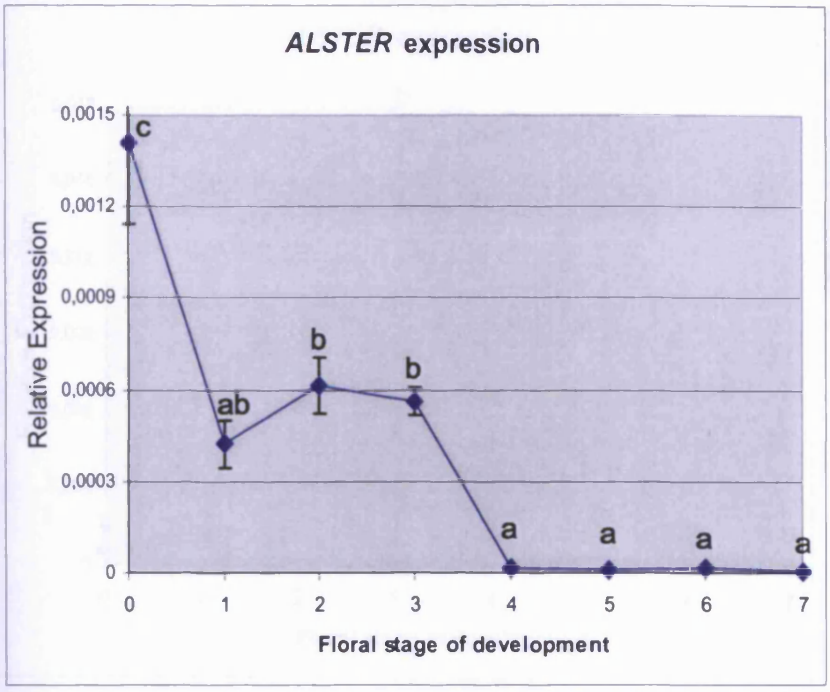


Figure 5.13. Means of *ALSTER* gene expression evaluated through real time qPCR on 8 stages of development of *A.* cv. 'Ajax' flowers (± SE, n=12). Results are expressed as relative units, given by the ratio (transcript level of gene / transcript level of 18S rRNA).

Alstroemeria cv. 'Sweet Laura' - Stages of development

Expression of *ALSTER* in *Alstroemeria* cv. 'Sweet Laura' showed a strong peak at stage 3, coinciding with the starting of the maturation of floral reproductive organs, showing a value close to 0.015 which was statistically significantly different with respect all the rest of the floral stages. The rest of the floral stages including those both before and after stage 3, showed values in the order of 10^{-4} to 10^{-3} with no statistically significant differences among them (Figure 5.14).

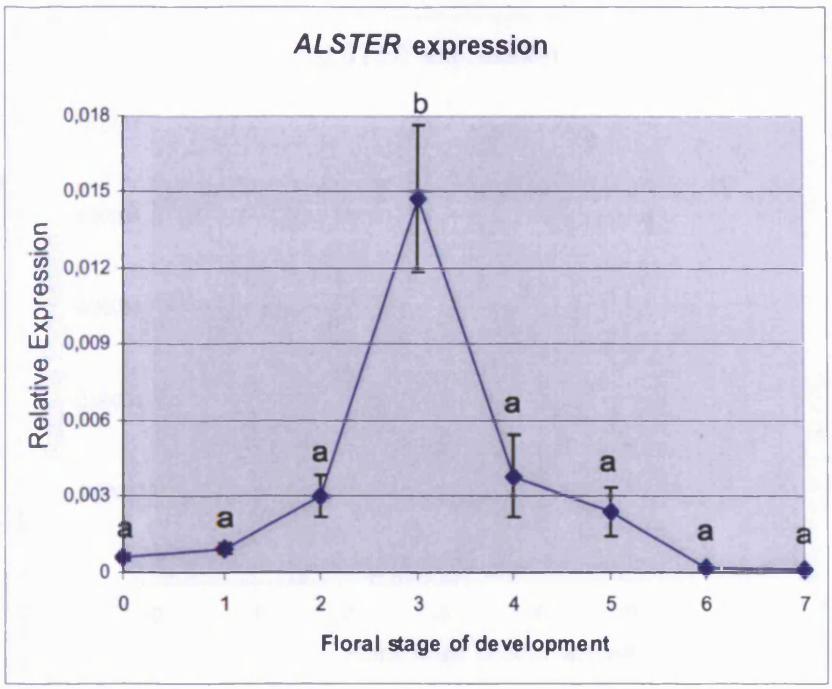


Figure 5.14. Means of *ALSTER* gene expression evaluated through real time qPCR on 8 stages of development of *A.* cv. 'Sweet Laura' flowers (± SE, n=12). Results are expressed as relative units, given by the ratio (transcript level of gene / transcript level of 18S rRNA).

Alstroemeria caryophyllaea - Stages of development

No statistically significant differences were found in the expression of *ALSTER* throughout the development of *Alstroemeria caryophyllaea* flowers. Thus all the stages of development showed relatively constant values in the order of 10^{-5}, with very small peaks at stages 2 and 7 (Figure 5.15).

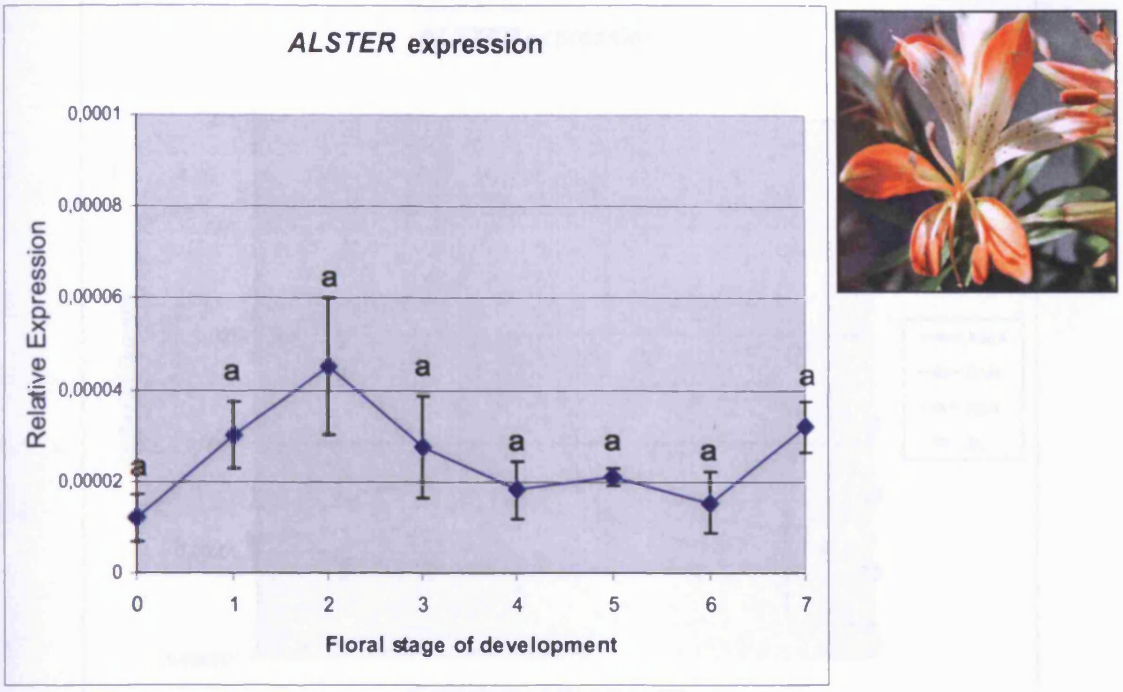


Figure 5.15. Means of *ALSTER* gene expression evaluated through real time qPCR on 8 stages of development of *A. caryophyllaea* flowers (± SE, n=12). Results are expressed as relative units, given by the ratio (transcript level of gene / transcript level of 18S rRNA).

Comparison of the four genotypes

In order to compare the expression of *ALSTER* throughout development in the four genotypes analyzed, a single graph was plotted including a logarithmic scale. *A.* cv. 'Sweet Laura' showed the highest expression throughout floral development while the lowest expression was observed in the non scented *A.* cv. 'Rebecca'. Values observed were up to 10^4 times higher in *A.* cv. 'Sweet Laura' (values around 10^{-2}) compared to those found in *A.* cv. 'Rebecca' (values around 10^{-6}). On the other hand *A.* cv. 'Ajax' and *A. caryophyllaea* were in an intermediate position in this analysis in terms of quantity, with values in the order of 10^{-5} to 10^{-3} (Figure 5.16).

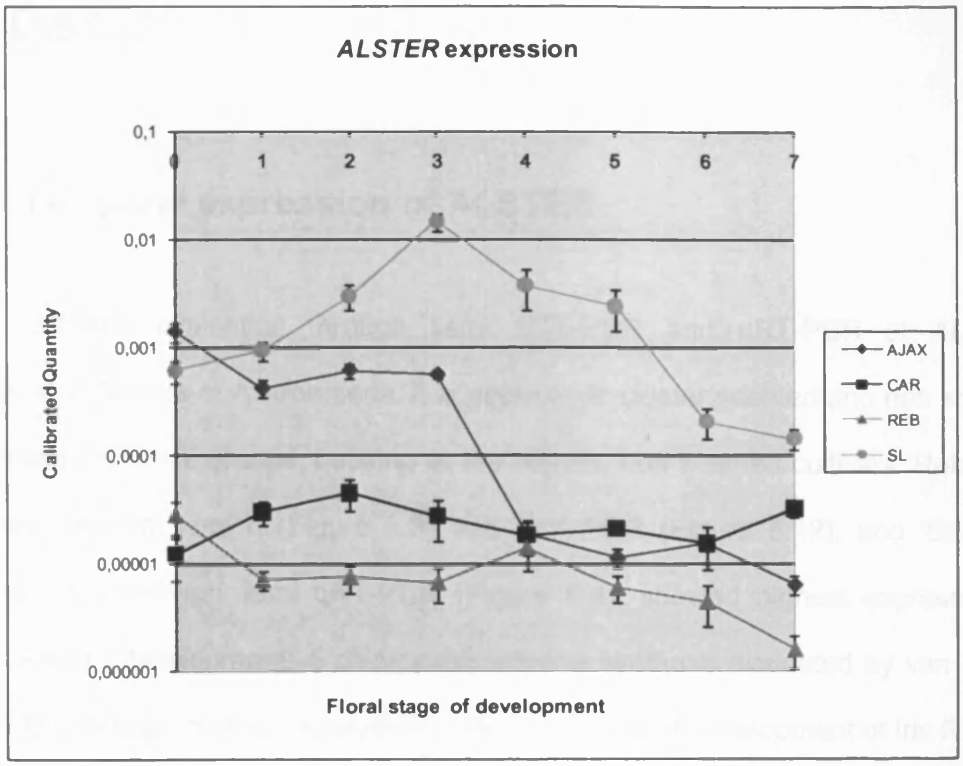


Figure 5.16. Means of *ALSTER* gene expression evaluated through real time qPCR on 8 stages of development of *A.* cvs. 'Ajax', 'Sweet Laura', 'Rebecca' and *A. caryophyllaea* flowers. Results are expressed as relative units, given by the ratio (transcript level of gene / transcript level of 18S rRNA). Scale has been adjusted to a logarithmic scale.

5.4. DISCUSSION

5.4.1. Temporal expression of ALSTER

Following evaluation through semi qRT-PCR and qRT-PCR of *ALSTER* expression in flowers of Alstroemeria, it is possible to cluster scented and non scented genotypes in different groups. Looking at the results, non scented cultivars 'Rebecca', evaluated through semi q (Figure 5.3) and qRT-PCR (Figure 5.12), and 'Samora', evaluated only through semi qRT-PCR (Figure 5.4), showed highest expression in early stages of development. A chloroplast terpene synthase evaluated by van Doorn *et al.* (2003) through northern analysis in different stages of development of Iris flowers, also showed highest expression of this gene in early stages of development. Since Iris has non scented flowers, the early expression of this chloroplast terpene synthase could be associated to other roles not necessarily linked with biosynthesis of volatile compounds. Many other physiological, metabolic, and structural functions have been described for terpenoids for example as photosynthetic pigments (phytol, carotenoids), hormones (gibberellins, abscisic acid), electron carriers (ubiquinone, plastoquinone), mediators of polysaccharide assembly (polyprenyl phosphates), and structural components of membranes (phytosterols) (McGarvey and Croteau, 1995).

On the other hand, the high levels of *ALSTER* expression found at around stages 2 and 4 in the scented genotypes through semi q (Figures 5.5, 5.6) and qRT-PCR (Figures 5.13, 5.14 and 5.15), coincides with high scent emission perceived and also with the maturation of reproductive organs. This fits with previous reports suggesting attraction of pollinators as the main function of scent emission in flowers (Ando *et al.*, 2001; Reinhard *et al.*, 2004; Dudareva *et al.*, 2004; Jürgens *et al.*, 2003) and considering that wild species of Alstroemeria are naturally cross pollinated by

Hymenopteran insects (Aizen and Basilio, 1998; Cavieres *et al.*, 1998; Botto-Mahan and Ojeda-Camacho, 2000), the expression pattern of *ALSTER* in scented Alstroemerias with a peak during anthesis would be consistent with a role in scent emission. Previous studies performed through Real time qRT-PCR have found in flowers of Petunia a high correlation between anthesis, the stage with the highest output of volatile compounds, and an up regulation of floral scent-related genes (Dexter *et al.*, 2007). Nagegowda *et al.* (2008) evaluated two TPS through qRT-PCR and semi qRT-PCR, obtaining also high expression of these genes during anthesis of snapdragon flowers. Moreover scented genotypes, particularly *A. caryophyllaea*, showed more protandry than other Alstroemerias studied (principally non scented cultivars); with the stigma still growing and not ready to be pollinated yet whilst anthers started to dry out and senesce, making cross pollination and attraction of pollinators even more necessary since self pollination is almost impossible.

Considering now the quantitative analysis of *ALSTER* performed in all the genotypes studied, no correlation between high expression of *ALSTER* and the scent character was found comparing the results obtained through semi qRT-PCR, since the non scented *A.* cv. 'Samora' reached the maximum expression while the scented *A. caryophyllaea* the lowest (Figure 5.9). On the other hand, real time qRT-PCR results showed higher levels of expression fairly well-correlated to scented flowers and *vice versa*, excepting the scented *A. caryophyllaea* which was similar to the non scented *A.* cv. 'Rebecca' (Figure 5.16).

Finally, excepting *A. caryophyllaea*, all the genotypes analyzed through semi q and qRT-PCR showed low levels of expression at stage 7, which is characterized by complete senescence of the flowers showing wilting of tepals and cell death (Wagstaff *et al*, 2001). Thus it is expected to find low expression of *ALSTER* during senescence due to the reduction of some metabolic processes at this stage.

5.4.2. Spatial expression of *ALSTER*

The evaluation of *ALSTER* expression in different organs of scented *A.* cv 'Sweet Laura' and *A. caryophyllaea* showed some differences. The data for leaves in *A. caryophyllaea* may be overestimated as in some replicates there was no band visible by gel electrophoresis for this organ and the value detected is probably only due to the software sensitivity to band intensity. Therefore *ALSTER* is essentially flower-specific for *A. caryophyllaea*, but not for *A.* 'Sweet Laura' in which expression of the gene was also observed in leaves, though very low expression was found in this organ.

In *A. caryophyllaea*, the specificity to flowers and the peak of expression in pistils support the association between *ALSTER* expression, reproductive organ maturation, and emission of scent. In both *A. caryophyllaea* and *A.* cv. 'Sweet Laura', pistils had high expression levels, however for 'Sweet Laura', the highest expression was observed in inner tepals. Investigations performed in other flowers (Tholl *et al.*, 2004) reported upper and lower lobes of the petals of snapdragon as almost the exclusive source of emission of volatiles and biosynthesis of a geranyl diphosphate synthase. Moreover, Pichersky *et al.* (1994) observed that two-thirds of the floral emission of linalool in Clarkia *sp.* comes from petals. However, another experiment performed by D'Auria *et al.* (2002) on *Clarkia breweri* evaluated the expression of an enzyme which catalyzes the formation of benzylbenzoate (BEBT) and pistil showed the highest expression, supporting the hypothesis in this investigation that expression of *ALSTER* could be further related to scent production.

The remarkably high expression found in both *A. caryophyllaea* and *A.* cv. 'Sweet Laura' in tepals (modified petals) tissues, is in agreement with Dudareva *et al.* (2000), who also found highest level of expression of scented related enzymes in this

tissue. Furthermore, these authors suggest that upper and lower lobes of the petals come into contact with the bee's body during the pollination enabling the bee to accumulate floral scent molecules on its body surface. This floral scent is then carried to the nest and might help the bee to recruit new foragers to locate the flowers.

5.4.3. Real Time qRT-PCR v/s Semi qRT-PCR

A comparative analysis between the methodologies used on this study to asses the expression of *ALSTER* has been carried out. All the data obtained through the Real time qRT-PCR have been expressed as percentage of maximum, in order to compare them with data obtained through the semi qRT-PCR (Figure 5.17).

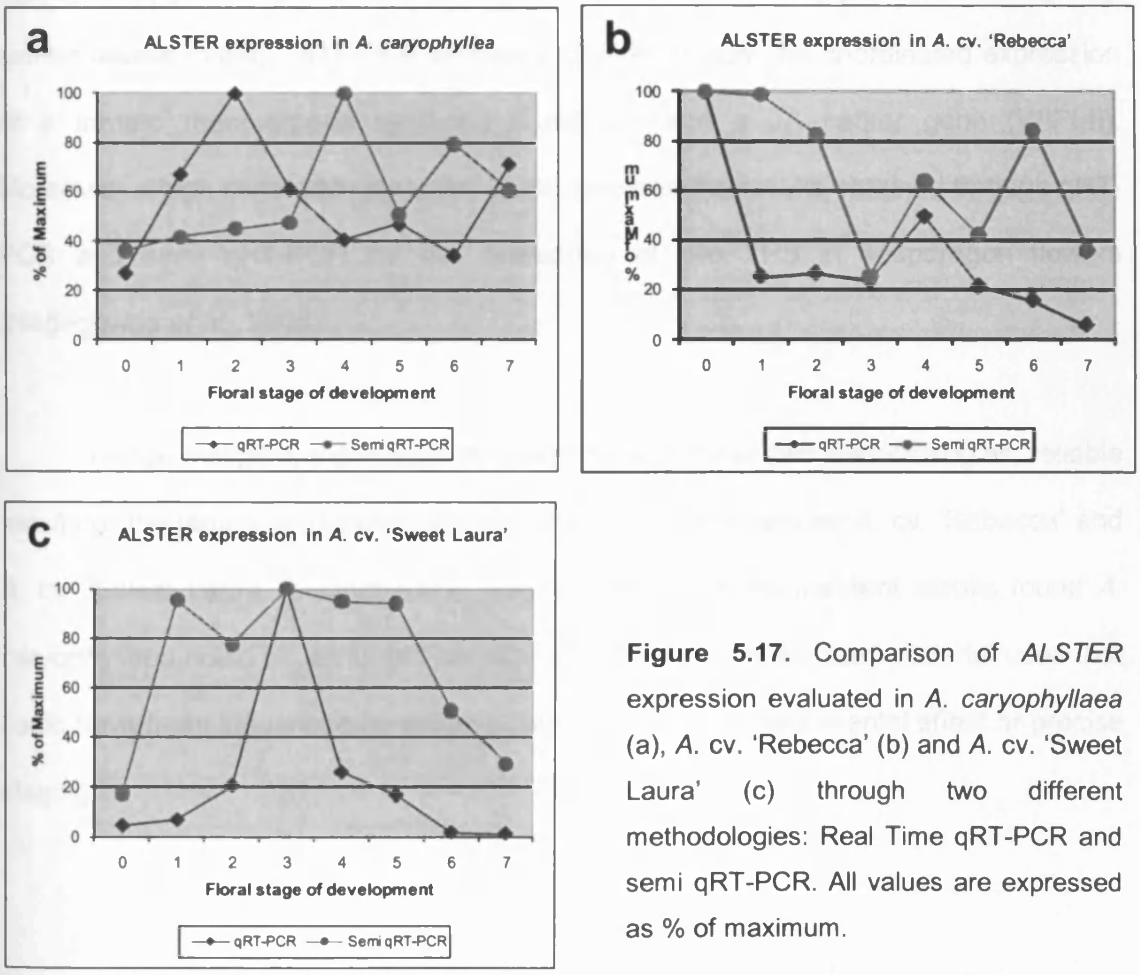


Figure 5.17. Comparison of *ALSTER* expression evaluated in *A. caryophyllaea* (a), *A.* cv. 'Rebecca' (b) and *A.* cv. 'Sweet Laura' (c) through two different methodologies: Real Time qRT-PCR and semi qRT-PCR. All values are expressed as % of maximum.

An very similar *ALSTER* expression pattern throughout floral development was found using both methodologies in *A.* cv. 'Rebecca', with a maximum expression at stage 0 and then a small second peak at stage 4. The expression of *ALSTER* in *A.* cv. 'Sweet Laura' appeared fairly similar by the two methodologies, with the highest expression observed at stage 3. However, while the up regulation detected by qRT-PCR reached a single peak, when using semi qRT-PCR the up regulation seems to be maintained from stage 2 until stage 5. More important differences were found in *A. caryophyllaea* where, although a similar behaviour of expression was observed with only one peak, this peak was detected two stages later through the semi qRT-PCR (S4), compared to the qRT-PCR (S2).

A study aimed at evaluating whether a linalool synthase is induced in tomato trichomes by jasmonic acid was carried out by van Schie *et al.* (2007), obtaining very similar results through qRT-PCR and semi qRT-PCR from the coordinated expression of a tomato monoterpene synthase (LeMTS1) and a JA-marker gene (WIPI-II). Moreover, a high correlation was also found between the results obtained through qRT-PCR and semi qRT-PCR for the evaluation of two TPS in snapdragon flowers (Nagegowda *et al.*, 2008).

Hence matching the results obtained through these two methodologies, reliable results of the temporal expression of *ALSTER* were obtained for *A.* cv. 'Rebecca' and *A.* cv. 'Sweet Laura', but not for *A. caryophyllaea* The inconsistent results found *A. caryophyllaea* could be attributed perhaps to differences in the plant material used that could have been influenced by external factors such as environmental effect or precise staging.

Thus this comparison based only on qualitative parameters showed many similarities between the two methodologies performed for the evaluation of *ALSTER* expression. Thus, considering the easier manipulation and lower cost involved in the semi qRT-PCR, it could be still useful as a first approach for gene expression studies. Nevertheless, is clear that a more accurate analysis, with more reliable qualitative and quantitative data is obtained through the Real time qRT-PCR.

CHAPTER VI.

FUNCTIONAL ANALYSIS OF A PUTATIVE ALSTROEMERIA TERPENE SYNTHASE (*ALSTER*)

VI. FUNCTIONAL ANALYSIS OF A PUTATIVE ALSTROEMERIA TERPENE SYNTHASE (*ALSTER*)

6.1. INTRODUCTION

Considering the terpenoid compounds as a large group of natural products, the number and diversity of enzymes involved in their synthesis is also immense. During the formation of terpenoid compounds, the primary mechanism starts when C_5 units of IDP and DMAPP are catalyzed by prenyltransferases (Koyama and Ogura, 1999; Liang *et al.*, 2002) to generate geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP), which are the main precursors of monoterpenes, sesquiterpenes and diterpenes respectively. These precursors are then modified through carbocationic reactions which are controlled by this large number of enzymes known as terpene synthases (TPS) (Trapp and Croteau, 2001).

Several monoterpene synthases have been identified with molecular weights (MWs) ranging from 60 to 70 kDa. For instance, a limonene synthase found in *Citrus unshiu* has a MW of 70 kDa (Shimada *et al.*, 2004), while a monoterpene synthase found in Arabidopsis producing 1,8-cineole has a MW of 60 kDa (Chen *et al.*, 2004). A myrcene synthase (MyrS) described by Fischbach *et al.* (2001) in *Quercus ilex,* has a fairly similar MW of 64.3 kDa.

The activity of TPS has been evaluated through enzymatic assays and many of them have been already identified and functionally characterized in many species. Thus monoterpene synthases producing myrcene (Bohlmann *et al.*, 1997; Fischbach *et al.*, 2001), limonene (Shimada *et al.*, 2004), pinene (Bohlmann *et al.*, 1997; Lu *et al.*, 2002) and sesquiterpene synthases producing aristochelene (Facchini and Chappell, 1992), valencene (Sharon-Asa *et al.*, 2003; Lucker *et al.*, 2004) and germacrene (Bennett *et al.*, 2002; Prosser *et al.*, 2004) among others, have been functionally characterized through the use of recombinant proteins and their activity with specific precursors (GPP and FPP).

The production of recombinant proteins is a useful technique to determine gene-product function and it has been successfully utilized in plants. Furthermore, with the increasing number of genes with unknown functions identified through random and genome sequencing projects, this technique has become even more important (Frommer and Ninnemann, 1995). A recombinant protein production process consists basically of gene cloning, expression and protein purification (Yokoyama, 2003). Thus, the target gene is first amplified by PCR and the products are subcloned into an expression vector which can be yeast or bacteria. The advantages of using bacteria (especially *Escherichia coli*) over yeast as the expression system are the availability of well known molecular tools and defined mutants, high growth rates and high yield of overproduced protein (Frommer and Ninnemann, 1995).

Perhaps one of the crucial steps during the production and further expression of recombinant proteins is the extraction and purification of these proteins. Different levels of purification are required for different purposes and a wide range of techniques have been described, most of them using chromatographic methods based on size exclusion, charge or hydrophobicity, ion exchange, among others (Scopes, 1994;

Amersham, 1999). However, partially purified proteins have demonstrated to be also suitable for functional analyses (Shimada *et al.*, 2004).

In the work described in this chapter, the consensus cDNA sequence of the Alstroemeria TPS previously described in the chapter IV, was used to obtain a full length cDNA clone encoding the pseudo mature protein (*ALSTER*). This was expressed in bacteria in order to investigate its enzymatic activity, which is another step towards elucidating the *in vivo* function of *ALSTER*.

6.2. METHODOLOGY

6.2.1. *ALSTER* amplification

The final primers were designed to PCR amplify the pseudo-mature ORF of ALSTER and included also the restriction sites *Bam*HI and *Sal*I to facilitate cloning, and some additional nucleotides in order to optimize the Tm and to ensure sufficient space for the restriction enzyme to bind to its target sequences (Figure 6.1).

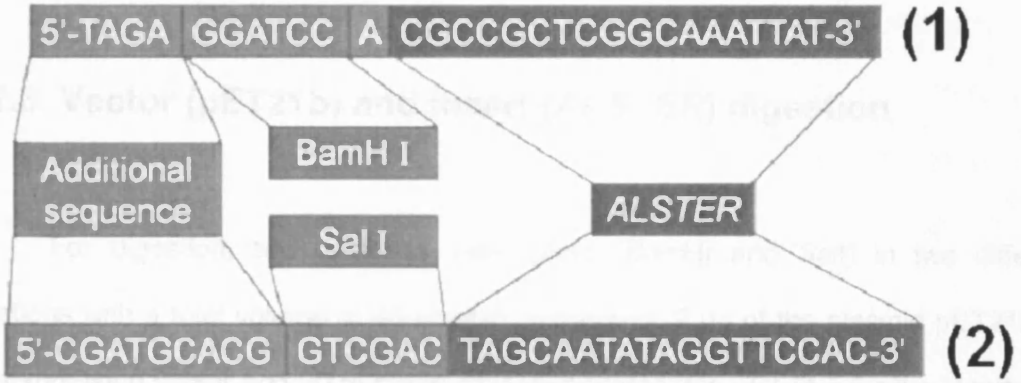


Figure 6.1. Primers designed for the amplification and further expression of *ALSTER* including the restriction sites (*Bam*HI and *Sal*I) and the additional nucleotides.
(1) and (2) stands for forward and reverse primers.

A PCR was performed using the protocol described before (Chapter II, section 2.2.4) and the fragments were purified using a QIAquick Gel Extraction Kit. Purified DNA was ligated into pGEM-T easy vector (Promega, Madison, WI, USA) as previously described (Chapter II, section 2.2.8). Afterwards the plasmid was sequenced using M13 forward (5'-GTAAAACGACGGCCAGT-3') and reverse (5'-AACAGCTATGACCATG-3') primers in order to check whether the insert was correctly amplified.

6.2.2. Vector (pET21b) preparation

Plasmid pET21b, used as a vector, was grown in DH5α *E. coli* in 10 ml tubes containing 5 ml of liquid LB medium with antibiotic (Ampicillin 100 $\mu g \cdot ml^{-1}$). Tubes were incubated over night at 37 ºC shaking at 100 rpm. After incubation the DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen). Purified DNA (3 µl) was analyzed on an ethidium bromide stained agarose gel (1.5 %) and DNA concentration (ng μl^{-1}) of the samples was quantified using a spectrophotometer (GeneQuant, Pharmacia).

6.2.3. Vector (pET21b) and insert (*ALSTER*) digestion

For digestion, two enzymes were used (*Bam*HI and *Sal*I) in two different reactions with a total volume of 40 µl each containing: 2 µg of the plasmid pET21b for the expression vector and 30 µl of the *ALSTER* ligated into pGEM-T for the insert; 4 µl of 10X buffer E (Promega, Madison, WI, USA) for *Bam*HI and buffer D (Promega, Madison, WI, USA) for SalI; 0.4 µl of BSA and 1 µl of each enzyme. The reactions were incubated for 90 min at 37 °C. Afterwards a PCR clean up was performed using QIAquick PCR Purification Kit, in order to remove the remaining buffer and enzyme.

A second digestion using 30 µl of each digested plasmid and insert was performed following the same protocol just described but swapping the reactions with the enzymes, that is the plasmid or insert previously digested with *Bam*HI was now digested with *Sal*I and *vice versa*. The reactions were incubated for 90 min at 37 °C.

The digested product was run on a gel to confirm that the insert was actually released from the pGEM-T and thus avoid a religation with itself. The band was

excised from the gel and purified using QIAquick Gel Extraction Kit as previously described in chapter II, section 2.2.7.

6.2.4. Phosphatase treatment for the vector

In order to minimize self-ligated vector in the transformation, a phosphatase treatment was performed on the digested plasmid. This should also improve the percentage of colonies with inserts after the transformation. A 100 µl reaction was set up containing the digested plasmid DNA, 10 µl of 10X CIAP buffer (Calf Intestinal Alkaline Phosphatase) and 1 µl of CIAP enzyme (1:100 diluted). The reaction was incubated for 30 min at 37 °C, afterwards an additional 1 µl of CIAP enzyme (1:100 diluted) was added and the reaction was finally incubated for other 30 min at 37 °C. The CIAP was stopped and removed by purification of the plasmid through QIAquick clean up columns.

6.2.5. Ligation

An overnight 15 µl ligation was set up at 4 °C using the purified digested insert and the phosphatased digested vector. Control ligations without enzyme (ligase) and insert were also included (Table 6.1).

Table 6.1. Ligations performed using buffer (10X Rapid ligation buffer, Promega, Madison, WI, USA), enzyme (Ligase, Promega, Madison, WI, USA), Vector (Plasmid pET21b) and the insert *ALSTER*.

	Ligation 1	Ligation 2	Ligation 3
Buffer (10X)	1.5 µl	1.5 µl	1.5 µl
Enzyme ($3u \cdot \mu l^{-1}$)	1.5 µl	-	1.5 µl
Vector ($100\ ng \cdot \mu l^{-1}$)	4.0 µl	4.0 µl	4.0 µl
Insert	8.0 µl	-	-
H_2O	-	9.5 µl	8.0 µl

6.2.6. Transformation of *E. coli* BL 21 competent cells

The ligations were transformed as previously described (Chapter II, section 2.2.9), into *E. coli* BL 21 competent cells, recommended for protein expression because of their T7-lac operator promoter which is inducible by isopropil-β-D-tio-galactósido (IPTG). Colonies were checked by PCR as described in Chapter II, section 2.2.10, and those that were positive were used for protein and enzymatic assays.

6.2.7. Protein extraction and purification

One of the single colonies obtained from the previous transformation was inoculated in 100 ml LB medium with antibiotic (Ampicillin 100 $\mu g \cdot ml^{-1}$). The culture was incubated overnight at 37 °C at 150 rpm. After the incubation, 20 ml from the overnight culture were used to inoculate 2 l of fresh LB medium with antibiotic (Ampicillin 100 $\mu g \cdot ml^{-1}$). The total volume was divided between 4 flask containing 500 ml each of fresh

LB and 5 ml of overnight culture, and the cultures were again incubated at 37 °C at 150 rpm until reaching an OD_{600} of 0.6 – 0.8.

Protein expression was induced by adding IPTG to a final concentration of 1 mM and cultures were incubated for an additional 4 hours. After the induction, the cultures were centrifuged for 10 min at 4,654 x g using a microcentrifuge (Minispin plus, Eppendorf) and the pellets were stored at -80 °C. In order to check the induction, samples of 1 ml each were collected from the cultures before and after induction and they were visualized on an SDS-PAGE 12 % gel, as described in Chapter II, section 2.2.12.

Pellets containing induced proteins were thawed on ice and then resuspended in 100 ml of cell lysis buffer (20 mM Tris, 5 mM EDTA, 5 mM β-mercaptoethanol, pH=8.0). Samples were vortexed and then sonicated (Bursts of 5 s sonication at 10 s intervals) on ice for 3 min to dissolve the pellets. Dissolved pellets in lyisis buffer were centrifuged at 4 °C for 30 min at 20,130 x g using an Avanti J-E centrifuge (Beckman Coulter). After checking that the protein was expressed as insoluble inclusion bodies, the supernatant was discarded and the pellet was resuspended in 50 mL of lysis buffer and dissolved by stirring at 4 °C for 30 min. The solution containing the completely dissolved pellets was denatured by raising the pH up to 12 with drops of 5M NaOH. The solution was again stirred at 4 °C for 30 min and then the pH was now adjusted to 8.0, where undesirable proteins and other impurities tend to precipitate. β-mercaptoethanol was added to a final concentration of 5 mM. After 30 min of stirring at 4°C, the solution was centrifuged (4 °C, 40,400 x g, 30 min) and the supernatant was dialysed overnight in 3 l of dialysis buffer (10 mM Tris-HCl, 5 mM β-mercaptoethanol, pH 7.0). After reduction of the volume the protein solution was tested for terpene synthase activity.

For the detection of the protein, a 12 % SDS-PAGE gel (as described in Chapter II, section 2.2.13) was cast and samples from different steps of the process of protein extraction and purification were loaded including: pellet and supernatant of the pellet, solution after pH adjustments ('raw material') and after dialysis.

6.2.8. Enzymatic assay

The extracted and purified recombinant protein obtained was used to set up an enzymatic reaction containing the recombinant protein *ALSTER*, incubation buffer (25 mM HEPES, 15 mM $MgCl_2$ and 5 mM DTT, pH 7.5) and GPP (2mM), natural substrate for the production of monoterpenes. FPP (2mM), main precursor of sesquiterpenes, was also tested in the reaction in order to check whether *ALSTER* is actually a monoterpene synthase and if so, its specificity when reacting with other precursors. Two negative controls for each substrate were also performed using (1) only buffer and substrate and (2) another non specific protein (BSA) (Table 6.2).

A layer of 1 ml of pentane was added to the top of each reaction in order to trap all the organic compounds resulting. The reaction was incubated for 24 h at 25 °C in completely sealed 1 ml-bottles. The olefin products were extracted with pentane (3 x 1 ml) and then passed through a short pad of silica gel (~500 mg). The resulting solution was ready to use for GC-MS analysis.

Table 6.2. Enzymatic reactions performed aimed at the expression of *ALSTER* as a monoterpene synthase. Negative control 1 included another enzyme (BSA) instead of *ALSTER* and negative control 2 included only incubation buffer and precursors (GPP and FPP).

	Incubation buffer	Enzyme	GPP (2 mM)	FPP (2 mM)	BSA (10 µg/ml)
For monoterpene production	400 µl	100 µl	15 µl	-	-
For sesquiterpene production	400 µl	100 µl	-	20 µl	-
Negative control 1	400 µl	-	15 µl -	- 20 µl	25 µl
Negative control 2	400 µl	-	15 µl -	- 20 µl	-

6.2.9. GC-MS analysis

The products obtained from the enzymatic reaction were analyzed with a GC-MS consisting of a Hewlett Packard 6890 GC fitted with a J&W scientific DB-5MS column (30 m x 0.25 mm internal diameter) and a Micromass GCT Premiere detecting in the range m/z 50-800 in EI+ mode with scanning once a second with a scan time of 0.9 sec. Injections were performed in split mode (split ratio 5:1) using the following temperature programme: Starting with oven temperature at 50 °C, increasing temperature at a rate of 4 °C · min^{-1} up to 250 °C, and then at 20 °C · min^{-1} for 5 min (250 °C final temperature).

Fresh flowers of *A.* cv. 'Sweet Laura' were also analyzed through GC-MS in order to compare the data obtained with the incubation of *ALSTER* + GPP. Moreover, water from the bottom of the jars containing the flowers and cut pedicels of the flowers (Figure 6.2) were analyzed through GC-MS, to check for any contamination in the

chromatograms using flowers. All the analyses were performed using the same protocol described previously in the Chapter III, section 3.2.

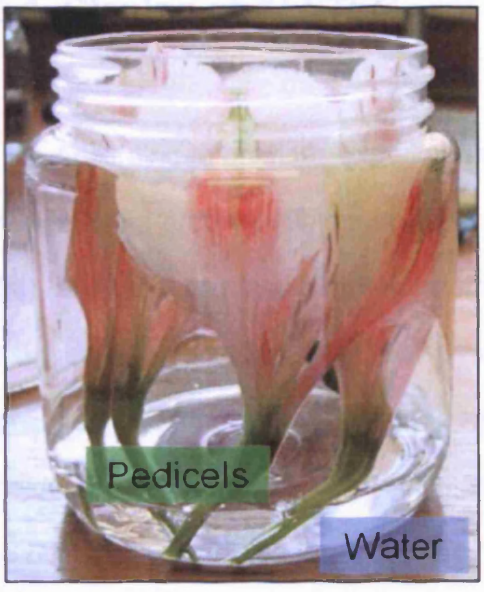


Figure 6.2. Illustration of the fresh flowers analyzed through GC-MS, including the description of the negative controls: 'pedicels' and 'water'.

Synthetic standards for 3-carene (Fluka), ocimene (Fluka) and myrcene (Aldrich) were also analyzed through GC-MS as positive controls and, together with the retention time, to confirm the identification of the peaks produced from the enzymatic reactions and the flowers. Finally, a sample containing only pentane was also used as solvent control.

6.3. RESULTS

6.3.1. Cloning of *ALSTER*

According to previous experiments using TPS sequences for further expression of recombinant proteins (Shimada *et al.*, 2004; Shimada *et al.*, 2005; Lee and Chapell, 2008), the pseudo mature protein starts with the R(R)X_8W motif. Furthermore, the conserved N-terminal of the R(R)X_8W motif was shown not to be required for functional expression and removing this sequence the activity of isolated enzymes improved considerably (Williams *et al.*, 1998). Hence an ORF of 1619 bp corresponding to *ALSTER* was cloned into the expression vector pET21b for the further production of a recombinant protein utilized in the functional analysis. The ORF was fully sequenced (Figure 6.3) and the predicted amino acid sequence was 98% homologous to the fragments obtained by RACE in Chapter IV (Fig 6.4).

```
      cgccgctcggcaaattatacgccgaccgtctggaacaacaactatttgcaaacattagagagcgaattcattgga
        R  R  S  A  N  Y  T  P  T  V  W  N  N  N  Y  L  Q  T  L  E  S  E  F  I  G
atggaatgtgctgccaggctcgagaagctgaaatccgaggcaaaaagccttatcgccgggaccacctccttggtagagaag
 M  E  C  A  A  R  L  E  K  L  K  S  E  A  K  S  L  I  A  G  T  T  S  L  V  E  K
ctggagctggtggacacgctgcgacagctcgggttggcgtatcactttgaggaggagatcatggatgtcctggccgccatc
 L  E  L  V  D  T  L  R  Q  L  G  L  A  Y  H  F  E  E  E  I  M  D  V  L  A  A  I
ttacaatcagcggatttggactcggttgcacgacaactagatggcctgcatgctactgccctactattcaggctgctaagg
 L  Q  S  A  D  L  D  S  V  A  R  Q  L  D  G  L  H  A  T  A  L  L  F  R  L  L  R
gagcatggttttgaaatctcacaagacatattcaggtggttccatgacgaaacaacaggtgggttcaaagcttgcattacc
 E  H  G  F  E  I  S  Q  D  I  F  R  W  F  H  D  E  T  T  G  G  F  K  A  C  I  T
cgtgacattaagggattattgagcttttacgaagcttcctatgttgccatcgaagaggagaacataatggatgatgcaaga
 R  D  I  K  G  L  L  S  F  Y  E  A  S  Y  V  A  I  E  E  E  N  I  M  D  D  A  R
gagttcacaaccaaacatctcaaagatttcatcgagaattctacagaaccatggctacgtgagcgagccttacatgcttta
 E  F  T  T  K  H  L  K  D  F  I  E  N  S  T  E  P  W  L  R  E  R  A  L  H  A  L
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 E  L  P  L  N  W  R  F  Q  R  L  H  S  R  W  F  I  D  M  Y  E  R  G  T  D  T  N
ctttgtctacttgaactcgcaaagctagacttcaacattgtccaaggcgtatacaagacggaaatcagacaactctcaaag
 L  C  L  L  E  L  A  K  L  D  F  N  I  V  Q  G  V  Y  K  T  E  I  R  Q  L  S  K
tggtgggccgatctcgaccttattggagataagttgagctttgctagagacaggttgctagagtgctacttgtgggcggca
 W  W  A  D  L  D  L  I  G  D  K  L  S  F  A  R  D  R  L  L  E  C  Y  L  W  A  A
ggtgggtcgcctgagcccgaatcctggagatgcagacaagtatttaccaagtgcatttgcttagcaacaatcattgatgac
 G  G  S  P  E  P  E  S  W  R  C  R  Q  V  F  T  K  C  I  C  L  A  T  I  I  D  D
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 I  Y  D  V  Y  G  T  L  E  E  L  E  L  F  T  K  A  V  E  R  W  E  V  S  A  I  E
cagcttccagattacatgaagatatgtgttctagcactcttcaacacgtttaatgagattgcctacaaaaccttaaaagaa
 Q  L  P  D  Y  M  K  I  C  V  L  A  L  F  N  T  F  N  E  I  A  Y  K  T  L  K  E
aaggggttggatatcattccattcttaagaaaagcatggtcggatctttgcaatgcatatttggtggaagcaaagtggtat
 K  G  L  D  I  I  P  F  L  R  K  A  W  S  D  L  C  N  A  Y  L  V  E  A  K  W  Y
tacaagggtcattcaccgccattcggggagtaccttgagaatgcagggatttcgataggggaacatctaatactcactctt
 Y  K  G  H  S  P  P  F  G  E  Y  L  E  N  A  G  I  S  I  G  E  H  L  I  L  T  L
gctttctttgttaatgactatgtatctgtagaatctgtagaacgcttcaaagcatatcaaagtttgatgtgttggtcgggt
 A  F  F  V  N  D  Y  V  S  V  E  S  V  E  R  F  K  A  Y  Q  S  L  M  C  W  S  G
```

```
attatagttcgactttatgacgacttggcaacttcagaggcagaaggagagagaggtgatgtttccaaagccatccaatgc
 I  I  V  R  L  Y  D  D  L  A  T  S  E  A  E  G  E  R  G  D  V  S  K  A  I  Q  C
tacatgcatgaaacgggtgcttcagaggtgatggctagcatgtttataagagacatgatagctgataaatggaaaatattg
 Y  M  H  E  T  G  A  S  E  V  M  A  S  M  F  I  R  D  M  I  A  D  K  W  K  I  L
aacaaagaccgcaaggctagtaccaaacatgagaagtacttcaaaagtgttgccataaactccgtacaaacagcacaatgg
 N  K  D  R  K  A  S  T  K  H  E  K  Y  F  K  S  V  A  I  N  S  V  Q  T  A  Q  W
tcctatcaacatggagacggatttggagaacctcaacatagaacaaaggacacaatattggcattgttggtggaacctata
S  Y  Q  H  G  D  G  F  G  E  P  Q  H  R  T  K  D  T  I  L  A  L  L  V  E  P  I
ttgcta
 L  L
```

Figure 6.3. Nucleotide sequence, and its amino acid deduced sequence, obtained from the sequencing of the full length ORF of *ALSTER*. Highlighted are the conserved motifs R(R)X_8W and DDXXD.

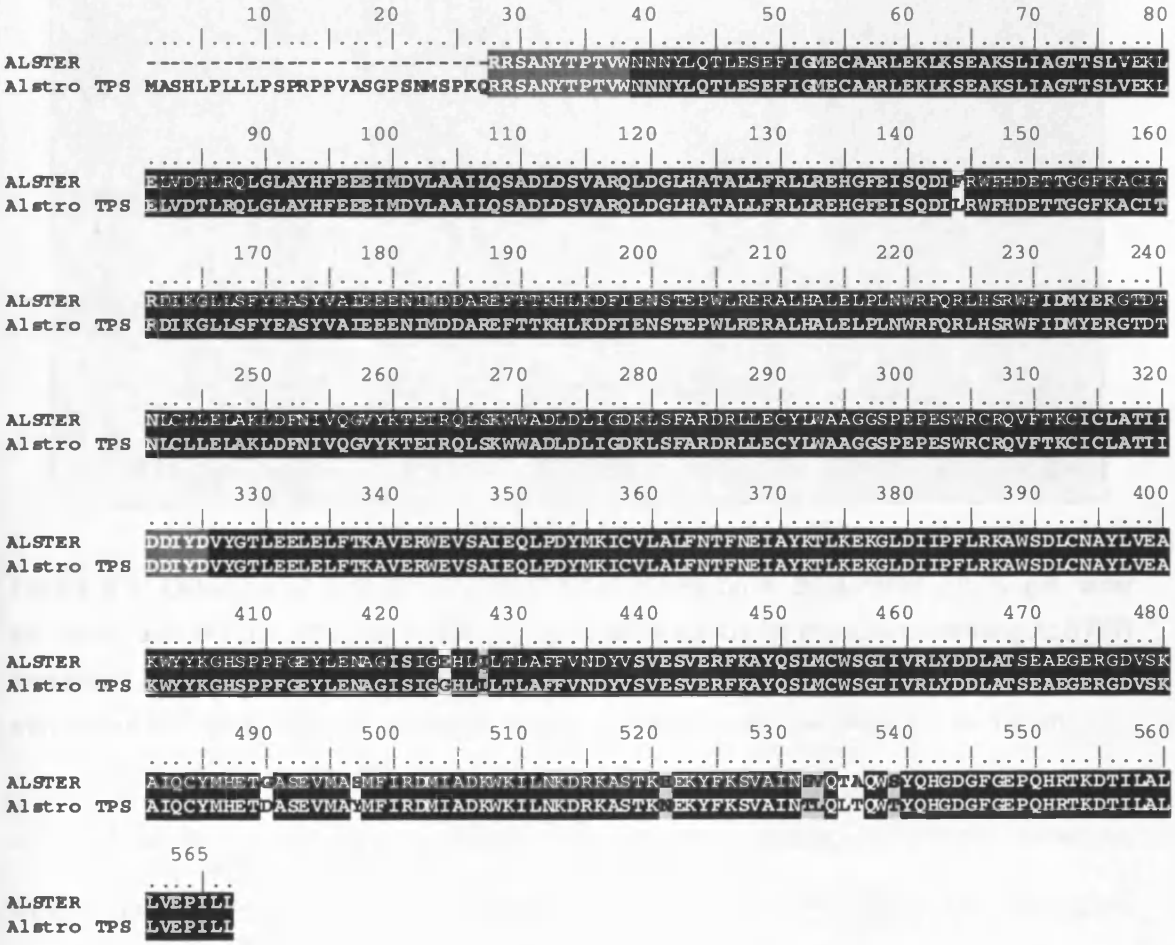

Figure 6.4. Alignment of the amino acid deduced sequences of the full length ORF of *ALSTER* (*ALSTER)* and the consensus of the Alstroemeria TPS obtained from the RACE products (Alstroemeria TPS). Highlighted are the conserved motifs R(R)X_8W and DDXXD.

6.3.2. Protein extraction and purification

After the induction of the cultures of *E. coli* BL 21 competent cells containing *ALSTER*, the SDS-PAGE (12%) gel performed showed two induced bands at around 63 and 50 kDa (Figure 6.5).

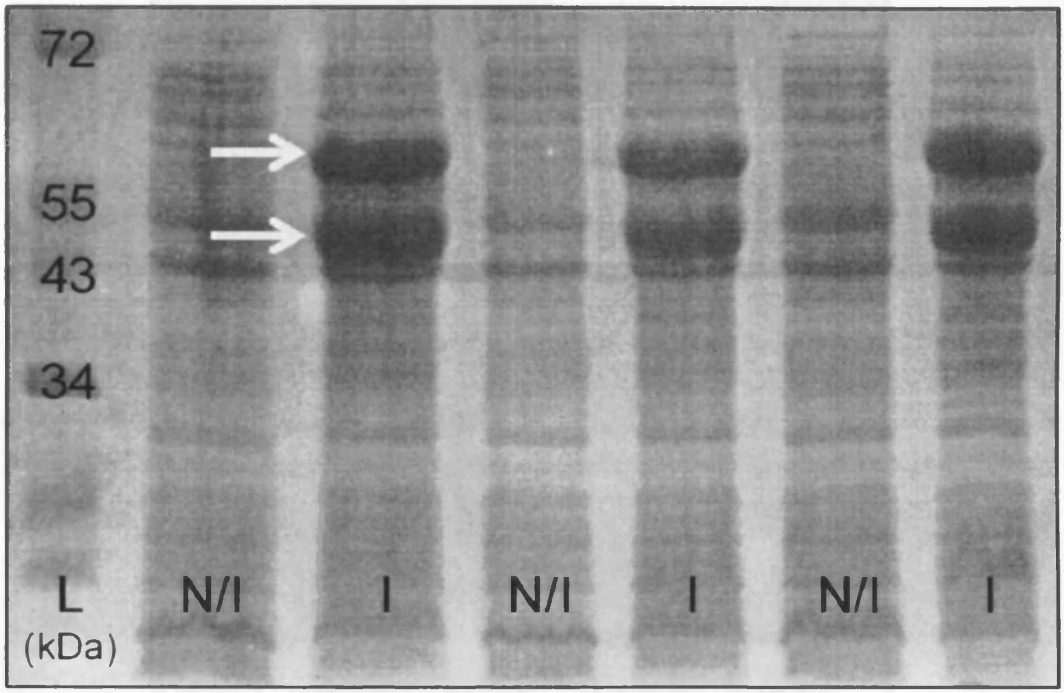

Figure 6.5. Detection of induced (I) proteins (*ALSTER*) on a SDS-PAGE 12 % gel, after incubation with IPTG 1 mM. Non induced (N/I) proteins stands for medium containing *ALSTER* expressed in *E.coli* BL 21, collected before the induction. The two induced bands are shown with arrows and their molecular weight (MW) was estimated using the ladder (L) on the left.

According to softwares available on line for the prediction of protein molecular weight (http://www.bioinformatics.org/sms/prot_mw.html and http://expasy.org/tools/), using the deduced amino acid sequence found in section 6.3.1., *ALSTER* weighs 65.3 kDa. Monoterpene synthases previously identified have shown a similar MW (Shimada *et al.*, 2004; Chen *et al.*, 2004, Fischbach *et al.*, 2001), as described in the introduction, therefore the band detected at around 63 kDa was identified as the target recombinant protein induced.

Although a complete purification of the protein was not achieved, a fairly clean and concentrated band was observed after the dialysis. Further enzymatic analysis demonstrated that this level of purification was enough to perform a functional analysis using this recombinant protein (Figure 6.6).

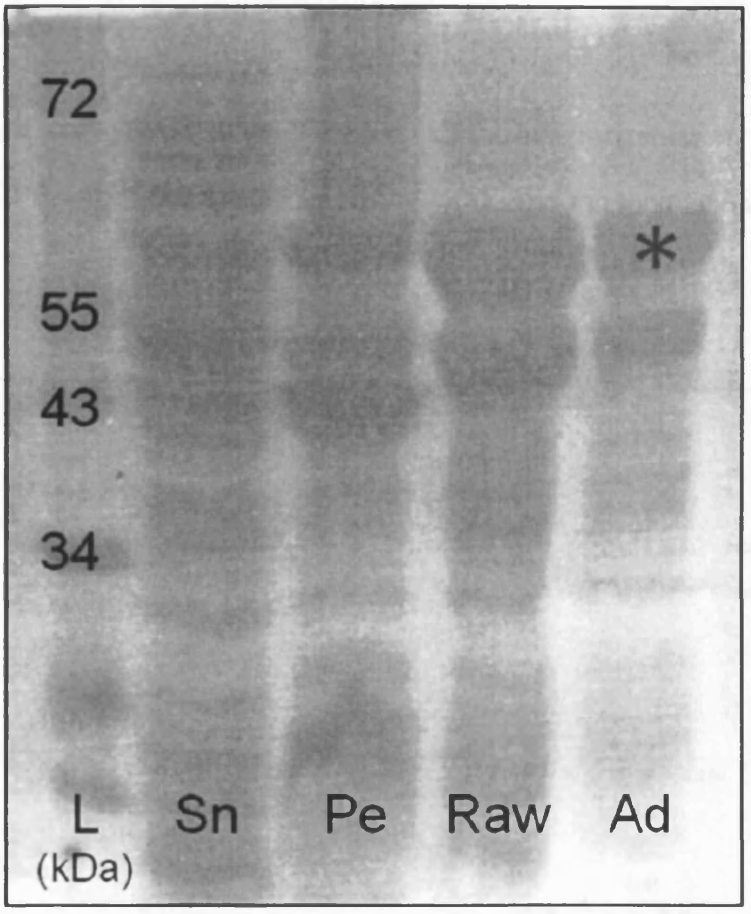

Figure 6.6. SDS-PAGE 12 % gel showing the presence and purity of the recombinant protein (*ALSTER*) at different steps during the extraction and purification process, including supernatant (Sn), pellet (Pe), raw material (Raw) and after dialysis (Ad).
(*) stands for the band corresponding to the putative monoterpene synthase.

6.3.3. Enzymatic analysis: GC-MS results

From all the experiments performed, only fresh flowers and incubation of *ALSTER* with GPP resulted in clear peaks in the chromatograms during the analysis by

GC-MS. Incubation of *ALSTER* with FPP did not show any clear peak, so no sesquiterpene compounds were detected and all the peaks observed were identified as monoterpenes.

6.3.3.1. Negative controls

No major compounds were detected above expeted noise in samples from incubation of GPP with a non specific protein (BSA), and the incubation of GPP alone (Figure 6.7).

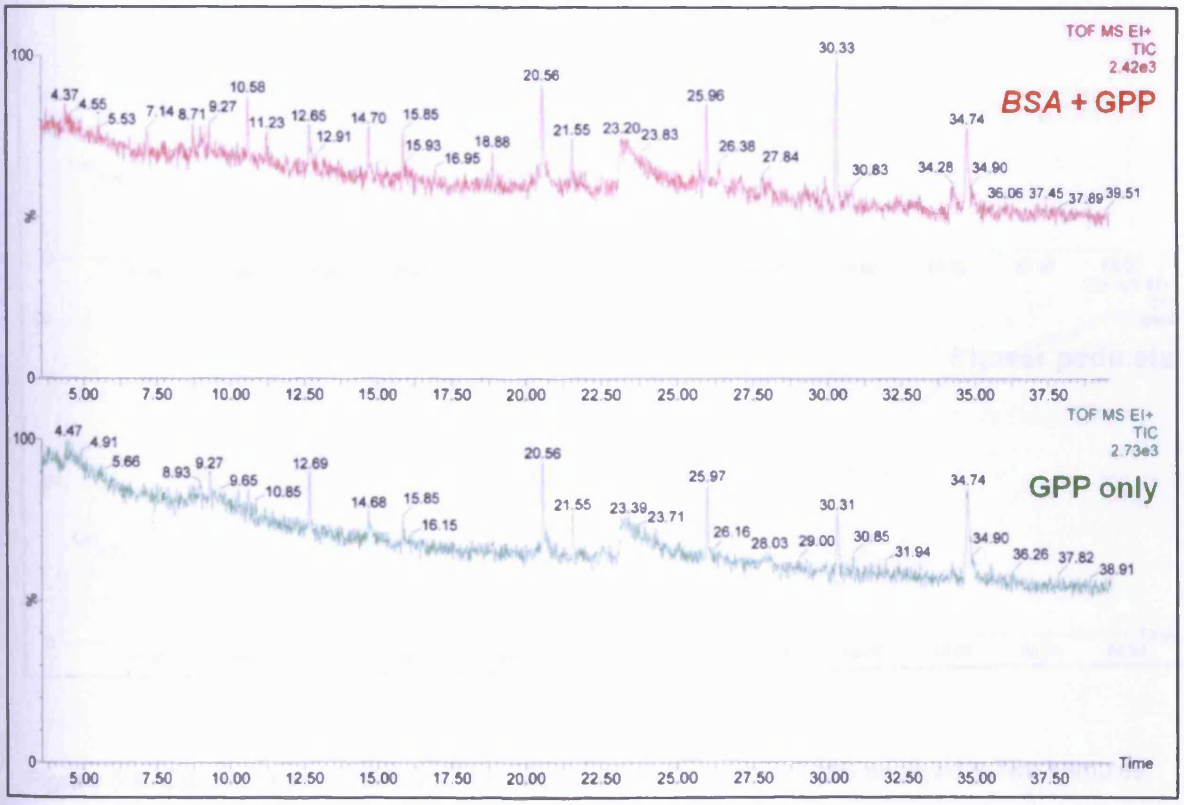

Figure 6.7. Chromatogram obtained from the analysis through GC-MS assayed in two reactions used as negative controls, including the product obtained after the incubation of BSA with GPP and the product of the incubation of GPP only, both reactions with incubation buffer.

The negative controls used for the analysis of the fresh flowers also no major peaks in the chromatograms from the GC-MS analysis of either water or pedicels (Figure 6.8).

These results confirm that the products obtained after the incubation of *in vitro* expressed *ALSTER* with GPP and from the flowers are genuine and contamination as the source of the peaks can be eliminated.

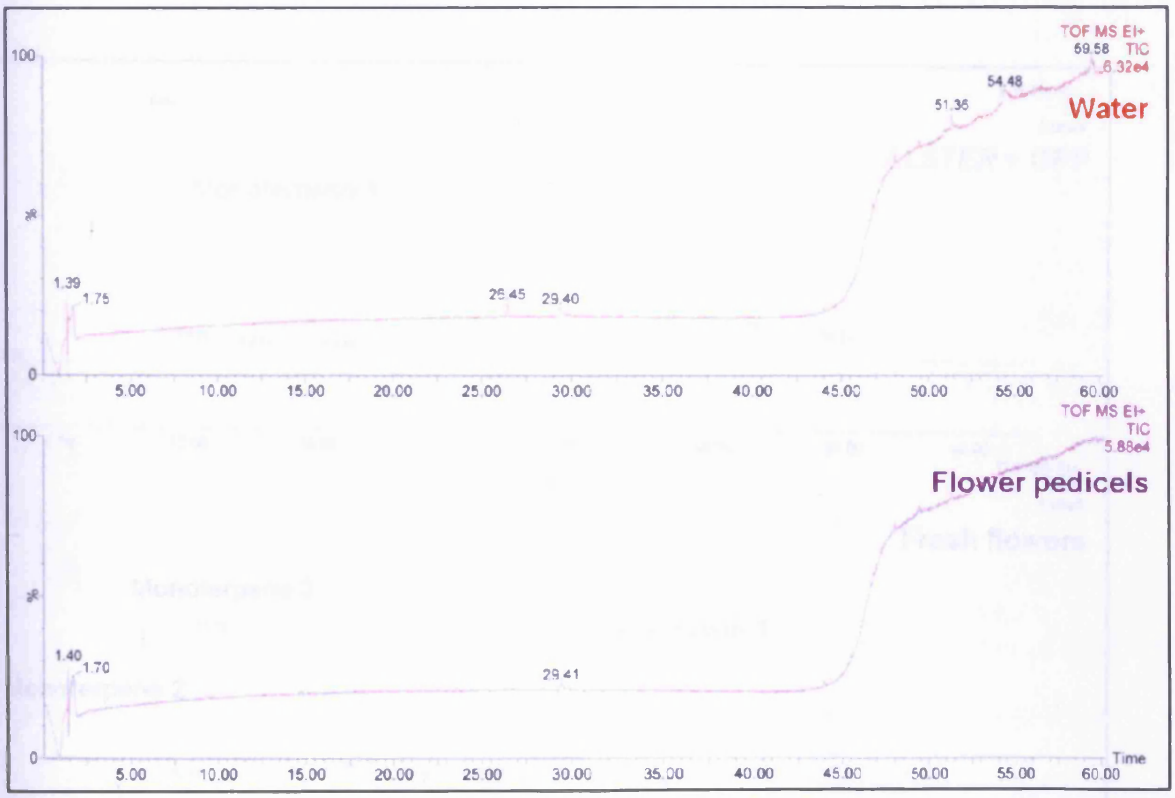

Figure 6.8. Chromatogram obtained from the analysis through GC-MS assayed in two samples (water and flower pedicels) used as negative controls for the analysis of fresh flowers.

6.3.3.2. GC-analysis of products from *in vitro* expressed *ALSTER* incubated with GPP as a substrate v/s fresh flowers

Different peaks were observed in the chromatograms obtained after the analysis of *ALSTER* + GPP and fresh flowers of Alstroemeria. Whilst a single product (Monoterpene 1) was obtained from the *ALSTER* enzyme following incubation with GPP, several peaks were observed in the chromatogram of fresh flowers, from which two of them (Monoterpene 2 and 3) were analyzed because of their similarity and possible correspondence with Monoterpene 1 (Figure 6.9).

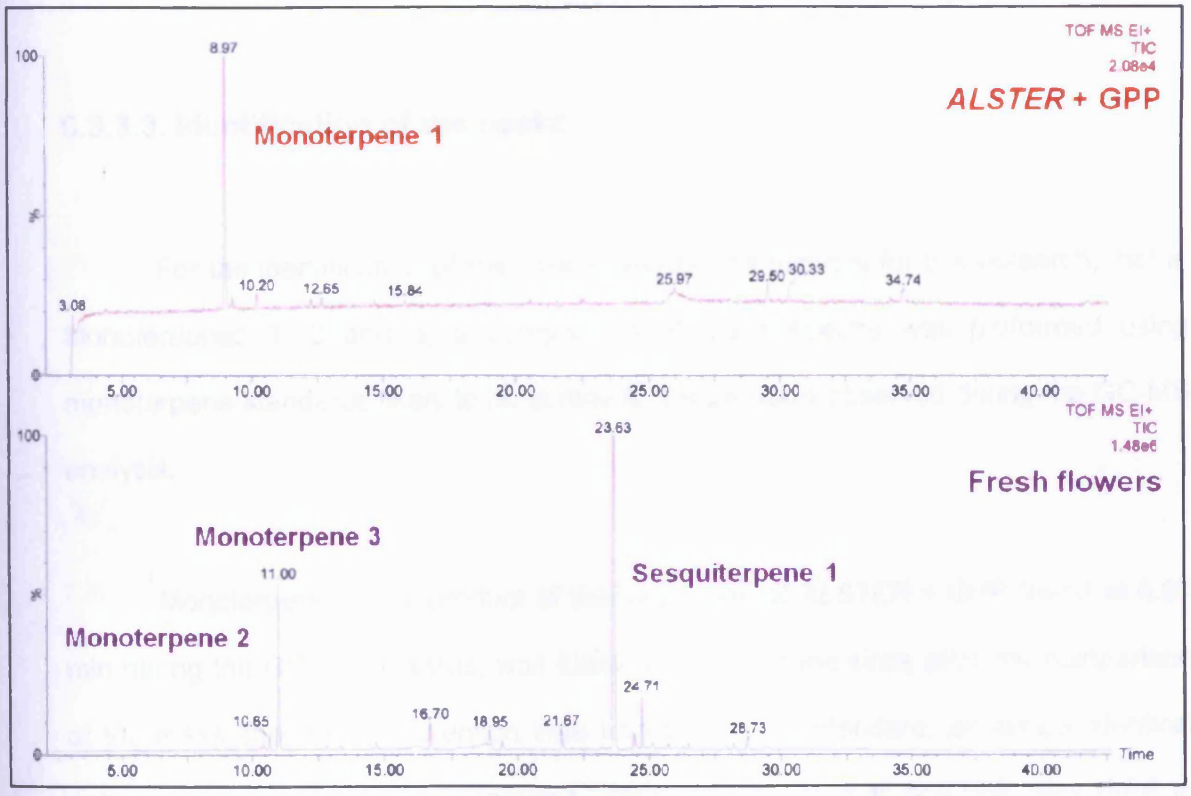

Figure 6.9. Chromatogram obtained from the analysis through GC-MS of products obtained from the incubation of *ALSTER* with GPP, and in fresh flowers of *Alstroemeria* cv. Sweet Laura'. Monoterpene (1 and 2) and Sesquiterpene (1) stand for peaks analyzed afterwards through comparison with standards.

The highest peak observed in the chromatograms of the flowers was a sesquiterpene identified as caryophyllene (Sesquiterpene 1 on Fig. 6.9), coinciding with the analysis previously performed in the Chapter III. However this peak was not considered for further analysis in this chapter, as *ALSTER* showed activity only with GPP and not with FPP and, thus, this enzyme was acting as a monoterpene synthase. Further analysis of the mass spectra observed was therefore focused on the identification of the monoterpene product obtained (Monoterpene 1 on Fig. 6.9) and its correspondence with the products obtained from the fresh flowers (Monoterpene 2 and 3, on Fig. 6.9).

6.3.3.3. Identification of the peaks

For the identification of the peaks selected as relevant for this research, that is Monoterpenes 1, 2 and 3, a comparison of mass spectra was performed using monoterpene standards likely to be similar to the products observed during the GC-MS analysis.

Monoterpene 1, the product of the incubation of *ALSTER* + GPP found at 8.97 min during the GC-MS analysis, was identified as myrcene since after the comparison of the mass spectra and retention time with that of the standard, an almost identical pattern was observed in both samples. Thus considering that *ALSTER* uses GPP to produce myrcene, this enzyme can be considered now as a myrcene synthase (Figure 6.10).

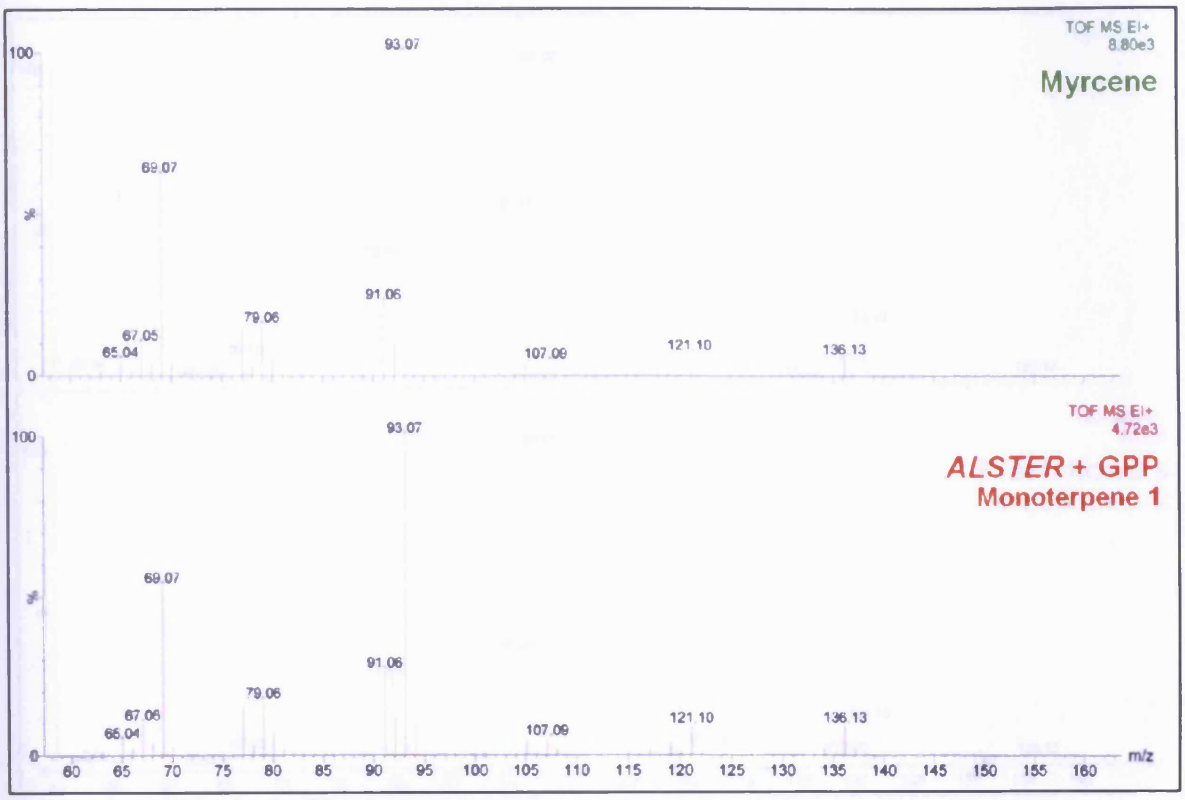

Figure 6.10. Mass spectra obtained through GC-MS of the standard myrcene and the peak 'Monoterpene 1' observed in the chromatogram of the reaction *ALSTER* + GPP (Figure 6.9).

The mass spectrum of the peak observed at 10.65 min (Monoterpene 2) in the chromatogram of fresh flowers was compared with the mass spectrum of ocimene, finding an almost identical pattern and very similar retention time. Hence ocimene is one of the monoterpenes emitted by fresh flowers of *A.* cv 'Sweet Laura' (Figure 6.11).

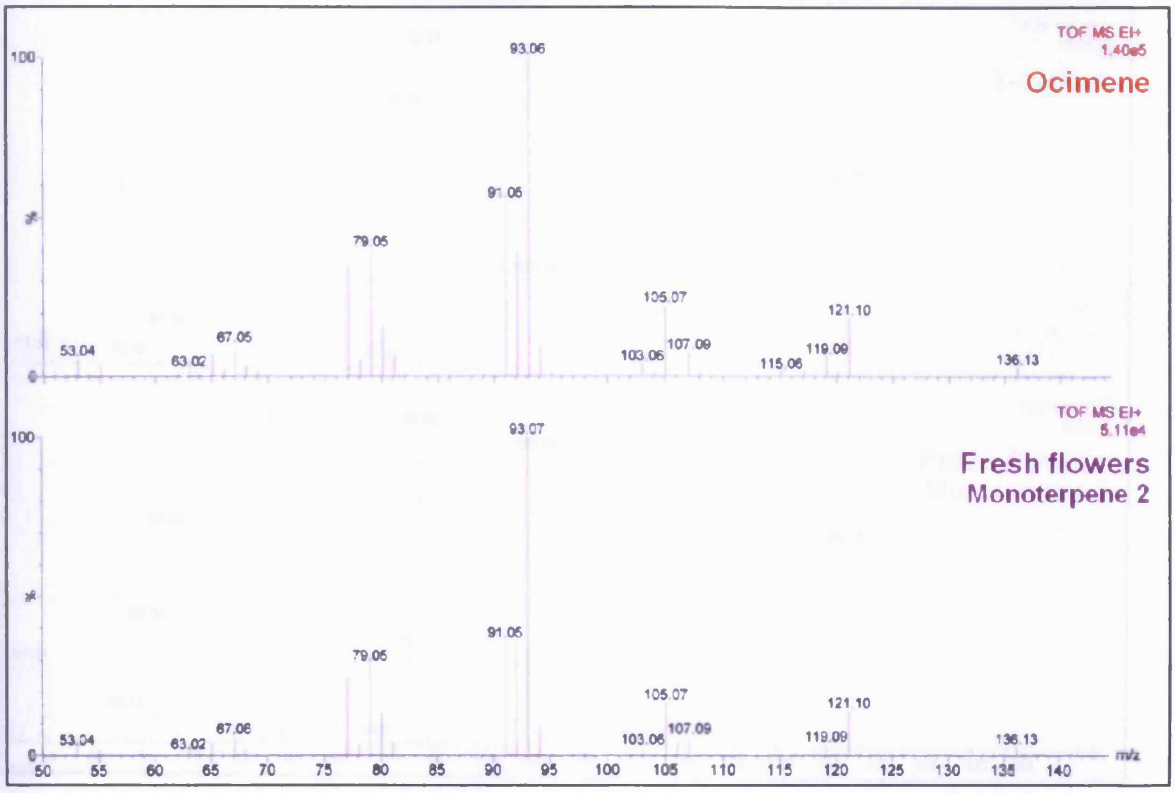

Figure 6.11. Mass spectra obtained through GC-MS of the standard ocimene and the peak 'Monoterpene 1' observed in the chromatogram of fresh flowers (Figure 6.9).

The peak observed at 11.00 min (Monoterpene 3) during the GC-MS analysis of fresh flowers of Alstroemeria was compared with the standard 3-carene finding that even though their mass spectra were not identical, fairly similar patterns were observed. After testing other monoterpene standards, none matched better than 3-carene, therefore the volatile compound emitted by flowers of Alstroemeria was identified putatively as a 3-carene (Figure 6.12).

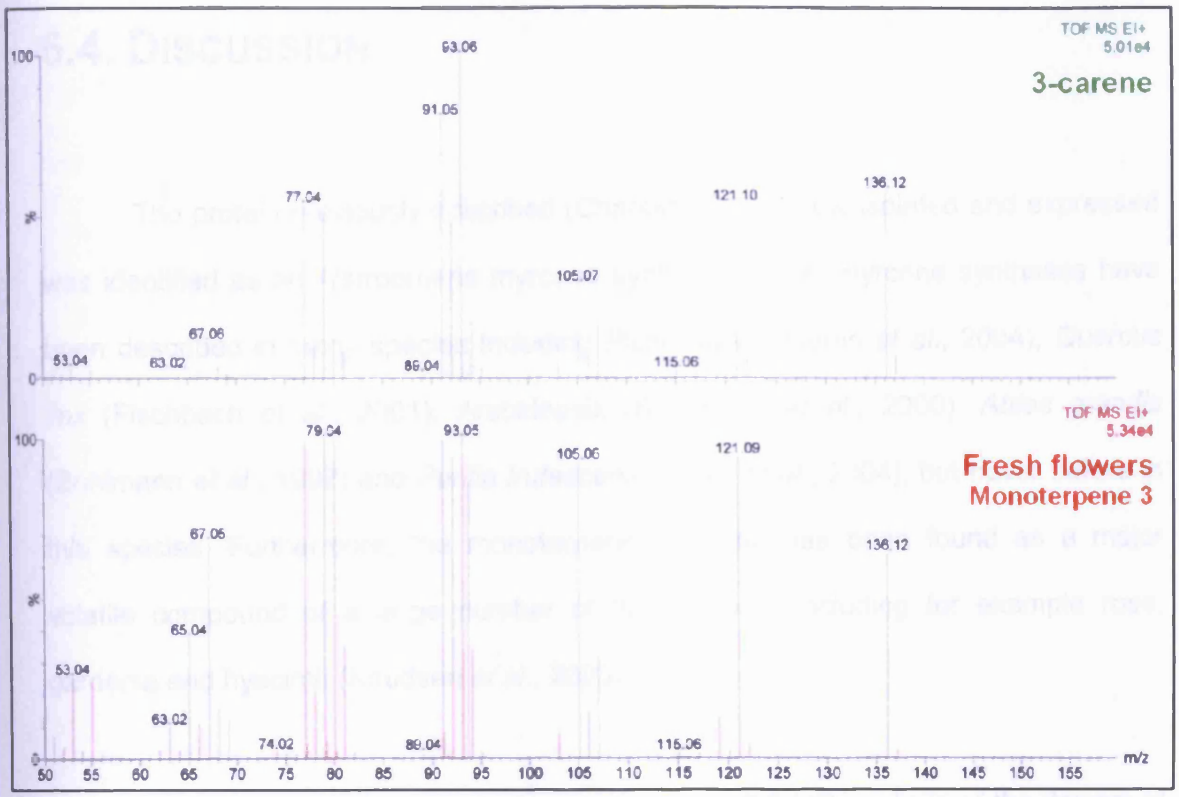

Figure 6.12. Mass spectra obtained through GC-MS of the standard 3-carene and the peak 'Monoterpene 2' observed in observed in the chromatogram of fresh flowers (Figure 6.9). Results are expressed as % of maximum peak value.

6.4. DISCUSSION

The protein previously described (Chapter IV) and now isolated and expressed was identified as an Alstroemeria myrcene synthase. Other myrcene synthases have been described in many species including *Picea abies* (Martin *et al.*, 2004), *Quercus ilex* (Fischbach *et al.*, 2001), Arabidopsis (Bohlmann *et al.*, 2000), *Abies grandis* (Bohlmann *et al.*, 1997) and *Perilla frutescens* (Hosoi *et al.*, 2004), but never before in this species. Furthermore, the monoterpene myrcene has been found as a major volatile compound of a large number of floral scents including for example rose, gardenia and hyacinth (Knudsen *et al.*, 2003).

Considering now the results found during the GC-MS analysis of the flowers of Alstroemeria, the products observed (3-carene and ocimene) differ from the myrcene obtained by the reaction of GPP with *ALSTER*. This fact is in contrast with the expected scenario where the same volatile compounds are produced from the same enzyme *in vivo* (fresh flowers) and *in vitro* (*ALSTER*). Thus, myrcene was expected to be one of the volatile compounds emitted by flowers of Alstroemeria since the myrcene synthase *ALSTER* was derived from RNA extracted from the same tissue. Previous investigations have found similar volatile compounds produced by *in vivo* material such as Arabidopsis flowers (Chen *et al.*, 2003b), Maize leaves (Schnee *et al.*, 2002), snapdragon flowers (Dudareva *et al.*, 2003), *Quercus ilex* leaves (Fischbach *et al.*, 2001), among others, and their respective recombinant enzymes.

The results of this study lead to the conclusion that *ALSTER* could be a monoterpene synthase that produces myrcene *in vitro* and perhaps 3-carene and/or ocimene *in vivo*, since still there is a possibility that other enzymes are involved in the biosynthesis of these monoterpenes. The monoterpene 3-carene is a cyclic

monoterpene while ocimene and myrcene are acyclic (Figure 6.13). Although some functional analyses of acyclic monoterpene synthases have found cyclic monoterpenes produced as minor products (Dudareva *et al.* 2003; Bohlmann *et al.*, 2000), according to a recent review published by Degenhardt *et al.* (2009) cyclic and acyclic monoterpenes are synthesized by completely different pathways. Therefore the cyclic 3-carene is unlikely to be one of the products of *ALSTER*.

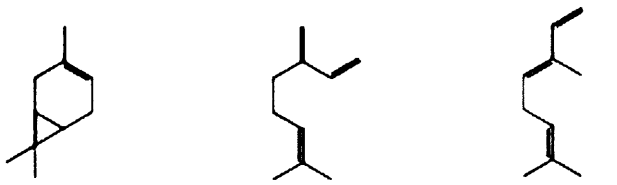

Figure 6.13. Molecular structures of the monoterpenes identified through GC-MS present in flowers of Alstroemeria (3-carene and ocimene) and in the product of the incubation GPP+*ALSTER* (myrcene).

Concerning now whether the monoterpene synthase *ALSTER* could be involved in the biosynthesis of both ocimene and myrcene, a previous investigation carried out by Dudareva *et al.* (2003), described two closely related (92% identical) monoterpene synthases producing ocimene and myrcene respectively. Furthermore, an Arabidopsis terpene synthase (*AtTPS10*) was described and functionally characterized finding that this recombinant enzyme was able to synthesize both myrcene and ocimene (Bohlmann *et al.*, 2000). This enzyme (*AtTPS10*) was identified as one of the TPS most closely related to *ALSTER* in its amino acid sequence in the alignment previously performed in the chapter IV (Figure 4.10). The mechanism proposed by Bohlmann *et al.* (2000) for *AtTPS10* as the enzyme involved in the production of myrcene and

ocimene, describes both products coming from the deprotonation (loss of the H^+) of the intermediate product linalyl cation. Thus, linalyl cation can undergo deprotonation at the C3 methyl group to form myrcene or at the C4 methylene group to form ocimene (Figure 6.14).

However, two other reactions (phosphorylation and isomerization) are necessary to convert the GPP into the linalyl cation and considering that functional analyses of monoterpene synthases are carried out by incubating a single enzyme with GPP, without the presence of any other enzyme, then the problem remains of how this can occur in the *in vitro* system.

A more recent publication (Degenhardt *et al.* 2009) reviewing monoterpene and sesquiterpene synthases, however, proposes two alternative ways for the biosynthesis of myrcene and ocimene, one coming from the geranyl cation and another coming from the linalyl cation. Hence looking at the biosynthesis pathways described by these authors, the suggested activity of *ALSTER* could be in the deprotonation at the C3 methyl group of the geranyl cation to produce myrcene (Figure 6.14 (a)). Alternatively via the linalyl cation, *ALSTER* could possibly produce both myrcene and ocimene by deprotonation of the linalyl cation at the C3 and C4 methyl groups respectively (Figure 6.14. (b) and (c)).

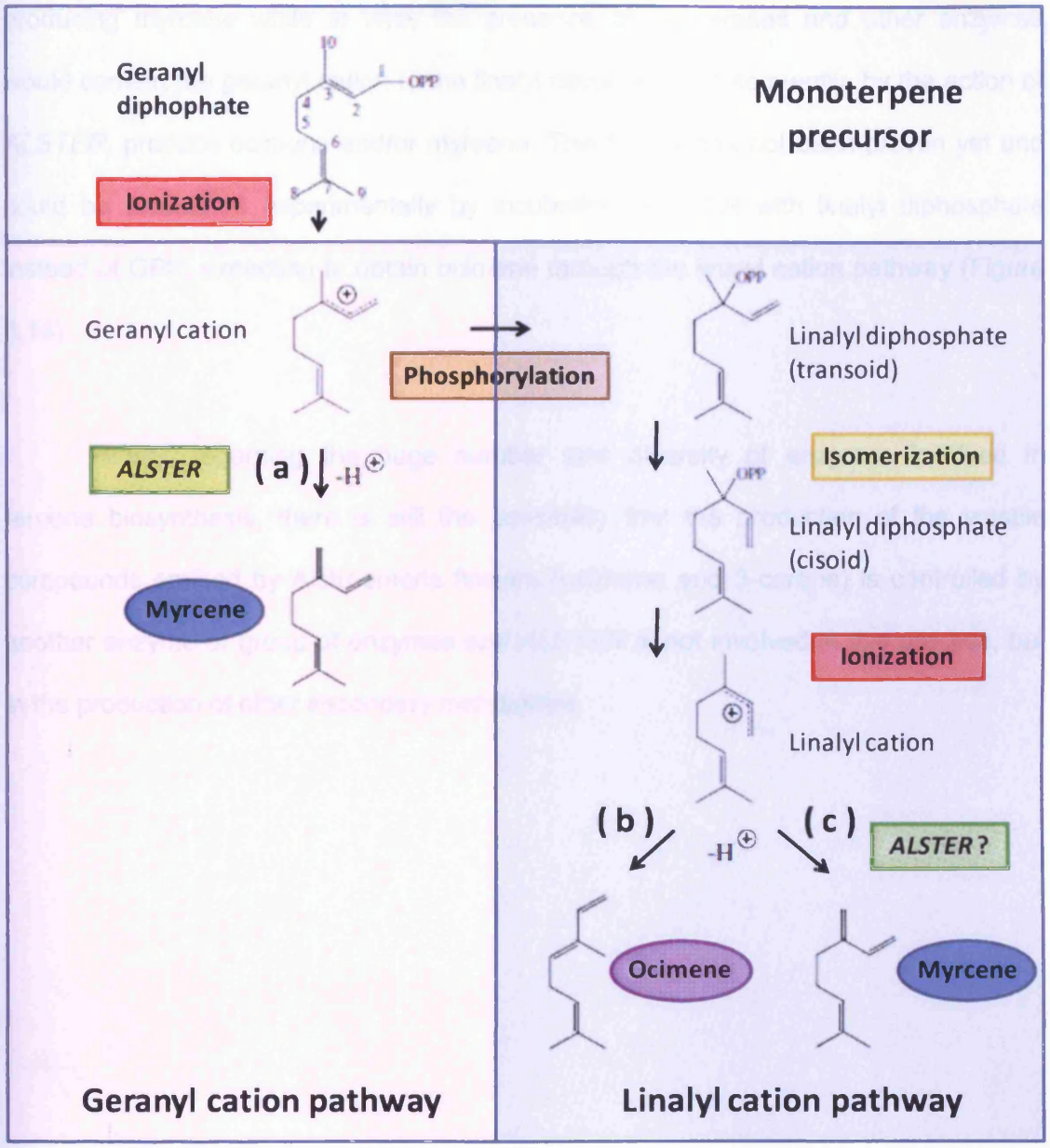

Figure 6.14. Description of the proposed mechanism of reaction of *ALSTER* to produce myrcene and ocimene, based on the biosynthesis pathways suggested by Degenhardt *et al.* (2009). Both geranyl and linalyl cation pathways are shown and reactions are boxed in each step of the metabolic route. Highlighted letters stand for the deprotonations undergone by geranyl cation (a) and linalyl cation (b) and (c).

These suggestions could explain the differences found between the GC-MS results of the recombinant enzyme and the fresh flowers. Thus in *in vitro* conditions, the monoterpene synthase *ALSTER* would be active through the geranyl cation pathway

producing myrcene while *in vivo*, the presence of isomerases and other enzymes would convert the geranyl cation to the linalyl cation and subsequently, by the action of *ALSTER*, produce ocimene and/or myrcene. This theory has not been proven yet and could be elucidated experimentally by incubating *ALSTER* with linalyl diphosphate instead of GPP, expecting to obtain ocimene through the linalyl cation pathway (Figure 6.14).

Finally, regarding the huge number and diversity of enzymes involved in terpene biosynthesis, there is still the possibility that the production of the volatile compounds emitted by Alstroemeria flowers (ocimene and 3-carene) is controlled by another enzyme or group of enzymes and *ALSTER* is not involved in this process, but in the production of other secondary metabolites.

CHAPTER VII.

EVALUATION OF NEW LINES OF SCENTED ALSTROEMERIAS

VII. EVALUATION OF NEW LINES OF SCENTED ALSTROEMERIAS

7.1. INTRODUCTION

Alstroemerias belong to the genus Alstroemeria described by Linnaeus in 1762 and subsequently were classified within an independent family called *Alstroemeriaceae,* by Dumortier in 1829. This family is native to South America and includes about 60 described species (Bayer, 1987; Muñoz and Moreira, 2003). Despite their rich diversity, the commercial value of the native species is poor because many of them present a long period of dormancy and their flowers are often small and insignificant with a short postharvest life (Bridgen *et al.*, 2009).

Many techniques have been used for Alstroemeria breeding and one of the first applied was the induction of mutagenesis, by irradiating rhizomes with x-rays (Broertjes and Verboom, 1974) and then gamma rays (Przybyla 1992; Przybyla, 2000) . The use of polyploidization by colchicine as a breeding method has been also used in Alstroemeria, principally to improve vigour in the new selections (Takayuki, 1999; Bridgen *et al.*, 2009). Howevever, interspecific hybridization and the subsequently embryo rescue, has been the most widely used technique in breeding programmes of Alstroemeria because of the high diversity observed in the new lines produced (Burchi *et al.*, 1997; Lu and Bridgen, 1997; Buitendijk *et al.*, 1995; Kristlansen, 1995). Although still in development, transformation of Alstroemeria could also be a very efficient technique to improve specific characters (Akutsu *et al.*, 2004).

All these methods applied in Alstroemeria breeding have been supported by molecular breeding techniques like the construction of linkage maps (Han *et al.*, 2002), and the use of molecular markers such as RAPDs, for evaluation of genetic variation evaluation of new selections (Anastassopoulos and Keil, 1996) and studies of genetic diversity observed among cultivated and wild species of Alstroemerias (Dubouzet *et al.*, 1998; Aros *et al.*, 2006).

Hence, all these techniques have been implemented in breeding programmes to obtain new cultivars of Alstroemeria and not surprisingly, at the moment this flower is one of the major products in the cut flower industry (Kamminga, 2008). However, almost all the new cultivars of Alstroemeria have been selected based on conventional characters (aesthetic value, vase life longevity and productivity) and not many efforts have been made to obtain fragrant cultivars. This is probably because floral scent is a very variable trait, easily acquired or lost through evolution (Dudareva *et al.*, 1996), and also because normally floral scent has been unintentionally selected against due to the negative correlation found between longevity of the flowers and fragrance (Porat *et al.*, 1993). Thus the cultivars previously described in this study: *A.* cv. 'Sweet Laura' and *A.* cv. 'Ajax', are the only well known scented Alstroemerias, and they both share a common ancestor: the scented species *A. caryophyllaea*.

Alstroemeria caryophyllaea was first identified and fully described by Jacquin in 1804 and later on by Foster (1945). More recently Assis (2004), identified this plant as native and endemic to Brazil, particularly to humid and shaded zones in the regions of Minas Gerais and Rio de Janeiro. However this species is rarely found in the wild nowadays and it is mostly cultivated in all the south east region of Brazil, propagated routinely by rhizomes. Easily recognized by its fragrance, *A. caryophyllaea* presents erect stems, resupinate and elliptic to spatulate leaves, and flowers arranged in simple umbels. The flowering period is during June and July in the wild (Brazil) according to

Assis (2004), while Foster (1945) described it as an almost ever-blooming plant, grown under favourable conditions in the state of Florida, USA.

Considering the importance of *A. caryophyllaea* as the only known source of scent for the production of new cultivars of Alstroemeria, new lines have been obtained in the present study through self pollination with the objective of characterizing and evaluating them as potentially new cultivars or selecting them as promising starting points for breeding purposes. Although the inbreeding technique used for this study is not an efficient method to obtain high variability in the offspring, it has been very useful in Alstroemeria at early stages of a breeding program, because it assures the production of new lines which can be selected for future hybridization with other species (Meijles, 2008; Bridgen, 2008). The evaluation criteria used have been adjusted to the requirements and objectives of this study. Thus, a taxonomical characterization was discarded because the information obtained through this methodology does not fit the characters required by the market. The characterization and classification of Alstroemeria species based on UPOV descriptors (UPOV, 2003) performed by Aros *et al.* (2006), was also discarded because the high level of similarity of the new lines obtained in this study would not have made possible a substantial distinction among them.

7.2. METHODOLOGY

7.2.1. Self pollination of *A. caryophyllaea* flowers

Cut flowers of *A. caryophyllaea*, collected from vegetatively propagated plants grown in a commercial crop field, were manually self-pollinated by collecting mature anthers and approaching them to mature pistils, that is showing stigmatic exudates on the surface of the stigma (Figure 7.1). Because of the protandric condition of these flowers, in some cases the pollen was collected from mature anthers and stored in Eppendorf tubes at 4 °C, for no more than 3 days to keep its viability. In this case the pollination was made by approaching the Eppendorf tubes to the mature stigma.

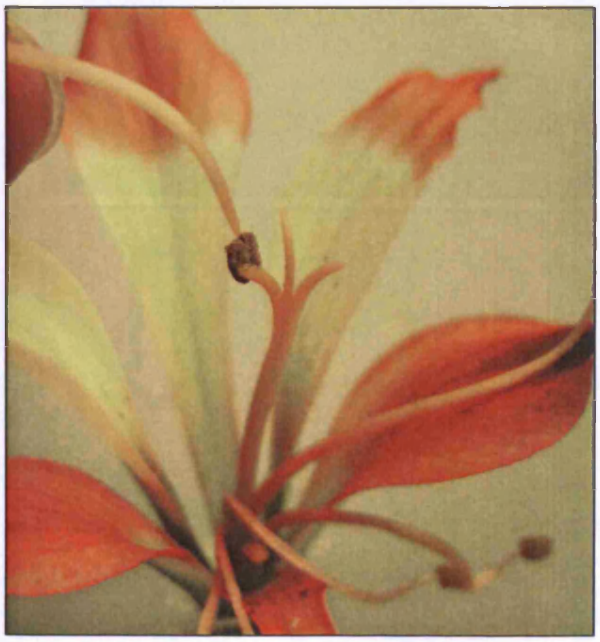

Figure 7.1. Self pollination of *A. caryophyllaea* flowers performed manually.

Flowers were kept at room temperature after the pollination in vases with water and the fruits were visually evaluated after 5, 10 and 15 days after pollination (DAP) (Figure 7.2).

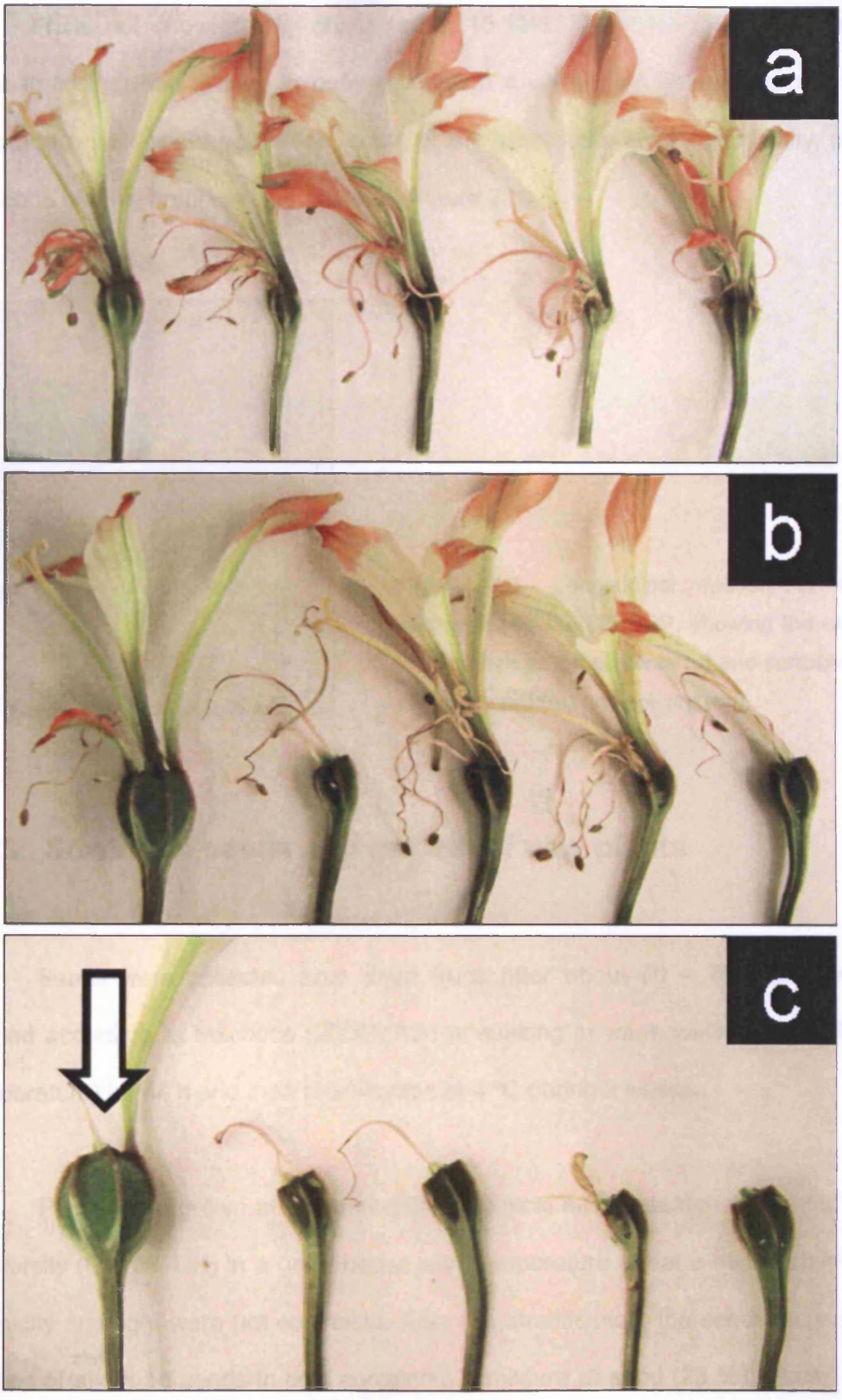

Figure 7.2. Example of five fruits of *A. caryophyllaea* growing 5 DAP (a), 10 DAP (b) and 15 DAP (c). A downward arrow in the bottom picture indicates the fruit which probably has been successfully pollinated.

Fruits not showing any change after 15 DAP were discarded, keeping only those fruits that had grown. In order to confirm a correlation between growth of the fruits and a successful pollination, some of the fruits were cut longitudinally, and the presence of early embryos was checked (Figure 7.3).

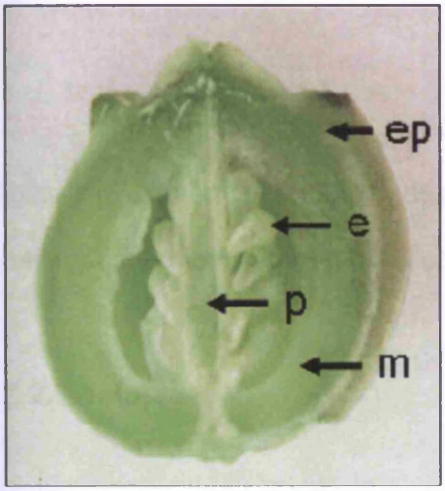

Figure 7.3. Longitudinal section of an *A. caryophyllaea* fruit 15 DAP, showing the embryos (e) attached to the placenta (p) and surrounded by the mesocarp (m) and epicarp (ep).

7.2.2. Sowing of seeds and growth of new plants

Seeds were collected from dried fruits after about 60 – 75 DAP and were treated according to Machuca (2006), that is soaking in warm water (30-40 ºC initial temperature) for 48 h and then stratification at 4 ºC during 2 weeks,.

Plants were grown at the University Botanical and Research Garden of Cardiff University (Cardiff, UK) in a greenhouse with temperature set at a minimum of 14 ºC. Humidity and light were not controlled. After the stratification, the seeds were sown in groups of about 15 seeds in pots containing a mixture of sand (25 %), coarse grit (25 %) and compost (50 %). Once the seeds germinated, after a variable period of time, the new seedlings were transplanted to individual pots containing the same mixture of

soil as above. Finally, every new seedling was identified by an alphanumerical code starting with DANCAR 001 up to DANCAR 017 (Appendix G).

7.2.3. Morphological evaluation of the new lines of *A. caryophyllaea*

The new plants were evaluated morphologically, when flowers developed, by following different parameters depending on the organ studied. All the observations and measurements were taken during two seasons of flowering ('08/'09 and '09/'10).

7.2.3.1. Leaves

Shape of the leaves was assessed according to Hickey and King (1997).

7.2.3.2. Stem

Stem length (cm): the distance between the soil level and the highest flower was measured. Quality was assessed in relative terms. Relative values were assigned in each case using the following scale: 1: Weak; 2: Medium weak; 3: Medium; 4: Medium strong and 5: Strong. Thus, the best stem quality found was rated 5, the worst 1, with all the other values relative to these. The measure of the diameter of the stem (in mm) as a more objective parameter was discarded, since the strength of the stem was not always correlated to diameter. Therefore not just the diameter but also the firmness of the stem was considered in the global evaluation of quality.

7.2.3.3. Flowers

Flower size was measured in terms of length x width (cm). Width was considered as the maximum distance between the external margins of the two higher inner tepals or between the external margins of the two basal outer tepals. Length was considered as the distance between the margins of the higher outer tepal and the basal inner tepal (For details see figure 2.5 in Chapter II).

Flower shape was assessed in terms of the ratio calculated from the relationship 'height:width'. Thus, values close to 1 represent a round-shaped flower while values higher or lower than 1 represent elliptic-shaped flowers.

Flower colour was assessed as the main two or three colours covering the six tepals. Flower markings were assessed by the identification of dots/stripes covering the tepals in terms of location (inner or outer tepals), abundance (rare, medium or abundant) and shape (dots or stripes).

7.2.3.4. Anthers

Pollen abundance was assessed as abundant, medium or poor. Colour of the anthers before and after dehiscence was also recorded.

7.2.4. Phenological evaluation of the new lines of *A. caryophyllaea*

Phenology of the plants was tracked and recorded during two seasons ('08/'09 and '09/'10), in terms of duration of the vegetative period and flowering season for each of the new lines obtained.

7.2.5. Evaluation of the productivity of the new lines of *A. caryophyllaea*

Productivity was evaluated for each line by counting the number of floral stems obtained per plant per flowering season ('08/'09 and '09/'10) and the number of individual flowers per floral stem.

7.2.6. Sensorial evaluation of the new lines of *A. caryophyllaea*

7.2.6.1. Subject

A total of seventy persons including males and females with no restriction of age were included in the evaluation of five different DANCAR lines scent. They were advised via e-mail and their participation was absolutely voluntary. Samples were individually evaluated by each volunteer and the analysis was carried out in a laboratory at Cardiff University, School of Biosciences. An ethical approval was obtained from Cardiff University, BIOSI Research Ethics Guidance Group, confirming that ethical issues and health and safety measures were in accordance with University policy and the research ethics guidelines.

7.2.6.2. Stimuli

Floral scents from the five following lines of *Alstroemeria caryophyllaea* were evaluated: DANCAR 001, DANCAR 004, DANCAR 010, DANCAR 013 and DANCAR 017. In each case, three floral stems were cut in proportion to their natural length and then placed in a 500 ml measuring cylinder with water. Samples were identified by a three-digit numerical code (Figure 7.4).

7.2.6.3. Procedure

The procedure was separated into two phases (I and II) including several surveys for each. In addition an 'Information sheet' (Appendix F) was provided to the evaluators before the sensorial analysis in order to give them some instructions and explain the main considerations related to the evaluation.

Figure 7.4. Presentation of the new lines of *A. caryophyllaea* (DANCAR) during the sensorial analysis.

I) Subjects were asked to complete a survey (Appendix F) including general questions (Sex, age, etc.) and another survey (Appendix F), in order to assess relative importance they give to floral scent as a character when buying or looking at flowers. The question asked was – 'When you buy/look at flowers the character you appreciate more is' – and the scale included: 'strongly agree'; 'agree'; 'neutral'; 'disagree' and strongly disagree'.

II) The second phase included evaluations of the samples presented (DANCAR 001, DANCAR 004, DANCAR 010, DANCAR 013 and DANCAR 017). Volunteers were asked to approach each sample randomly and for each of them complete three surveys including:

Evaluation of the floral scent. Volunteers were asked to smell each sample and, without considering the appearance of the flower presented, evaluate only the floral scent by ticking a box on a response sheet (Appendix F) following the hedonic scale: 'Like extremely'; 'like very much'; 'like moderately'; 'like slightly'; 'neither like nor dislike'; 'dislike slightly'; 'dislike moderately'; 'dislike very much'; 'dislike extremely'. Volunteers were asked to wait for about one minute between evaluating each sample.

Evaluation of the intensity of the floral scent. Volunteers were asked to rate the intensity of the floral scent just perceived, by ticking a box on a response sheet (Appendix F) following the scale: 'Extremely high'; 'very high'; 'moderately high'; 'slightly high'; 'neither high nor low'; 'slightly low'; 'moderately low'; 'very low'; 'extremely low'.

Evaluation of the aesthetic value/appearance of the flower presented. Volunteers were asked to evaluate the overall liking of the flowers presented, considering only their aesthetic value or appearance but without considering the floral scent previously

perceived. The same hedonic scale described for the evaluation of the floral scent was used, changing only the main question (Appendix F).

7.2.6.4. Statistical analysis

Both the hedonic scale and the agreement scale were translated into scores. For the hedonic scale the scores were: Like extremely = 9, dislike extremely = 1, and for the agreement scale the scores were: Strongly agree = 5, strongly disagree = 1. In each case, standard deviation (STEDV) and standard error (SE) were calculated. Analysis of variance (ANOVA) was performed through SPSS 17.0 for Windows, using Tukey's HSD (Honestly Significant Difference) test with a significance level of 0.05.

7.3. RESULTS

From all the self pollinations performed, 17 plants were obtained from which 13 of them were in bloom during seasons '08/'09 and '09/'10. Thus all the following evaluations were made on these 13 new lines during the flowering period which lasted for about 14 weeks in the first season ('08/'09) and 22 weeks in the second season ('09/'10). Despite these restricted periods of flowering, leaves on the plants remained green also throughout the vegetative period, and dormancy was never observed.

7.3.1. Leaves

The most common leaf shape was lanceolate, observed in 8 out of the 13 lines evaluated (DANCAR 001, 002, 006, 009, 010, 013, 014 and 016). Elliptic leaves were observed in 4 of the lines (DANCAR 004, 005 and 008), while linear leaves were observed only in DANCAR 003 and DANCAR 017 (Table 7.1; Figure 7.5).

Table 7.1. Leaf shape observed in 13 new lines of *A. caryophyllaea*.

Line	Leaf shape
DANCAR 001	Lanceolate
DANCAR 002	Lanceolate
DANCAR 003	Linear
DANCAR 004	Elliptic
DANCAR 005	Elliptic
DANCAR 006	Lanceolate
DANCAR 008	Elliptic
DANCAR 009	Lanceolate
DANCAR 010	Lanceolate
DANCAR 013	Lanceolate
DANCAR 014	Lanceolate
DANCAR 016	Lanceolate
DANCAR 017	Linear
Parent plant	Linear to lanceolate

The size of the leaf was not considered in the evaluation because due to its high variability depending on environment and management conditions, a comparison with the parent plant would be difficult. However substantial differences were established between the different lines. Thus DANCAR 004 showed, in general, the largest leaf size compared to the other lines (Figure 7.5).

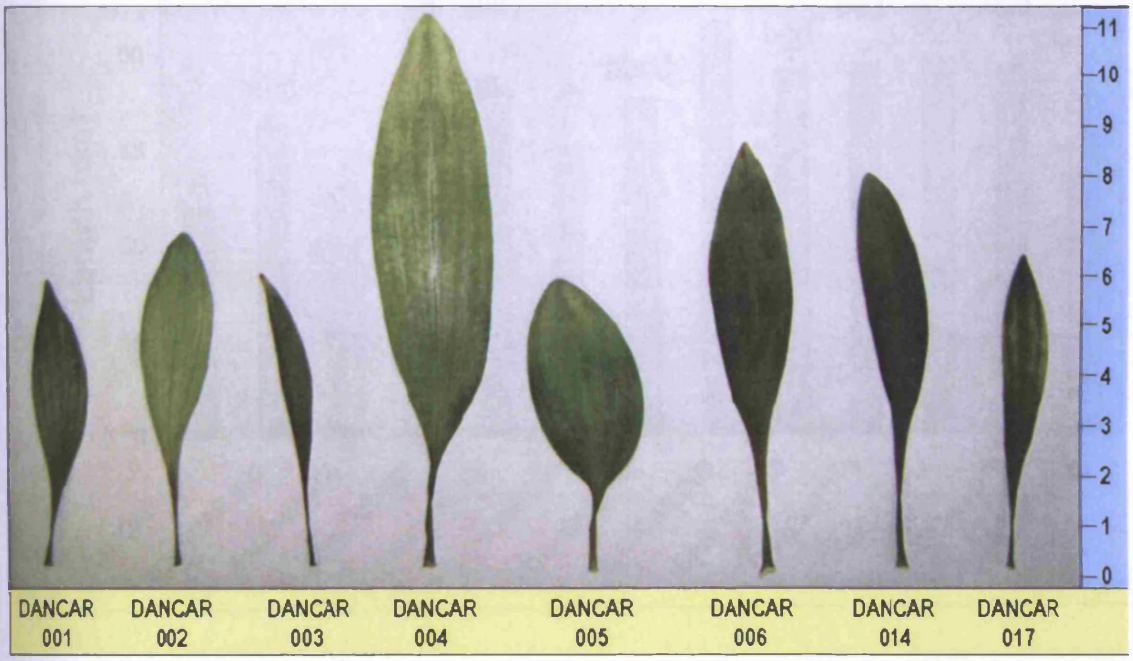

Figure 7.5. Leaf morphology observed in 8 lines of *A. caryophyllaea*: lanceolate (DANCAR 001, 002, 006 and 014), linear (DANCAR 003 and 017) and elliptic (DANCAR 004 and 005). Leaves were collected at floral stage from floral stems, and a representative sample was chosen in each case. On the right, a ruler in cm stands for leaf sizes.

7.3.2. Stem

Stem length was measured and recorded during two seasons ('08/'09 and '09/'10) for each line. DANCAR 004 showed the longest stem with an average of 70.6 cm (n=49), that was statistically different to six of the other lines, among them

DANCAR 001 and 003, which showed the shortest stems with averages of 35.5 (n=34) and 39.1 cm (n=37) respectively (Figure 7.6).

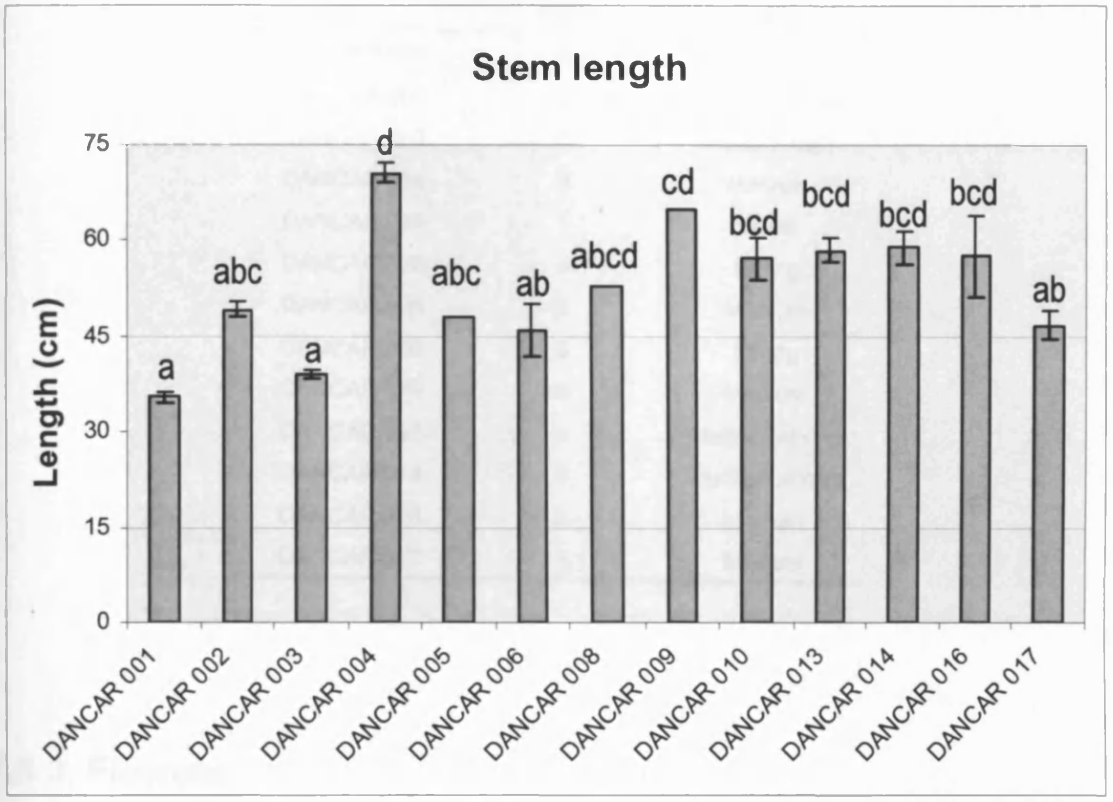

Figure 7.6. Average stem lengths (±SE, n=3 to 49)* evaluated on 13 new lines of *A. caryophyllaea,* during two periods of flowering ('08/'09 and '09/'10).

* The number of stems evaluated (n) depends on the productivity of each line.

Considering the relative quality of the stem, the highest quality in terms of firmness and thickness was found in DANCAR 006 and 009, rated as 'strong', while the lowest value was assigned to DANCAR 005 whose stem was evaluated as 'weak', and all the rest of the lines were rated with values relative to these (Table 7.2).

Table 7.2. Quality of the stem evaluated on 13 new lines of *A. caryophyllaea*, during two periods of flowering ('08/'09 and '09/'10).

DANCAR Line	Stem Quality	
	Rate	Quality
DANCAR 001	4	Medium strong
DANCAR 002	2	Medium weak
DANCAR 003	4	Medium high
DANCAR 004	3	Medium
DANCAR 005	1	Weak
DANCAR 006	5	Strong
DANCAR 008	3	Medium
DANCAR 009	5	Strong
DANCAR 010	3	Medium
DANCAR 013	4	Medium strong
DANCAR 014	4	Medium strong
DANCAR 016	3	Medium
DANCAR 017	3	Medium

7.3.3. Flowers

Flowers of the new lines were very similar to the wild species *A. caryophyllaea*, showing very narrow and non overlapping tepals. In terms of flower colour, all the lines were 'white' in background colour, while different intensities of pink and even red and green were also seen as cover colour of the margins of the tepals (Table 7.3 and Figure 7.7).

Six lines presented dots and seven lines presented stripes, with both stripes and dots abundant in the majority of the cases excepting DANCAR 001, 006 and 009 where the dots/stripes were evaluated as 'rare' (Table 7.3 and Figure 7.7). Characterization of the anthers showed one sterile line with no pollen (DANCAR 006), while anthers in DANCAR 001, 003 and 014 were abundant in pollen. 'Dark pink' was

the dominant colour in the anthers before dehiscence (8 out of 13) while different intensities of brown was the most common colour in anthers after dehiscence (Table 7.3).

Table 7.3. Characterization of the flowers of 13 new lines of *A. caryophyllaea*, considering flower colour, and description of the dots/stripes and the anthers. (B=before; A=after)

DANCAR LINE	FLOWERS	DOTS / STRIPES			ANTHERS		
	Colour	Position	Dots or Stripes	Abundance	Pollen	Colour	
						B / dehiscence	A / dehiscence
DANCAR001	Pale pink / White	Inner tepals	Dots	Rare	Abundant	Dark Pink	Light brown
DANCAR002	Light pink / White	Higher inner tepals	Stripes	Abundant	Poor	Dark pink	Dark brown-Purple
DANCAR003	Light pink Salmon / White	Inner tepals / Higher outer tepal	Stripes	Abundant	Abundant	Dark Pink	Light brown
DANCAR004	Light pink Greenish / White	Inner tepals / Higher outer tepal	Stripes	Medium	Poor	Dark pink	Dark brown-Purple
DANCAR005	Redish / White	Inner tepals	Dots	Medium	Poor	Dark pink	Purple
DANCAR006	Strong pink / White	Inner tepals / Higher outer tepal	Dots	Rare	No	White	Dark pink
DANCAR008	Red / White	Inner tepals / Higher outer tepal	Dots	Abundant	Poor	Pale orange	Light brown
DANCAR009	Strong pink Greenish / White	Inner tepals	Dots	Rare	Poor	Pale orange	Dark pink
DANCAR010	Pink / White	Inner tepals / Higher outer tepal	Stripes	Abundant	Medium	Dark pink	Light brown
DANCAR013	Pink Salmon/ White	Inner tepals / Higher outer tepal	Stripes	Abundant	Medium	Pale orange	Light brown
DANCAR014	Redish / White	Inner tepals / Higher outer tepal	Stripes	Abundant	Abundant	Dark Pink	Purple
DANCAR016	Pale pink / White	Inner tepals / Higher outer tepal	Stripes	Abundant	Medium	Dark Pink	Brown
DANCAR017	Red / White	Inner tepals / Higher outer tepal	Dots	Abundant	Medium	Red	Pale brown

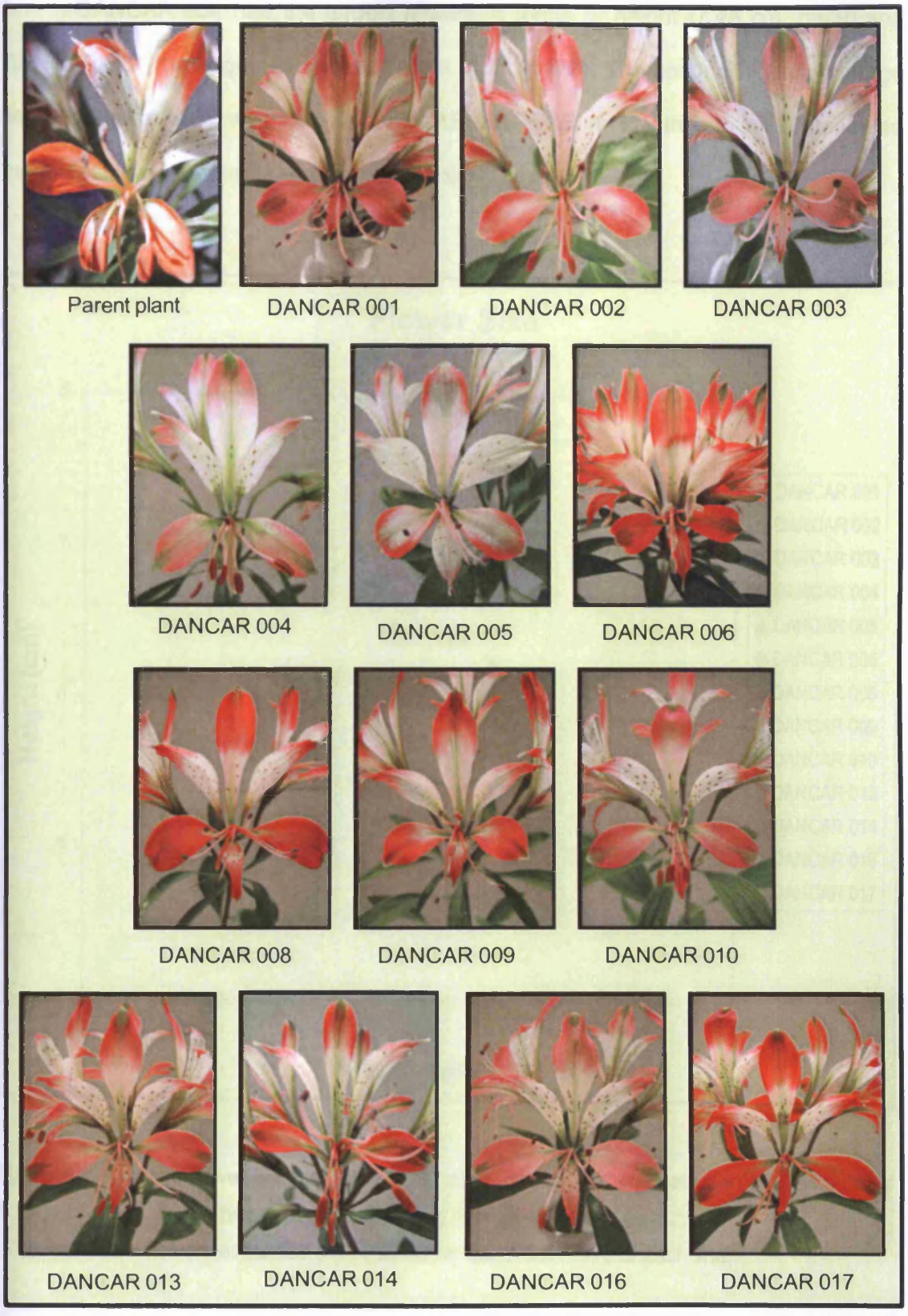

Figure 7.7. Flowers observed in 13 new lines of *A. caryophyllaea* and the parental line.

DANCAR 004 had the largest flowers in terms of height (7.48 cm, n=49) and DANCAR 001 the largest in terms of width (5.51, n=38). The second highest average value for flower width was found in DANCAR 004 (5.45, n=49), thus this line presented the largest flowers considering 'height x width' (Figure 7.8).

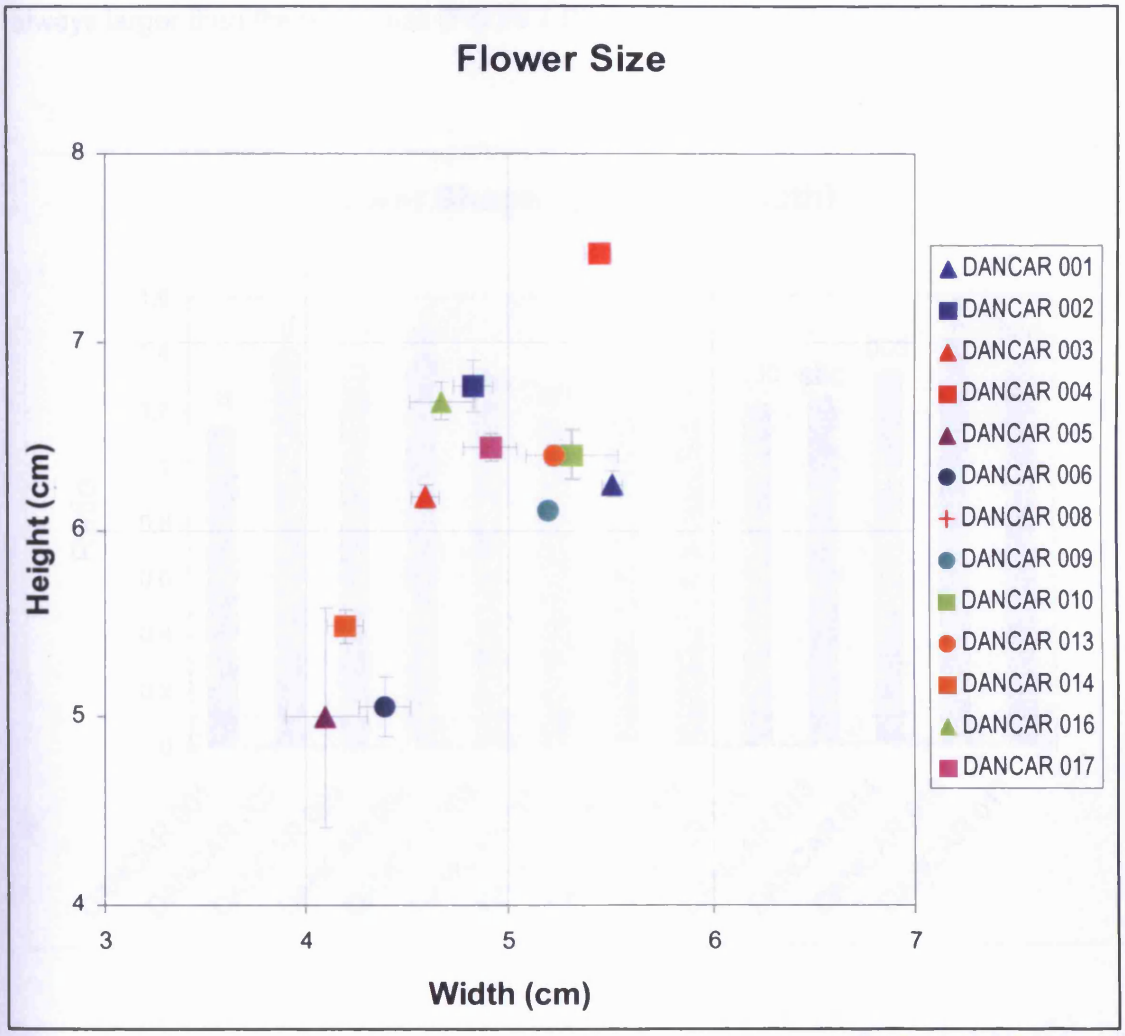

Figure 7.8. Average flower heights and widths (±SE, n=4 to 54)* evaluated on 13 new lines of *A. caryophyllaea*, during two periods of flowering ('08/'09 and '09/'10).

* The number of flowers evaluated (n) depends on the productivity of each line.

The smallest flowers were observed in DANCAR 005, 006 and 014., Both mean height and width were lowest finding DANCAR 005 with averages of 5.0 and 4.1 respectively although there was a lot of variability especially in height and thus the

flower size of DANCAR 005 was not statistically distinguishable from the other two lines.

The ratio between height and width was also considered part of the evaluation, finding average values > 1 in all the cases. That means, for all the lines the height was always larger than the width size (Figure 7.9).

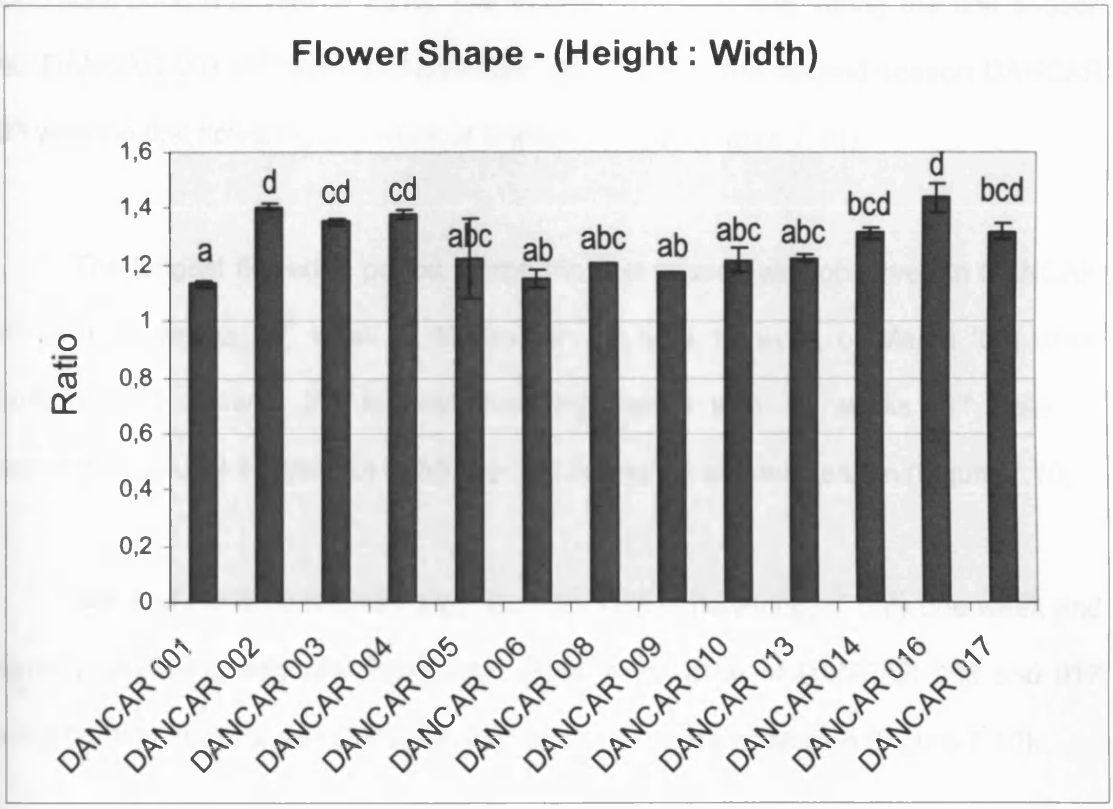

Figure 7.9. Average ratios calculated from the relationship between flower height and width (±SE, n=4 to 54)* evaluated on 13 new lines of *A. caryophyllaea,* during two periods of flowering ('08/'09 and '09/'10).

* The number of flowers evaluated (n) depends on the productivity of each line.

Although not statistically significantly different to most of the rest of the lines, the flower showing the lowest average value for the ratio height : width was DANCAR 001 (1.13, n=38), while the highest value was observed in DANCAR 016 (1.44, n=7). In

other words, DANCAR 001 presented the most round-shaped flowers while DANCAR 016 the most elliptic-shaped flowers.

7.3.4. Phenology

Two seasons of flowering were recorded for each line during the period starting November 2008 until March 2010. The earliest flowering line during the first season was DANCAR 001 (4th week of November '08), while in the second season DANCAR 003 was the first flowering (3rd week of September '09) (Figure 7.10).

The longest flowering period during the first season was observed in DANCAR 001 with 14 weeks (4th week of November '08 until 1st week of March '09) while DANCAR 003 showed the longest flowering period with 22 weeks (3rd week of September '09 until 4th week of February '10) during the second season (Figure 7.10).

Some of the lines showed very short periods of flowering, of only one week and normally producing only one floral stem. That is the case of DANCAR 005 and 017 during the first season, and DANCAR 009 during the second season (Figure 7.10).

DANCAR 005, 006 and 014 produced flowers only during the first season ('08/'09), while DANCAR 008, 009, 010, 013 and 016 produced flowers only during the second season ('09/'10). All the rest of the lines were productive during both seasons of flowering (Figure 7.10).

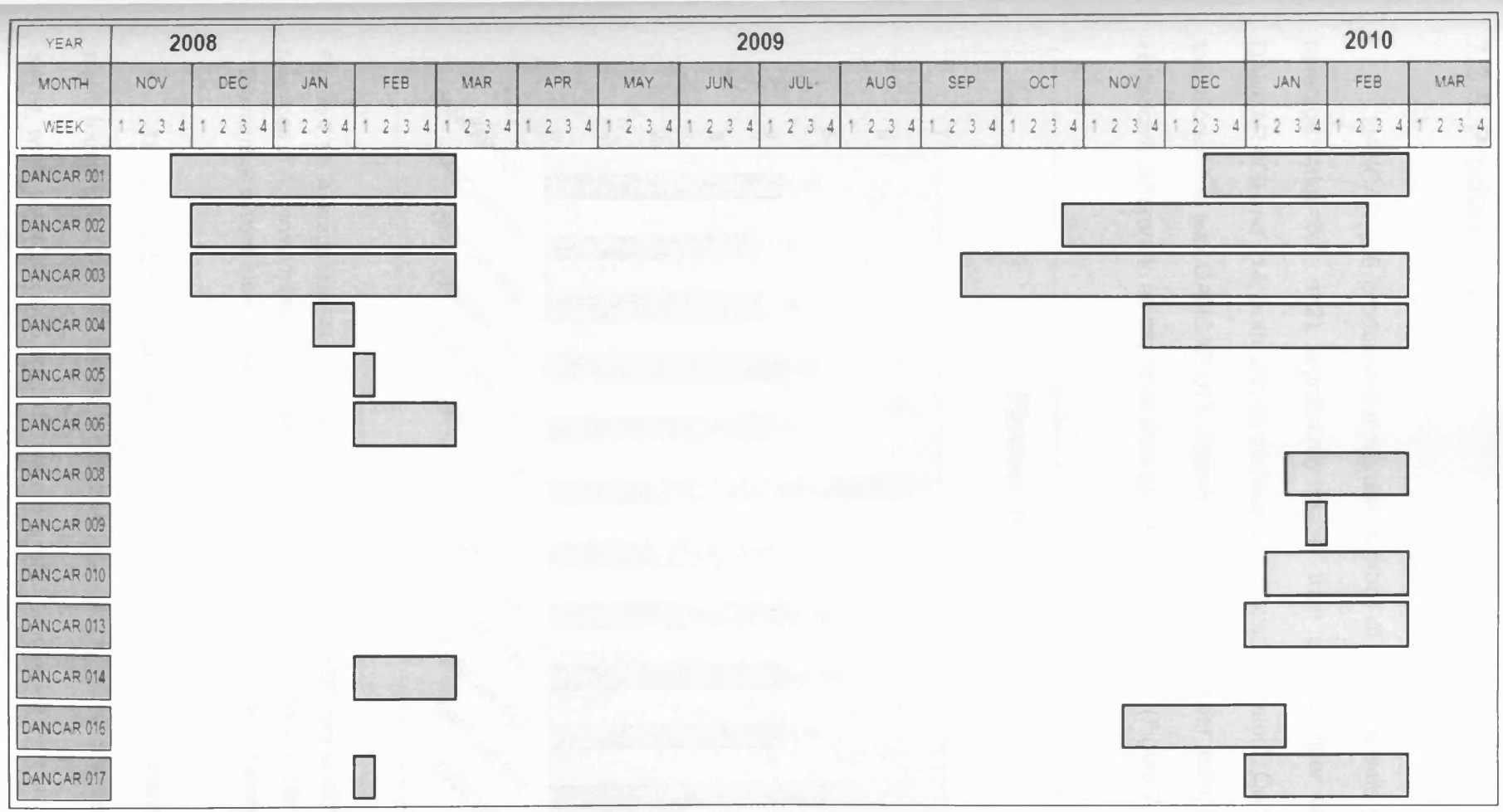

Figure 7.10. Scheme showing the flowering periods (coloured rectangles) observed on 13 new lines of *A. caryophyllaea*, during two seasons ('08/'09 and '09/'10).

7.3.5. Productivity

DANCAR 006 produced the highest number of single flowers per floral stem (average value=6.5, n=2), significantly higher than all the other lines, excepting DANCAR 009 and 014, both with an average of 5 flowers per stem. On the other hand, the poorest line was DANCAR 017, showing only 3.6 flowers per stem, but actually not statistically different to all the other lines except DANCAR 006 (Figure 7.11).

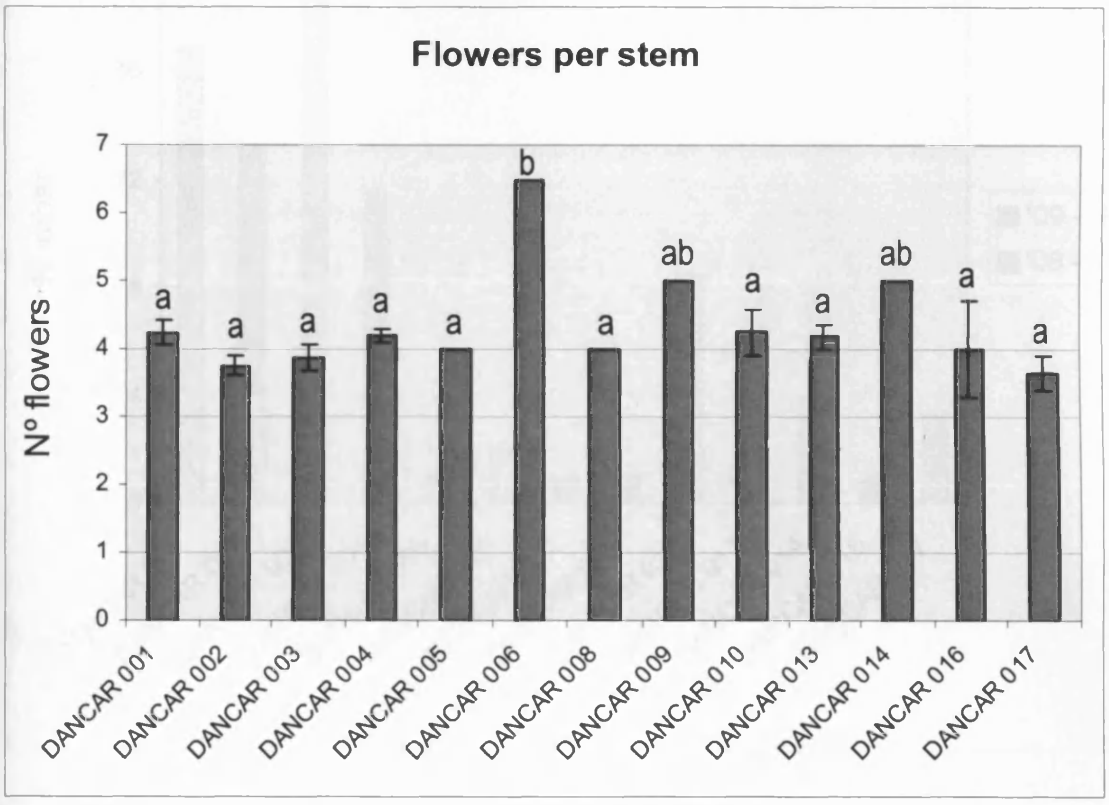

Figure 7.11. Average number of flowers produced per stem (±SE, n=3 to 49)* evaluated on 13 new lines of *A. caryophyllaea,* during two periods of flowering ('08/'09 and '09/'10).

* The number of floral stems evaluated (n) depends on the productivity of each line.

The productivity evaluated through the number of floral stems produced per plant, found DANCAR 001 as the most productive during the first season with 10 floral stems, while DANCAR 005 and 017 were the least productive with only one floral stem during the season '08/'09 (Figure 7.12).

The highest number of floral stems per plant during the season '09/'10 was found in DANCAR 003, being the most productive with 10.3 floral stems. DANCAR 008, 009 and 016 were the least productive with only one floral stem per plant per season (Figure 7.12).

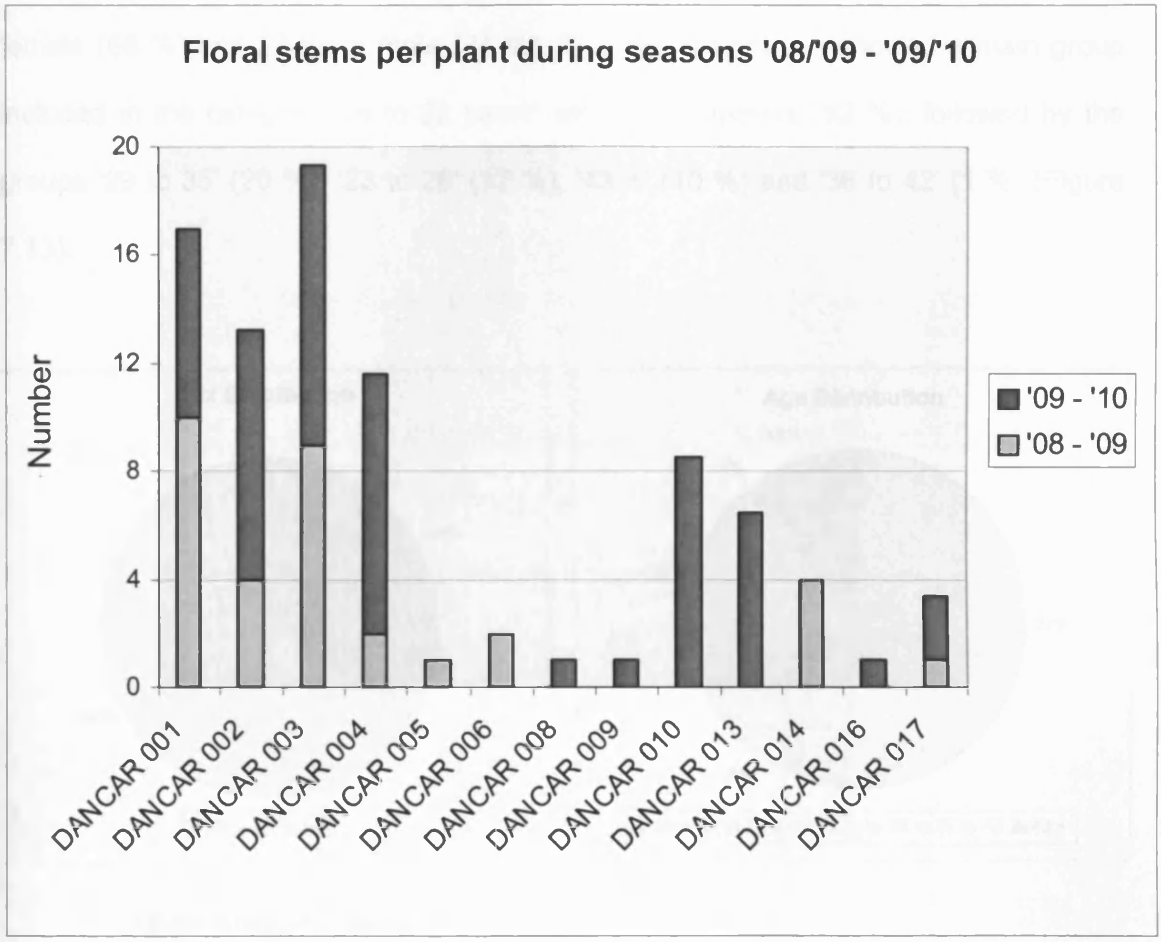

Figure 7.12. Average number of floral stems produced per plant per season, evaluated on 13 new lines of *A. caryophyllaea*, during two periods of flowering ('08/'09 and '09/'10).

Considering now both seasons ('08/'09 and '09/'10), DANCAR 003 was the most productive line with an average of 9.7 floral stems per plant per season while the least productive were DANCAR 005, 008, 009 and 016 with only 1 floral stem (Figure 7.12).

7.3.6. Sensorial Analysis

7.3.6.1. Characteristics of the population

Forty six of the seventy people participating in the sensorial analysis were female (66 %) and 24 were male (34 %). The age distribution showed a main group included in the category '18 to 22 years' with 36 volunteers (52 %), followed by the groups '29 to 35' (20 %), '23 to 28' (17 %), '43 +' (10 %) and '36 to 42' (1 %) (Figure 7.13).

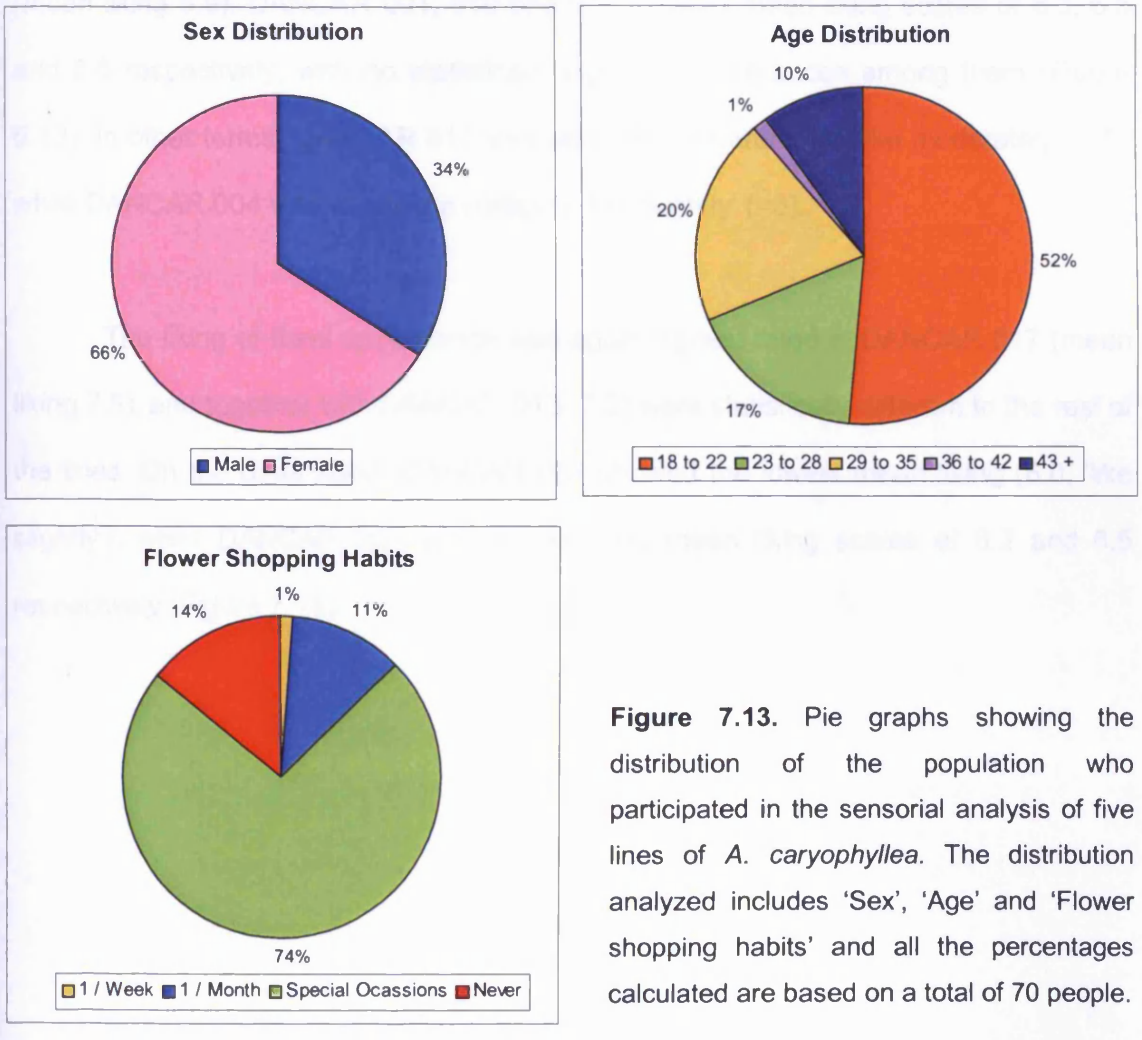

Figure 7.13. Pie graphs showing the distribution of the population who participated in the sensorial analysis of five lines of *A. caryophyllea*. The distribution analyzed includes 'Sex', 'Age' and 'Flower shopping habits' and all the percentages calculated are based on a total of 70 people.

Asked the question - 'How often do you buy flowers?' – the vast majority of the volunteers declared to buy flowers only for 'special occasions' (74 %) while only one person affirmed to buy flowers every week (1 %) (Figure 7.13).

7.3.6.2. Liking of floral scent and appearance

The liking of the floral scent evaluated on five lines of *A. caryophyllaea*, rated DANCAR 017 highest with a mean liking score of 6.9 (out of 9). However this value was statistically different only with the poorest floral scent rated found in DANCAR 004 (mean liking 5.9). DANCAR 001, 010 and 013 showed mean liking scores of 6.3, 6.5 and 6.6 respectively, with no statistically significant differences among them (Figure 5.13). In other terms, DANCAR 017 was close to the parameter 'like moderately' (=7), while DANCAR 004 was below the category 'like slightly' (=6).

The liking of floral appearance was again highest rated in DANCAR 017 (mean liking 7.5), and together with DANCAR 013 (7.2) were statistically different to the rest of the lines. On the other hand, DANCAR 001 showed the lowest mean liking (6.0, 'like slightly'), while DANCAR 004 and 010 showed mean liking scores of 6.2 and 6.5 respectively (Figure 7.14).

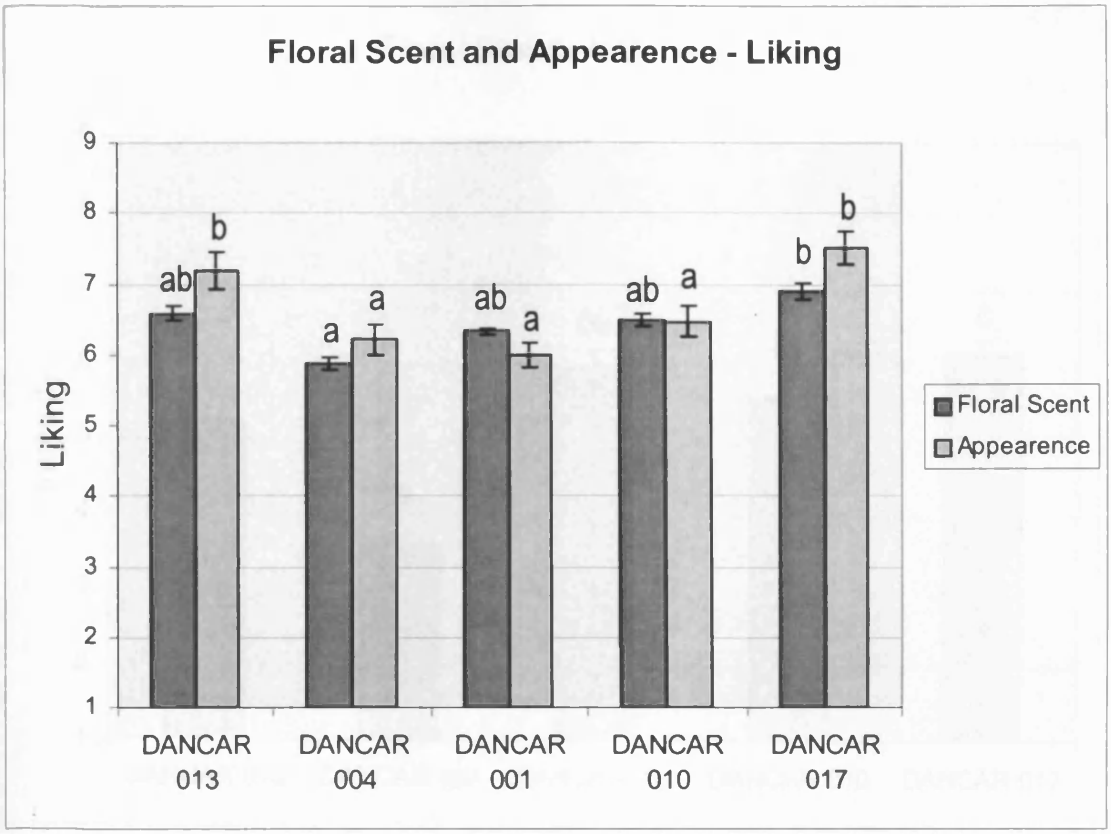

Figure 7.14. Means of floral scent and floral appearance liking (± SE, n=70) evaluated on 5 new lines of *A. caryophyllaea*. The scale ranges from 'like extremely (= 9) to 'dislike extremely' (= 1).

7.3.6.3. Floral scent intensity

The intensity of the floral scent was also evaluated, and the highest average value was observed in DANCAR 017 (6.2, out of 9). The lowest intensity was found in DANCAR 004 with an average value of 3.6, and it was statistically different to the rest of the samples. Thus the intensity of the floral scents perceived in DANCAR 001, 010 and 013 were evaluated with scores of 6.0, 5.6 and 5.2 respectively and no statistically significant differences were found among them (Figure 7.15).

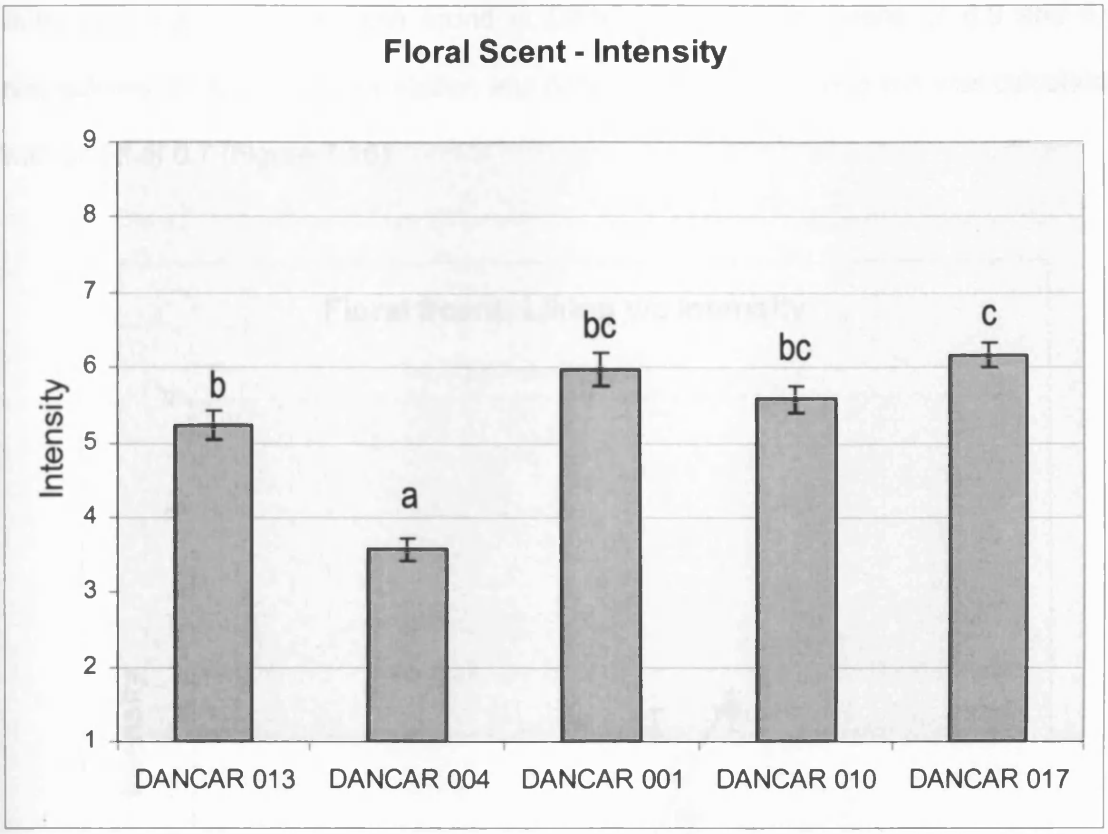

Figure 7.15. Means of floral scent intensity (± SE, n=70) evaluated on 5 new lines of *A. caryophyllaea*. The scale ranges from 'extremely high (= 9) to 'extremely low' (= 1).

In other terms, the intensity of the floral scent perceived in DANCAR 017 was evaluated just above the parameter 'slightly strong' (=6), while the floral scent of DANCAR 004 was assessed between the groups 'slightly low' and 'moderately low' (=3 and =4).

7.3.6.4. Correlation: floral scent liking v/s floral scent intensity

A consistent correlation was found between intensity and liking of scent, with the higher the intensity the higher the liking. Thus DANCAR 004 showed the lowest values for both floral scent liking (5.9) and intensity (3.6), while the highest values for

liking and intensity were both found in DANCAR 017 with means of 6.9 and 6.2 respectively. This positive correlation was plotted and a linear trend line was calculated with an R^2 of 0.7 (Figure 7.16).

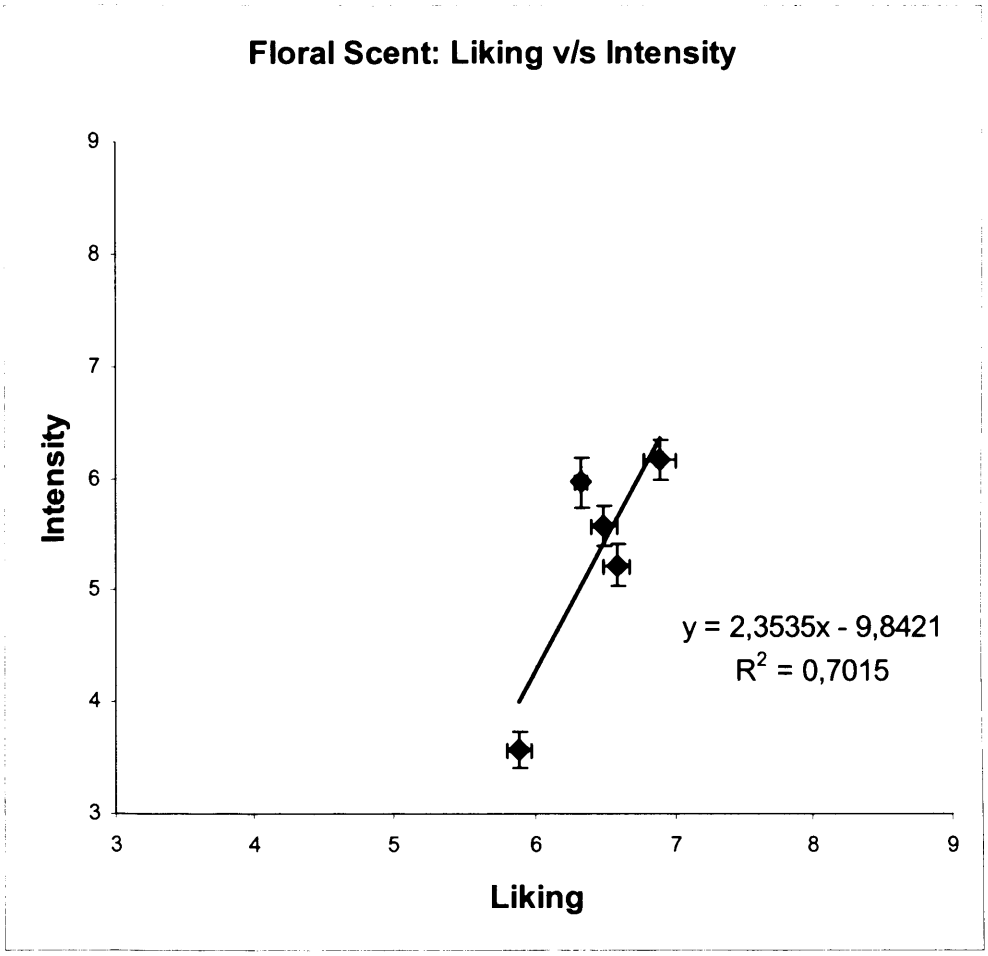

Figure 7.16. Means of floral scent liking and floral scent intensity (± SE, n=70) evaluated on 5 new lines of *A. caryophyllaea*. The liking scale ranges from 'like extremely (= 9) to 'dislike extremely' (= 1) and the intensity scale ranges from 'extremely high (= 9) to 'extremely low' (= 1).

7.3.6.5. Correlation: floral scent liking v/s appearance liking

A positive correlation was also found when comparing floral scent liking versus appearance liking. Again DANCAR 017 was the highest rated in both liking of floral

appearance and floral scent with mean values of 7.5 and 6.9 respectively. In this case, the lowest value for floral liking did not match with the lowest value observed in floral appearance and *vice versa*, however a consistent linear trend was calculated for the correlation of floral scent versus appearance liking, with an R^2 of 0.64 (Figure 7.17).

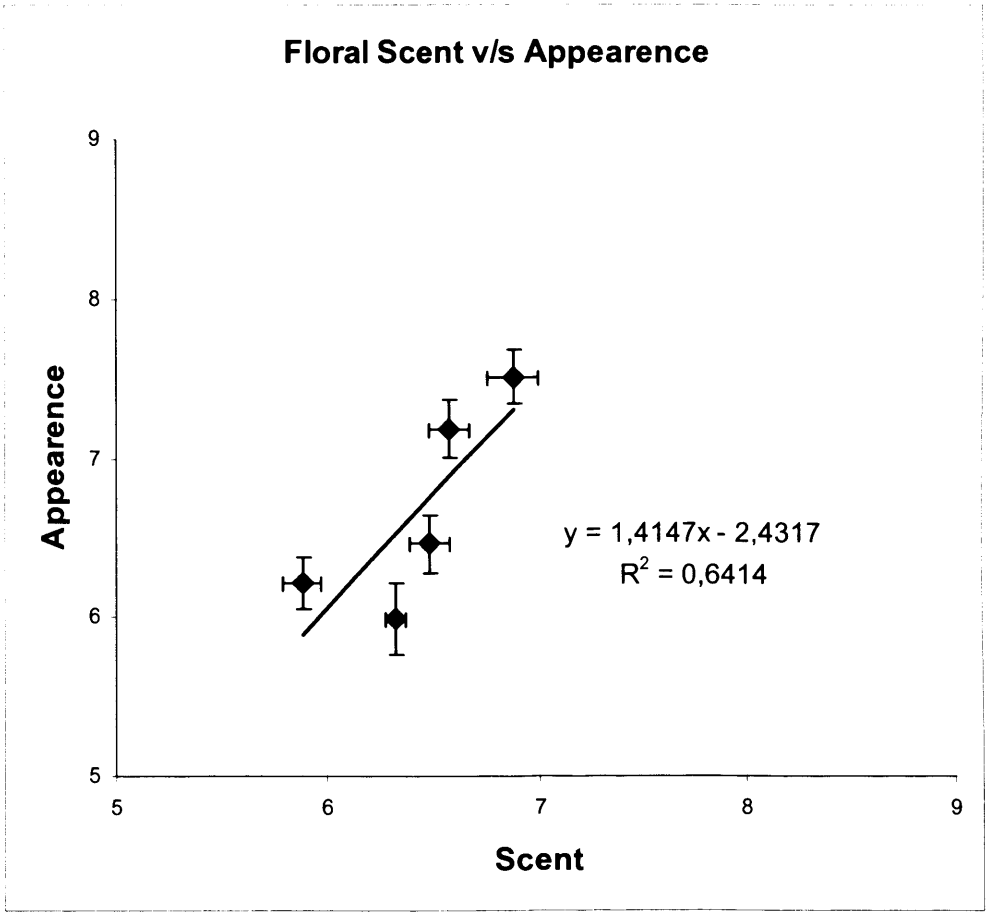

Figure 7.17. Means of floral scent and floral appearance liking (± SE, n=70) evaluated on 5 new lines of *A. caryophyllaea*. The scale ranges from 'like extremely (= 9) to 'dislike extremely' (= 1).

7.3.6.6. Characters more appreciated

The character most appreciated by the volunteers at the moment they buy or look at flowers was 'Flower colour' with an average value of 4.9 (out of 5). Floral scent was also highly rated with a value of 4.2 and was not statistically different from the character 'Flower colour'. Thus, the appreciations of these two characters were located

close to the answers 'strongly Agree' (=5) and 'agree' (=4), respectively. The character least appreciated was 'stem length' with an average value of 3.1, just above the answer 'neutral' (=3). The characters 'Flower size' and 'Vase life' showed mean values of 3.6 and 3.7 respectively (Figure 7.18).

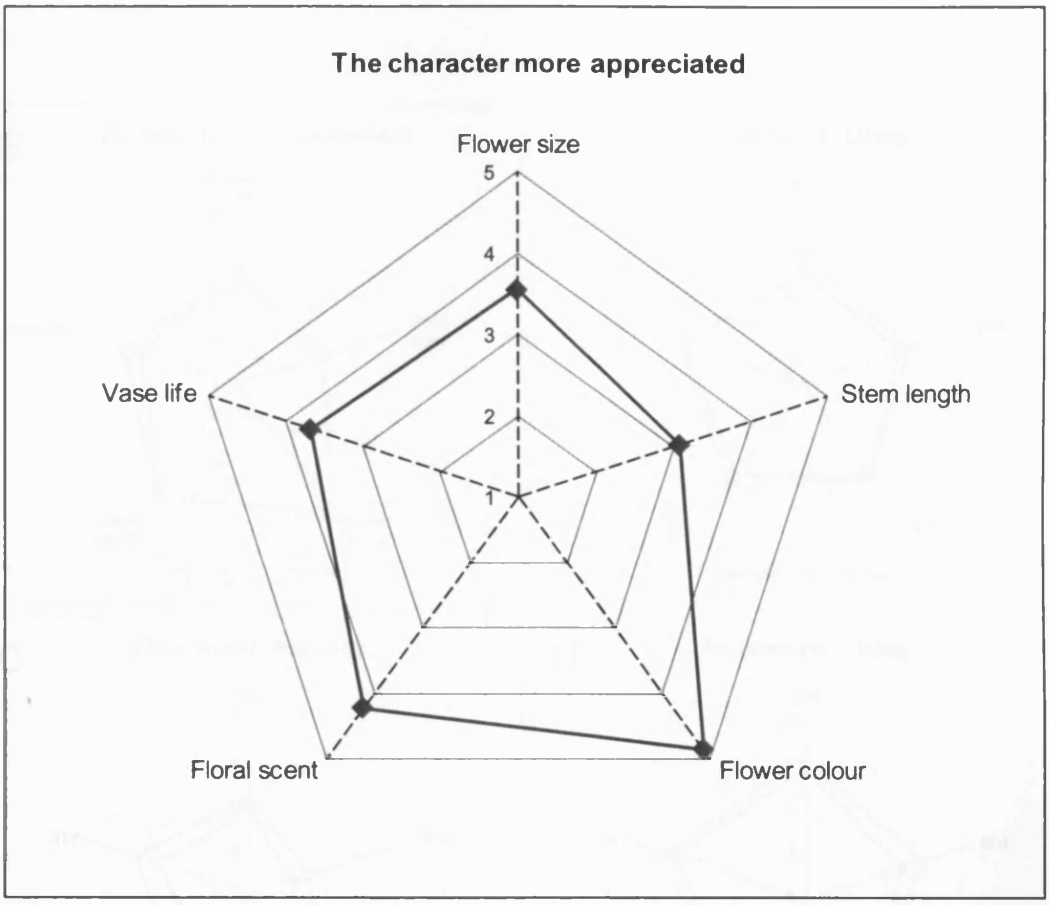

Figure 7.18. Radar graph showing the characters more appreciated by the volunteers when they buy or look at flowers. The scale ranges from 'strongly agree' (=5) to 'strongly disagree' (=1).

7.3.6.7. Differences by category

All the evaluations were analyzed individually by category (i.e. age, sex, 'shopping habits', etc), finding the most important results, with statistically significant differences, when comparing the surveys answered by male and female volunteers.

Thus, only excepting the 'Character more appreciated', where 'Flower size' and 'Stem length' were considered more highly by males rather than females, all the other categories including, 'Floral scent liking', 'Floral scent intensity' and 'Appearance liking', were always rated more highly by females rather than males for all the samples evaluated (Figure 7.19).

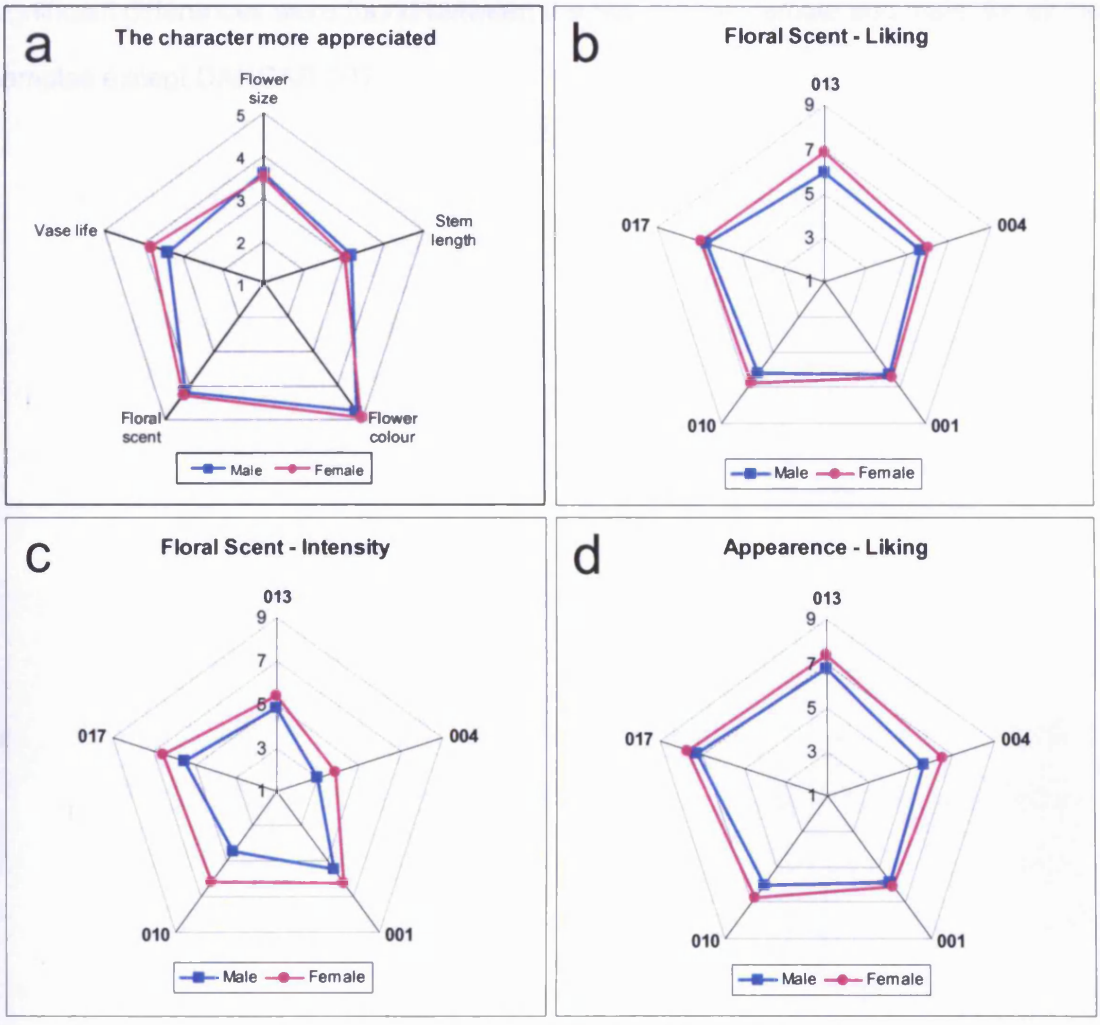

Figure 7.19. Radar graphs showing the sex distribution (male and female) of 'the characters more appreciated' (a), and the evaluation of 'Floral scent liking' (b), 'Floral scent intensity' (c) and 'Appearance liking' (d) on five new lines of *A. caryophyllaea* (DANCAR 001, 004, 010, 013 and 017).

Hence, for the floral scent liking, DANCAR 013 and 010 showed statistically significant differences between evaluations performed by male and female, while the rest of the flowers evaluated, although values were higher from female evaluations compared to male, no significant differences were found. The intensity of the floral scent perceived was statistically different between male and female evaluators for all the samples evaluated, excepting DANCAR 013. For appearance liking, statistically significant differences were found between the two groups, female and male, for all the samples except DANCAR 001.

7.4. DISCUSSION

7.4.1. Morpho-phenolocigal evaluation

Most of the leaves of the new lines were of lanceolate shape while the shape described by Assis (2004) for wild species of *A. caryophyllaea* at floral stage was linear to linear-lanceolate. In this study only DANCAR 003 and 017 showed linear leaves and three lines showed elliptical leaves, a shape not considered in previous descriptions. Environmental effects and management conditions could affect leaf shape and particularly in this study, the lack of light during the flowering period in Cardiff (September-March, 51° 28' N), could promote the growth of the leaves to elliptic shape. Thus to confirm this distribution, further assessments under different growth conditions should be made. The variability within and between lines could also be defined more thoroughly by taking measurements of leaf width and length. However these results indicate a possible underlying leaf shape heterozygosity within the parent species.

Although the flowers had the same basic morphology as their parental species and as those described by Assis (2004) and Foster (1945), the new lines of *A. caryophyllea* obtained showed some distinctive and attractive characteristics. Thus, although the most frequent colour found was the combination of pink and white, mostly white flowers (DANCAR 005) and combinations with red colours (DANCAR 008, 014 and 017) were also observed. The colour of the flowers deserves particular attention since this character was the most appreciated (4.9 out of 5) in the surveys answered during the sensorial analysis performed in this study. Furthermore, this character has also been targeted as one of the most important for Alstroemeria breeders (Bridgen, 2008, Kuipers, 2008, Meijles, 2008). The variability seen in the lines generated

indicates that further breeding could be used to select specific colour attributes from these lines considered of higher commercial value.

Regarding the size of the flowers, statistically significant differences were found among the new lines, and DANCAR 004 showed the largest flowers with averages of 7.48 x 5.45 cm (height x width), above the average of 5 to 6 cm described by Assis (2004). However, this size is still relatively small compared to the traditional commercial cultivars where as 7 to 8 cm is the normal size of most of the new cultivars developed by Royal Van Zanten and Könst, two of the most important Alstroemeria breeding companies. Furthermore, the lack of a proper overlapping between the tepals found in the flowers of all the new lines, has been highlighted as a serious limitation for the market since this character has been described as a requisite for a new cultivar of Alstroemeria (Kuipers, 2008; Meijles, 2008).

A. caryophyllaea has been described in the wild as a flower with dots or stripes only in the internal tepals (Assis, 2004) while during this evaluation, in nine out of thirteen lines assessed, dots or stripes were also found in the higher outer tepal. This is interesting and suggests the possibility of recessive alleles for outer tepal markings in the parent species revealed here.

Differences found in the shape of the leaves were also found and described. However more important in Alstroemeria seems to be the quality of the leaves during flowering, as many wild species show wilting and yellowish leaves before the end of the flowering period (Muñoz and Moreira, 2003; Bridgen, 2008). In all the lines evaluated foliage remained green until the very end of the flowering period suggesting that at least in this character the lines would be of value commercially.

Floral stem length showed significant differences among the different lines. However, although the longest average stem obtained was 70.6 cm (DANCAR 004), this length is still too short for the requirements of almost 100 cm for new cultivars of Alstroemeria cut flowers (Kuipers, 2008, Meijles, 2008). Assis (2004) described wild accessions of *A. caryophyllaea* as having stem length between 40 and 70 cm. Thus in order to improve this character, special crop management regimes or further interspecific crosses with more vigorous Alstroemerias would perhaps be necessary.

A very important parameter, particularly for growers although not for consumers, was also evaluated: productivity. In terms of number of flowers per floral stem, excepting DANCAR 006, 009 and 014, all the lines were under the average of five flowers per stem, necessary for commercialization. According to Royal Van Zanten and Könst, most of the new cultivars of Alstroemeria under optimal conditions, produce flowers all year round with production estimated at over 200 stems per m^2 per year. Although the production evaluated for the DANCAR lines is well below the standards for the commercial cultivars, is important to consider that these plants have been grown under standard conditions (i.e. no special program of fertilization or supplementary light), moreover the evaluations during the first season were made on flowers produced by new plants coming from seeds and not established crops. Although during the second season the productivity increased slightly, probably because the production came from more established plants, still the number of flowers produced was far below commercial standards (Figure 7.12).

Thus, the evaluations performed in this study lead to a categorization of all the new lines of *A. caryophyllaea* obtained as not suitable for commercial purposes according to the market standards. However, these lines could be considered as a promising starting point for further breeding through other methodologies (i.e. interspecific crosses) and thus, improve the characters spotted as deficient.

Furthermore, the introduction in the market of a completely new category of Alstroemeria could be an encouraging challenge to face since the market is currently looking for new types of Alstroemeria to commercialize not only as part of bouquets but also as single flowers (Kamminga, 2008). Hence, new cultivars with characteristics below the conventional standards required but with a distinctive trait, in this case scent, could be perhaps an opportunity to renew the Alstroemeria market.

7.4.2. Sensorial Analysis

7.4.2.1. Correlation: floral scent liking v/s floral scent intensity

A consistent correlation was found between intensity and liking of scent, with the higher the intensity the higher the liking (Figure 7.16). Correlations between intensity and hedonic judgement have been studied using synthetic fragrances, finding some fragrances with a positive correlation between liking and intensity, some with a negative correlation, and some others showing a variable inverted U-shaped function (Moskowitz, 1977; Moskowitz *et al.*, 1976; Doty, 1975). In this study the correlation found between intensity and liking of floral scent is clearly positive as found in a more recent and complex study performed by Distel *et al.* (1999) where intensity of everyday odours, perceived by different ethnic groups, was always positively correlated to hedonic judgment.

7.4.2.2. Correlation: floral scent liking v/s appearance liking

A positive correlation was also found when comparing floral scent liking versus appearance liking (Figure 7.17). Evidence of interactions between visual and olfactory stimuli have been previously described (Demattè *et al*, 2009; Walla, 2007), thus even though volunteers were advised to evaluate liking of the scent and liking of the appearance independently, a clear interaction between these two parameters has been detected. The positive correlation between scent and appearance is not surprising as these two stimuli (olfactory and visual) have been described to work on this way (Walla, 2007). Thus a positive visual perception will often be accompanied by a pleasant perception of odour, however not the other way round as there is no evidence of odour influencing visual perception (Thesen *et al.*, 2004).

Moreover, evidence of chromatic stimuli modulating odour responses in the human brain have been described (Demattè *et al*, 2006; Österbauer, 2005; Gilbert *et al*, 1996) although colour-smell associations are most likely acquired and may be subject to variation between cultures. However, in some cases these associations are quite clear (i.e. yellow—lemon). In this study an interaction between colour and scent might be present. Thus the only red flower evaluated (DANCAR 017) was the sample highest rated on both its appearance and floral scent, while the pale and light pink flowers of DANCAR 001 and 004 respectively, were the lowest rated on both their appearance and floral scent. Previous studies have found positive correlations between strong colours and scent intensity (Zellner and Kautz, 1990), thus the red colour of DANCAR 017 perhaps affected positively the perception of the intensity and subsequently, as discussed previously in 'liking v/s intensity', the liking of the scent. Conversely, pale and light tones of pink could affect negatively the perception of the intensity of the scent and thus, the liking of the scent.

7.4.2.3. Male v/s female floral scent perception

Differences in the sense of smell according to gender have been widely reported showing that women always perform better than men in olfactory tasks (Larsson *et al.*, 2003; Larsson *et al.*, 2004; Barber; 1997; Ship *et al.*, 1996). Supporting this information, Yousem *et al.* (1999) showed that women activate up to eight times more voxels in the frontal and temporal lobes than men when exposed to the same odorants. Hence the results obtained from this study (Figure 7.19) are consistent with previous studies, showing women rating higher both the intensity and liking of the floral scents evaluated in all the samples. This statement probably lead women to also rate highly the appearance of all the samples evaluated, according to the correlation between floral scent liking and appearance previously discussed.

CHAPTER VIII.

SENSORIAL ANALYSIS OF FLORAL SCENT

VIII. SENSORIAL ANALYSIS OF FLORAL SCENT

8.1. INTRODUCTION

It is well known that fragrance is ubiquitous in nature playing the important functions of both helping to locate food and attracting mates for reproduction. Considering these important roles it has been of increasing interest to understand food and fragrance choice based on odour and flavour preferences (Prescott *et al.*, 2007). In this sense, taste qualities are relatively fixed and innate and controlled by hedonic responses (Steiner *et al.* 2001). On the other hand odours seem to acquire their hedonic attributes by different learning mechanisms after exposure. Thus bad or good tastes or experiences are correlated with the smell sensory stimulus triggering negative or positive hedonic responses (Baeyens *et al.*, 1990; Zellner *et al.*, 1983).

A molecule is able to impart an odour sensation in humans when it has a minimum degree of volatility in order to reach the upper nasal cavity where the olfactory epithelium is located (Thomson, 1987). Since there is a large gene family of odorant receptors connected to the human olfactory system, we are able to recognize and discriminate between a vast variety, in the order of thousands, of odour molecules. Furthermore this recognition seems to be very specific as a single olfactory neuron is believed to express a single odour receptor gene (Zhao and Firestein, 1999). This specific recognition also depends on the concentration of the volatile compound, thus the organoleptic perception threshold can be extremely low detecting molecules at a concentration of 10^{-8} M or even less (Thomson, 1987).

In the particular case of floral scent, the human perception of a certain volatile compound in an aroma mixture does not necessarily reflect its absolute concentration in the mixture, but depends on the perception threshold. For some compounds our olfactory system could be more sensitive than analytical tools evaluating floral scent (Hinterholzer and Schieberle, 1998). Nevertheless human evaluation or sensorial analysis has also some disadvantages i.e. the lack of specific words to characterize specific scents, describing them only with subjective terms such as "woody," "fruity," "musty," etc. (Burdock, 1995). Hence an objective evaluation of floral scent may need specialized instruments like GC-MS, for example to establish correlations with the information obtained from sensorial analysis.

Little is known about sensorial analysis of floral scent, and to my knowledge, the only investigations have been published by Morinaka *et al.* (2001) and Yoichi *et al.* (2002), including the study of floral scents from different species including freesia, lily, rose, and carnation, among others.

The objective of this research was to evaluate the liking of different floral scents and its interaction with visual stimuli.

8.2. METHODOLOGY

8.2.1. Subjects

A total of forty persons including males and females with no restriction of age were included in the evaluation of five different floral scents. They were advised via e-mail and their participation was absolutely voluntary. Volunteers evaluated each sample individually and the analysis was carried out in a laboratory at Cardiff University, School of Biosciences. An ethical approval was obtained from Cardiff University, BIOSI Research Ethics Guidance Group, confirming that ethical issues and health and safety measures were in accordance with University policy and the research ethics guidelines.

8.2.2. Stimuli

The floral scents from two cultivars of Alstroemeria were evaluated: *A.* cv. 'Sweet Laura' (SL) and *A.* cv. 'Ajax' (AJ). Furthermore, the following flowers were evaluated in order to compare and differentiate them from Alstroemeria flowers: Freesia (white) (FR); Rose cv. 'Passion Kero II' (RO) and Peony cv. 'Shirley Temple' (PE). Freesia and Rose are well known scented flowers while Peony is novel species used as a cut flower and not well known as a scented flower.

The samples were identified by a three-digit numerical code and presented both as 'Covered' (Figure 8.1 A and B) and 'Exposed flowers' (Figure 8.2) in order to evaluate how visual stimulus affects the olfactory perception. 'Covered flowers' were

single flowers enclosed in 300-ml covered jars with 50 ml of water. Two replicates per sample were provided to the subjects asking them to smell randomly only one of the replicates.

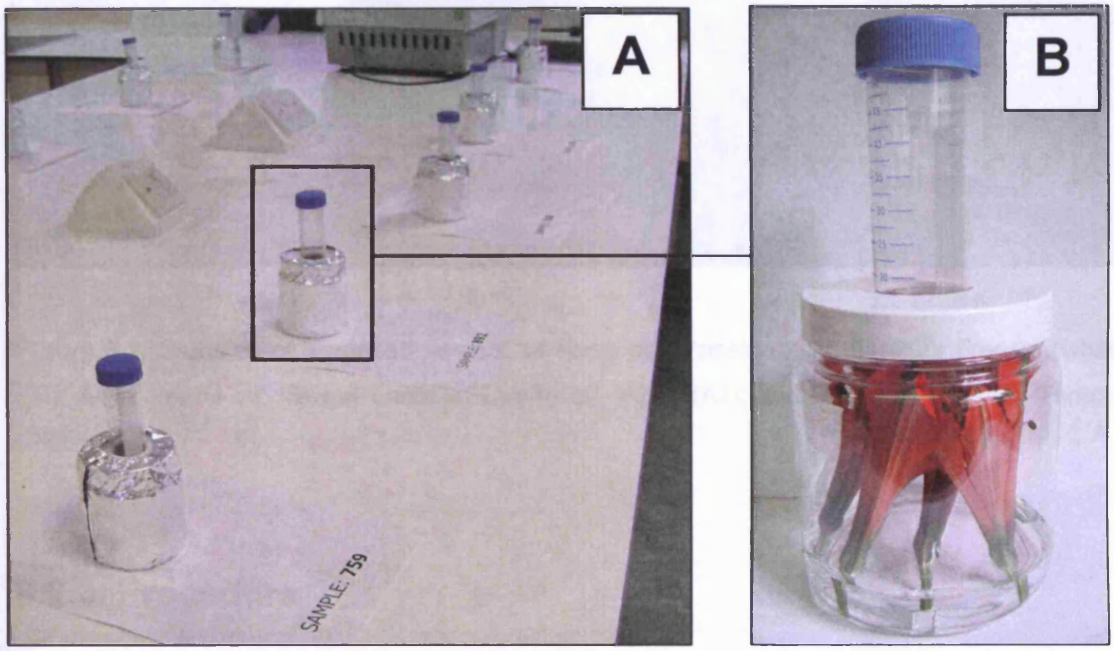

Figure 8.1. Samples of flowers enclosed and covered in jars (A) and before covering (B) with foil paper.

'Exposed flowers' were a bunch of flowers in vases with water and presented as they would normally be sold in the market. Only one replicate per sample was provided for scent assessment (Figure 8.2).

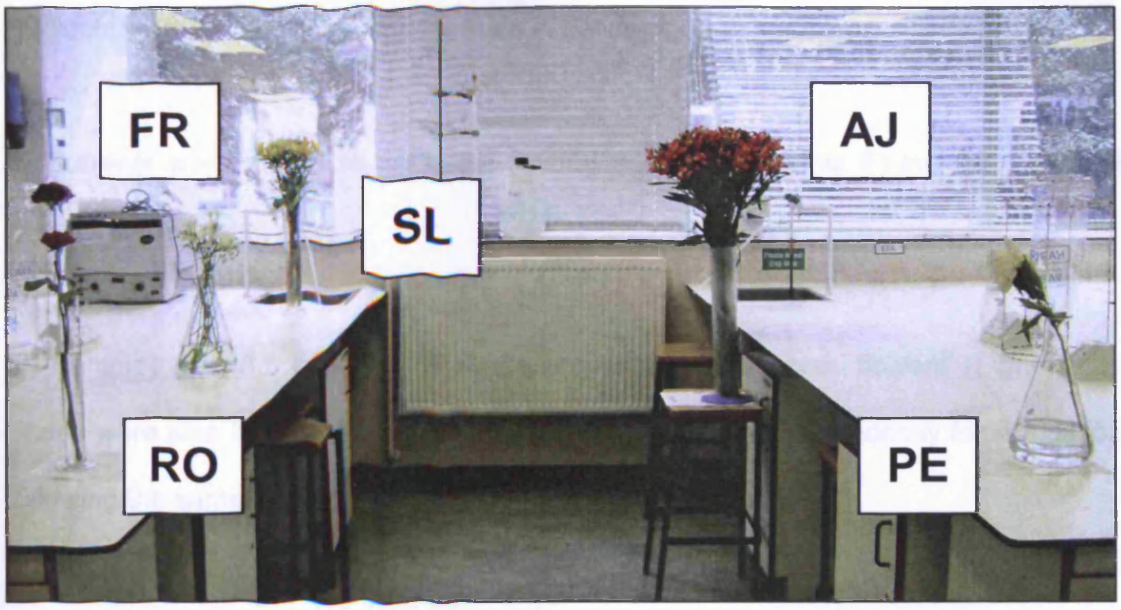

Figure 8.2. Samples of 'Exposed flowers' of Rose cv. 'Passion Kero II' (RO); Freesia (white) FR); *Alstroemeria* cv. 'Sweet Laura' (SL) and cv. 'Ajax' (AJ); and Peony cv. 'Shirley Temple' (PE).

8.2.3. Procedure

The procedure was separated into four phases (I to IV) including a survey for each. Moreover an "Information sheet" (Appendix F) was provided to the evaluators before the sensorial analysis to give them some instructions and explain the main considerations related to the evaluation.

I) Samples (SL, AJ, FR, RO, PE) enclosed in covered jars (Figure 8.1) were labelled with a 3 digit-number and presented randomly to be evaluated. Subjects were asked to smell each jar through a tube and then to evaluate the extent to which they liked the scent by ticking a box on a response sheet (Appendix F) with the following hedonic scale: 'Like extremely'; 'like very much'; 'like moderately'; 'like slightly'; 'neither like nor dislike'; 'dislike slightly'; 'dislike moderately'; 'dislike very much'; 'dislike extremely'.

Subjects were asked to wait for 1 minute in between each sample.

II) Subjects were asked to complete a short survey (Appendix F) including general questions (Sex, age, etc.).

III) Samples (SL, AJ, FR, RO, PE) were presented as 'Exposed flowers' (Figure 8.2). These were also labelled with a 3 digit-number and presented randomly for evaluation following the same procedure described in phase I (Appendix F).

IV) After this evaluation another survey was performed (Appendix F) in order to assess relative importance of floral scent as a character when buying or looking at flowers. The question asked was – 'When you buy/look at flowers the character you appreciate more is' – and the scale included: 'strongly agree'; 'agree'; 'neutral'; 'disagree' and strongly disagree'.

8.2.4. Statistical analysis

Both the hedonic scale and the agreement scale were translated into scores. For the hedonic scale the scores were: Like extremely = 9, dislike extremely = 1, and for the agreement scale the scores were: Strongly agree = 5, strongly disagree = 1. In each case, standard deviation (STEDV) and standard error (SE) were calculated. Analysis of variance (ANOVA) was performed through SPSS 17.0 for Windows, using Tukey's HSD (Honestly Significant Difference) test with a significance level of 0.05 (Appendix H, Tables H.1 to H.4).

8.3. RESULTS

8.3.1. Characteristics of the population

Forty persons were evaluated including 28 women (70%) and 12 men (30%) ranging from 22 to 51 years old with the following distribution: 49% between 22 and 28 years; 23% between 29 and 35 years; 13% between 36 and 42 years; and 15% of people older than 43 years old (Figure 8.3).

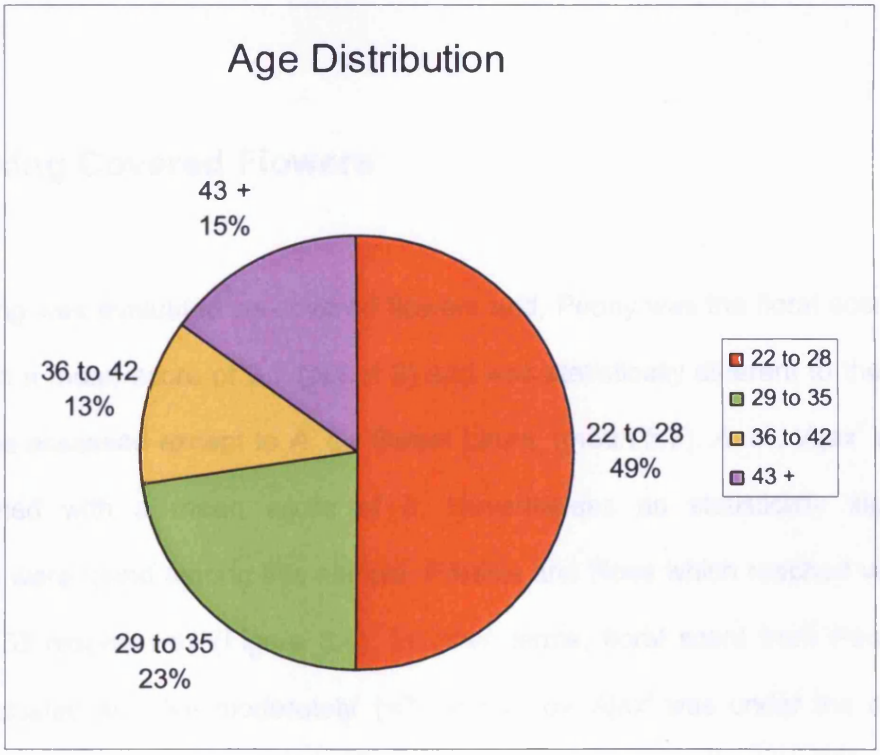

Figure 8.3. Pie graph showing the age distribution of the people who performed the sensorial analysis.

Regarding the activity of the subjects, the main group was 'Postgraduate students' (47%) and 'Non teaching staff' (30%). Teaching staff comprised 15% of the

sample and two minority groups also included were 'Undergraduate student' (3%) and 'Unemployed/Other' (5%) (Appendix H).

A group of 62% of the total sample declared being 'Non smoker' while 23% were 'Occasional smokers' and 15% were 'Regular smokers'.

When they were asked the question - 'How often do you buy flowers?' - only 5% answered 'once per week' (only women), 13% answered 'once per month' and the main group (74%) declared to buy flowers only for 'special occasions'. 8% of the sample replied they never buy flowers (Appendix H).

8.3.2. Liking Covered Flowers

Liking was evaluated on covered flowers and, Peony was the floral scent rated highest with a mean score of 6.7 (out of 9) and was statistically different to the rest of the samples assessed except to *A.* cv 'Sweet Laura' (mean 5.7). *A.* cv. 'Ajax' was the poorest rated with a mean score of 5. Nevertheless no statistically significant differences were found among this sample, Freesia and Rose which reached values of 5.3 and 5.53 respectively (Figure 8.4). In other terms, floral scent from Peony was almost evaluated as 'Like moderately' (=7) and A. cv 'Ajax' was under the category 'neither like nor dislike' (=5).

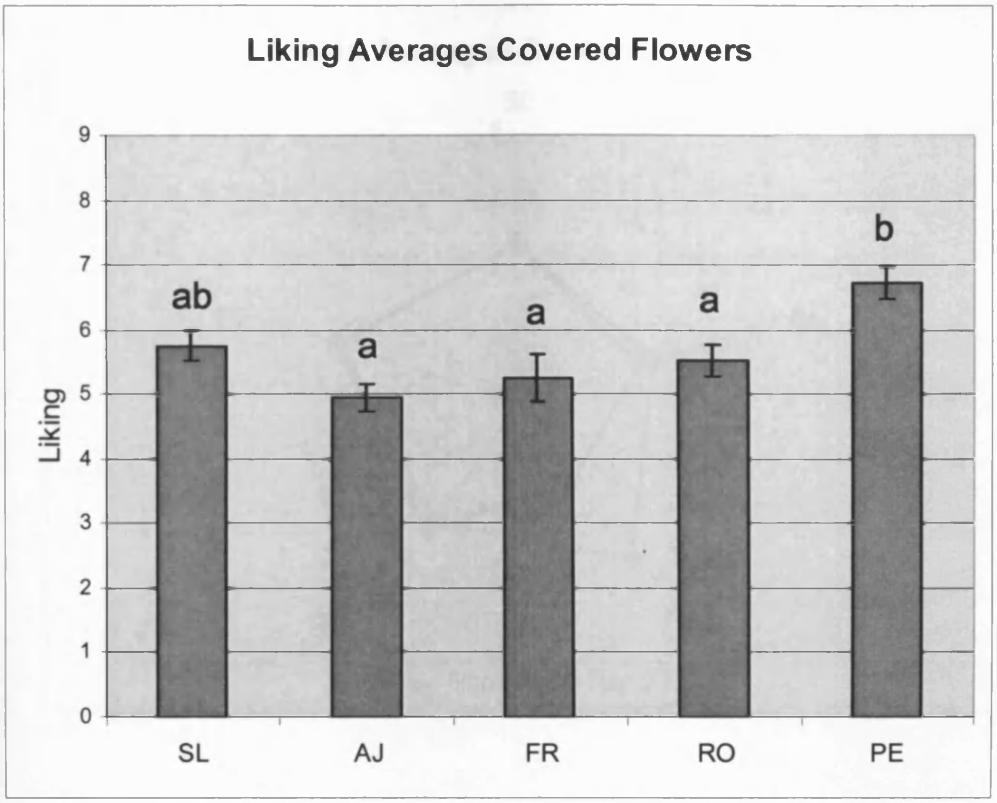

Figure 8.4. Means of floral scent liking (± SE, n=40) evaluated on covered flowers of *A.* cv. 'Sweet Laura' (SL), *A.* cv. 'Ajax' (AJ), Freesia (FR), Rose cv. 'Passion Kero II' (RO) and Peony cv. 'Shirley Temple' (PE). The scale ranges from 'Like extremely (= 9) to 'Dislike extremely' (= 1).

As mentioned in the methodology two replicates were included for each sample although only one replicate was assessed by each volunteer. The results from each replicate were analyzed independently, and no statistical differences were found between the two replicates in all the samples analyzed except in Freesia where a statistically significant difference was found between replicate 1 (3.9) and 2 (6.7) (Figure 8.5).

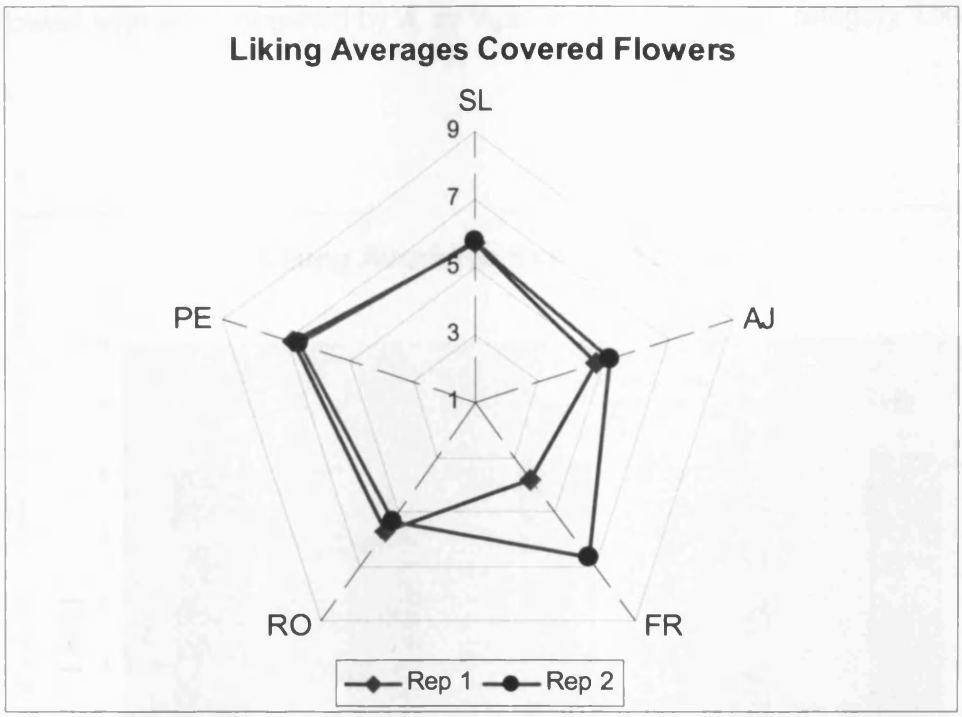

Figure 8.5. Radar graph showing floral scent liking from two replicates evaluated on covered flowers of *A.* cv. 'Sweet Laura' (SL), *A.* cv. 'Ajax' (AJ), Freesia (FR), Rose cv. 'Passion Kero II' (RO) and Peony cv. 'Shirley Temple' (PE). The scale ranges from 'Like extremely (= 9) to 'Dislike extremely' (= 1).

8.3.3. Liking Exposed Flowers

Liking evaluation of exposed flowers found Peony, Freesia and *A.* cv 'Sweet Laura' as the floral scents rated most highly with values of 7.2, 7.1 and 7.0 respectively and no significant differences were observed among them. The lowest values were for *A.* cv. 'Ajax' (5.9) and rose (6.6) although the value for rose was not significantly different to those from peony, freesia or A. cv. 'Sweet Laura'. (Figure 8.6).

Regarding the hedonic scale described in the methodology, floral scent from Peony, Fressia and *A.* cv 'Sweet Laura' were evaluated as 'Like moderately' (=7) and

the lowest evaluation obtained by *A.* cv 'Ajax' was almost in the category 'Like slightly' (=6).

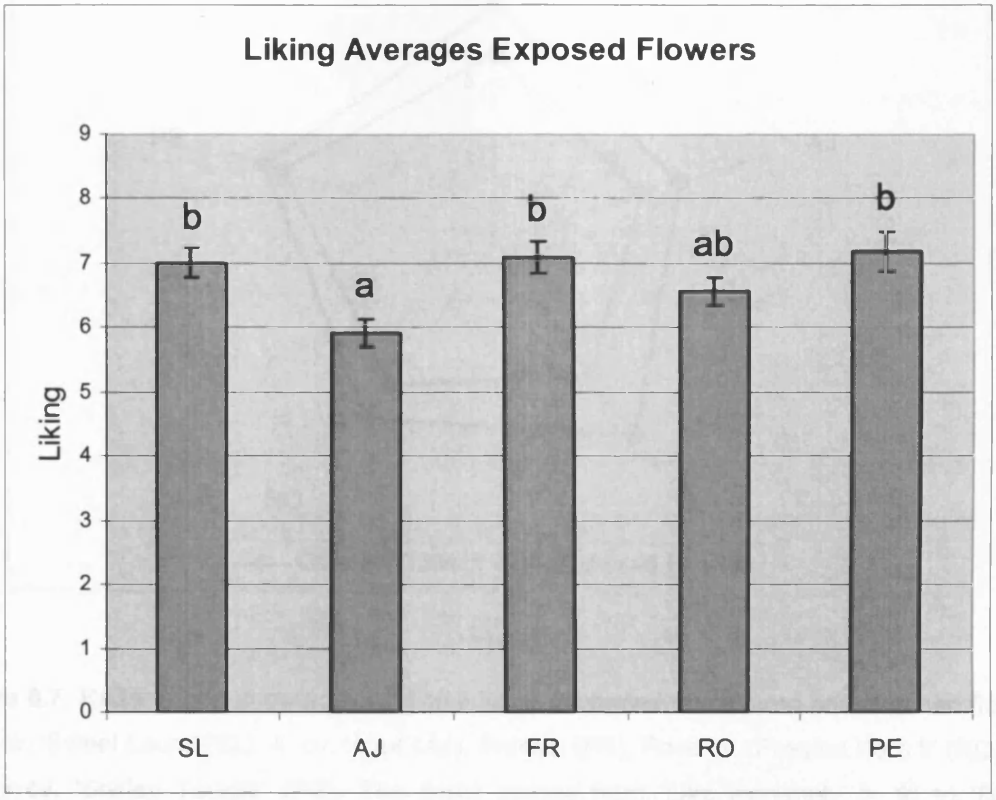

Figure 8.6. Means of floral scent liking (± SE, n=40) evaluated on exposed flowers of A. cv. 'Sweet Laura' (SL), A. cv. 'Ajax' (AJ), Freesia (FR), Rose cv. 'Passion Kero II' (RO) and Peony cv. 'Shirley Temple' (PE). The scale ranges from 'Like extremely (= 9) to 'Dislike extremely' (= 1).

8.3.4. Liking Exposed v/s Covered Flowers

Differences between values found during the evaluation of covered and exposed flowers were analyzed, finding that all the samples were evaluated more highly in exposed rather than covered flowers (Figure 8.7).

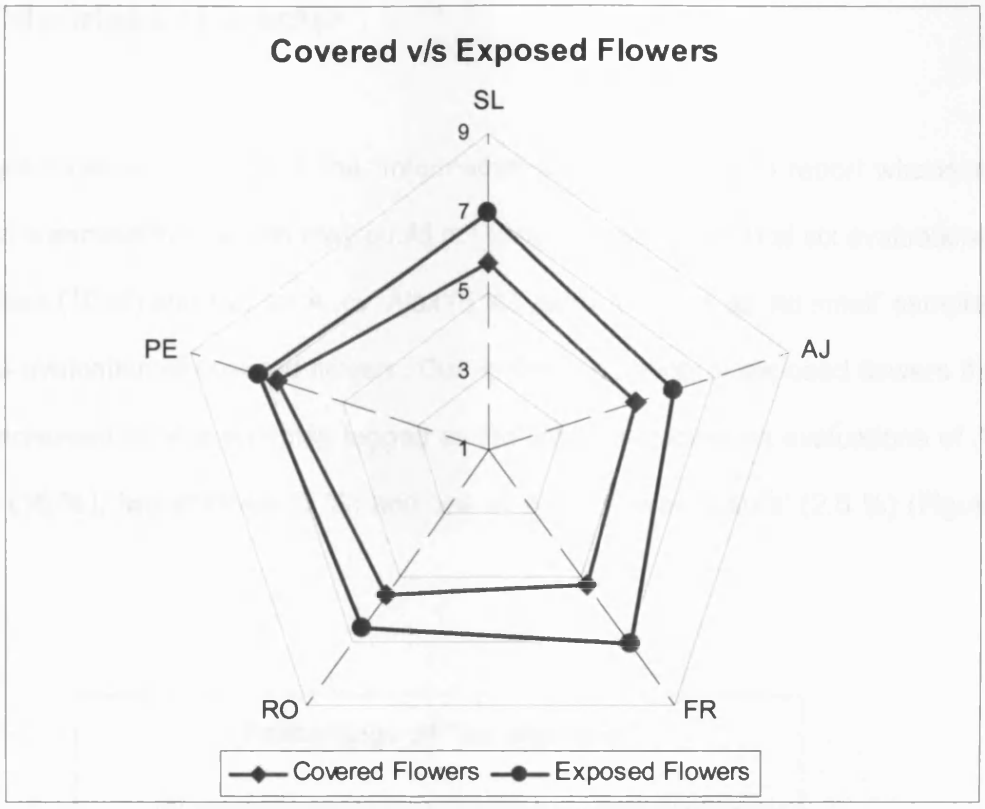

Figure 8.7. Radar graph showing floral scent liking evaluated on covered and exposed flowers of *A.* cv. 'Sweet Laura' (SL), *A.* cv. 'Ajax' (AJ), Freesia (FR), Rose cv. 'Passion Kero II' (RO) and Peony cv. 'Shirley Temple' (PE). The scale ranges from 'Like extremely (= 9) to 'Dislike extremely' (= 1).

In all cases the variation was positive and the highest variation was found in Freesia with a value of +1.83 while in Peony the lowest variation was observed with a difference of +0.45, probably because this floral scent was already the highest rated during the first analysis and remained as the highest in the second experiment. *A.* cvs. 'Sweet Laura' and 'Ajax' and Rose were also valued more highly during the analysis of exposed flowers showing values of +1.25, +0.96 and +1.04 respectively (Figure 8.7).

8.3.5. Odourless character

Evaluators were asked in the "Information sheet" provided to report whenever they found a sample from which they could not smell any scent. In total six evaluations, four for Rose (10 %) and two for A. cv. Ajax (5 %), were reported as 'no smell' samples during the evaluation of covered flowers. During the evaluation of exposed flowers the number increased to nine samples tagged as 'no smell' including six evaluations of A. cv. 'Ajax' (15 %), two of Rose (5 %) and one of A. cv. 'Sweet Laura' (2.5 %) (Figure 8.8).

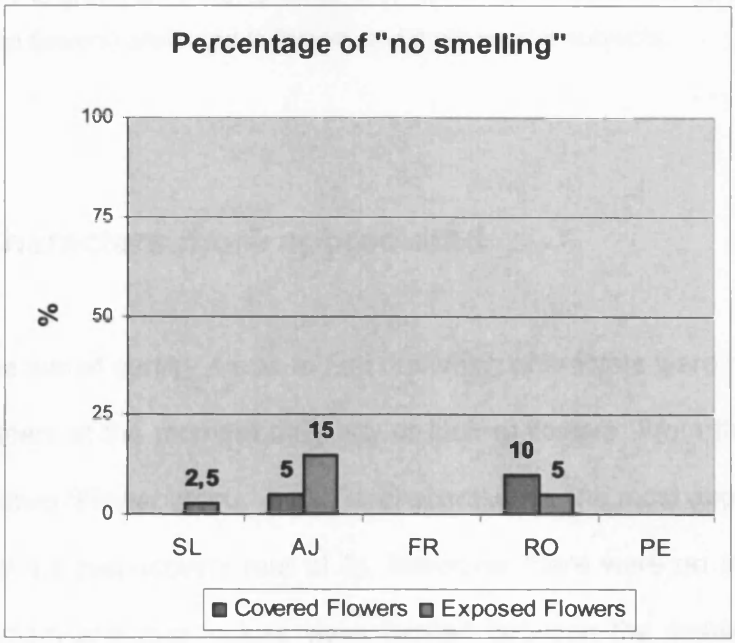

Figure 8.8. Graph showing percentage over the total of all the samples evaluated as "No smell" answers for surveys 1 and 3 (covered and exposed flowers) distributed by samples.

The 'No smell' character was also analyzed by sex finding interesting differences. From 15 'No smell' cases, 14 of them (93 %) were attributed to women while only one case (7 %) was from to men (Figure 8.9).

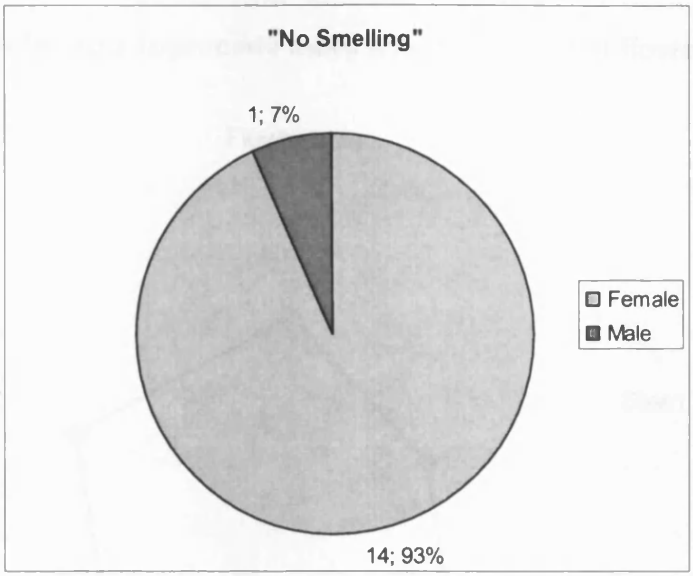

Figure 8.9. Pie graph showing percentage of "No smell" answers for surveys 1 and 3 (covered and exposed flowers) attributed to female and male gender subjects.

8.3.6. Characters more appreciated

The aim of survey 4 was to find out which characters were more appreciated by the volunteers at the moment they buy or look at flowers. From the five options listed the characters 'Flower colour' and 'Floral scent' were the most appreciated with values of 4.8 and 4.4 respectively (out of 5). Moreover there were no statistical differences between them and their values were located between the answers 'Strongly Agree' (=5) and 'Agree' (=4). 'Stem length' was the character least appreciated with a value of 2.75 just under the answer 'Neutral' and showing no statistical differences with 'Flower size' (3.28). The attribute 'Vase life' was rated with a value of 3.73, just below the answer 'Agree' (=4) (Figure 8.10).

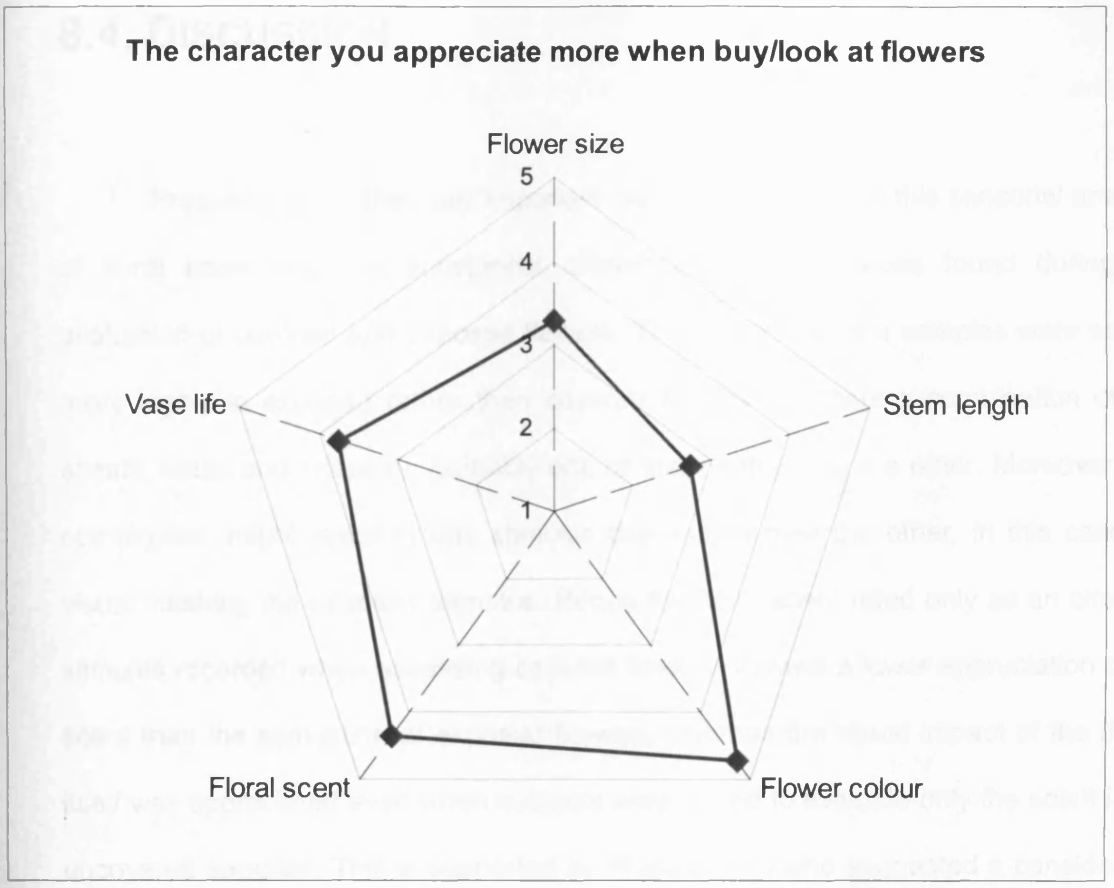

Figure 8.10. Radar graph showing the characters more appreciated by the volunteers when they buy or look at flowers. The scale ranges from 'Strongly agree' (=5) to 'Strongly disagree' (=1).

8.4. DISCUSSION

Probably one of the most important results obtained from this sensorial analysis of floral scent was the substantial difference between values found during the evaluation of covered and exposed flowers. The fact that all the samples were scored more highly in exposed rather than covered flowers suggests a combination of two stimuli, visual and olfactory, probably one of them enhancing the other. Moreover, this combination might result in one stimulus dominating over the other, in this case the visual masking the olfactory stimulus. Hence the floral scent rated only as an olfactory stimulus recorded when assessing covered flowers showed a lower appreciation of the scent than the evaluation of exposed flowers, whereas the visual impact of the flower itself was appreciated even when subjects were asked to evaluate only the scent in the uncovered samples. This is supported by Walla (2007) who suggested a considerable interaction between olfaction and vision based on anatomy since there are strong connections between virtually all cortical regions of the brain via corticocortical and thalamocortical pathways. However there is no evidence of negative or positive effects in the olfaction – visual interactions, whereas in this investigation a positive effect in the overall perception is suggested. Furthermore, Thesen *et al.* (2004) argued that visual-odour interactions seem to be a unidirectional effect as effects of vision on olfactory perception have been reported, but little is known about whether odours can influence visual perception. So we can assume that visual perception of the flowers may affect the floral scent evaluation but not the other way round. Thus even though the evaluators were told not to pay attention to the flowers but only to the floral scent, the positive effect on the floral scent evaluation is evident in the results, probably because of the positive influence of visual stimuli they received while they looked at the flowers.

Intensity of the scent it is an important factor that might affect the liking. This question was not formally included in any survey, however some of the evaluators commented about the intensity at the end of Survey 4. They found exposed and covered flowers of Rose as the floral scent perceived most poorly including a few comments about how weak the scent was and six 'no smelling' answers. Also some specific comments were made about how disappointed the subjects felt as rose is a familiar scent and they were expecting something much better and stronger. Moreover, Peony together with Freesia were identified as the most intense scent samples by the evaluators.

Concerning the survey related to evaluating the character most appreciated in flowers, it is possible that since evaluators were asked for a 'Floral scent evaluation' just before this survey, they may have overvalued this character. Despite this fact, floral scent could be an interesting attribute to consider during breeding programmes as most of the current programmes are focused on other qualitative and productive characteristics (Meijles, 2006).

CHAPTER IX.

FINAL CONCLUSIONS AND FURTHER WORK

IX. FINAL CONCLUSIONS AND FURTHER WORK

9.1. THE ALSTROEMERIA MYRCENE SYNTHASE *ALSTER*

A novel gene isolated from *Alstroemeria* cv. 'Sweet Laura' flowers (*ALSTER*) has been characterized considering its amino acid sequence, signal sequence, genomic organization (Chapter IV) and catalytic activity of the associated enzyme (Chapter VI) (Table 9.1).

Table 9.1. Summary of the different characterizations of the Alstroemeria myrcene synthase *ALSTER*.

Characterization	Description	Classification
Aminoacid sequence	567 aa	TPS-b / Angiosperm mono TPS (Bohlmann *et al*, 1998)
Signal sequence	27 aa	Plastid targeting sequence ('Predotar v. 1.03' and 'ChloroP 1.1 Server')
Genomic organization	5 introns and 6 exons	Class III / Angiosperm TPS (Trapp and Croteau, 2001)
Functional characterization	Production of myrcene when incubating with GPP	Alstroemeria myrcene synthase

Although only the functional characterization of *ALSTER* revealed the real properties of this enzyme as a myrcene synthase, all the previous characterizations

rightly classified it as an angiosperm mono TPS and angiosperm TPS through the amino acid sequence analysis and genomic organization respectively. Furthermore, the prediction of the targeting function of the *ALSTER* signal sequence was possibly correct as myrcene, like all the other monoterpenes, are supposed to be synthesized in the plastids (Douglas *et al.*, 1995). However, reporter protein localization studies should be performed to elucidate the actual target of the *ALSTER* signal sequence.

9.1.1. *ALSTER* as member of a TPS gene subfamily

Quantitative results obtained through real time qRT-PCR in Chapter V, revealed that the highest expression of *ALSTER* was in *A.* cv. 'Sweet Laura' (Figure 5.16). This agrees with the fact that a richer bouquet of fragrance was detected in these flowers by the GC-MS in Chapter III (Figure 3.5). Looking at the alignment of different clones obtained through the RACE performed (Figure 4.4) where some divergence was found, there is a possibility that the primers used to evaluate gene expression, although designed in well conserved regions, could still amplify alternative sequences with divergence in other regions of the ORF, corresponding perhaps to different genes. This hypothetical scenario would suggest that the expression evaluated in this study perhaps does not correspond exclusively to a single TPS but to a several TPS members of a closely related family gene, that coordinate the biosynthesis of some of these volatile compounds found in *A.* cv. 'Sweet Laura', producing an additive accumulation of transcripts.

This coordinated expression of different TPS genes suggested for *A.* cv. 'Sweet Laura' is less likely in *A.* cv. 'Ajax' or *A. caryophyllaea*, where only one volatile compound was detected by the GC-MS analysis in both cases (Figures 3.6 and 3.8

respectively). A good correlation between accumulation of terpenoid compounds and up regulation of a TPS has been found in Hop, where increasing levels of the sesquiterpenes caryophyllene and humulene detected by GC-MS, were closely correlated to high transcript levels of the hop sesquiterpene synthase 1 (HlSTS1), evaluated through real time qRT-PCR (Wang *et al.*, 2008).

In order to unravel this problem, more RACE should be performed using specific primers located to the divergent regions and subsequently obtain the full length sequence of these putative different clones.

9.1.2. *ALSTER* as a polyfunctional enzyme

Another hypothesis to explain the higher expression of TPS found in *A.* cv. 'Sweet Laura' compared to other scented genotypes during the qRT-PCR analysis (Chapter V), could be the possibility of considering *ALSTER* as a polyfunctional enzyme. That is a single terpene synthase controlling the biosynthesis of more than one terpenoid compound. Hence *ALSTER* could be responsible itself for the biosynthesis of more than one of the volatile compounds found in *A.* cv. 'Sweet Laura'. TPS controlling the biosynthesis of more than one volatile compound have been widely described in many species, for example a monoterpene synthase found in *Q. ilex* (*myrS*) was able to produce myrcene and four other cyclic monoterpenes (Fischbach *et al.*, 2001). Furthermore, TPS have been characterized in *M. grandiflora* (Lee and Chapel, 2008) and tomato fruits (Davidovich-Rikanati *et al.*, 2008) with double activity utilizing both GPP and FPP as substrates to produce mono and sesquiterpenes respectively. Thus, higher level of TPS transcripts might be related to the production of many terpenoid compounds, including perhaps both mono and sesquiterpenes.

During the functional analysis performed in this study (Chapter VI), *ALSTER* was able to convert GPP only into myrcene and no activity was observed with FPP. However, considering that this experiment was conducted under *in vitro* conditions, the *in vivo* scenario perhaps could lead *ALSTER* to produce more than one compound (Discussed in Chapter VI, section 6.4). Thus *ALSTER* might be potentially a polyfunctional enzyme, a hypothesis that could only be proven through more functional analyses, for example by feeding *ALSTER* with other intermediate products and not only GPP and FPP.

9.1.3. Compartmentalization of *ALSTER*

Another aspect that is still inconclusive is the target localization of the *ALSTER* signal sequence. Although in this study the signal sequence of *ALSTER* was predicted as a plastid targeting sequence (Chapter IV) and despite the fact that the monoterpene myrcene produced by this enzyme (Chapter VI) localizes its activity also in plastids (Douglas *et al.*, 1995), only by performing reporter protein localization studies can the actual target of this signal sequence be confirmed. Previous investigations have functionally tested the targeting nature of some TPS by using reporter genes and analysing the transient expression in model plants. Thus using a Green Fluorescent Protein (GFP) as reporter gene, a Magnolia mono TPS was observed in mitochondria and a chloroplasts of Tobacco epidermal cells (Lee and Chapel, 2008), and a snapdragon mono TPS and sesqui TPS were localized in the chloroplast and cytosol respectively by using Arabidopsis protoplasts (Nagegowda *et al.*, 2008).

9.1.4. Presence of *ALSTER* in non scented genotypes

As previously observed in Chapter V, at least some specific regions of *ALSTER* (clones CF421 and CF327 found in *A.* cv. 'Rebecca', section 4.3.1) are present in both scented and non scented genotypes of Alstroemeria. However, the functional analysis performed in Chapter VI confirmed that *ALSTER* isolated from a scented genotype it is definitely able to catalyze the production of the volatile compound myrcene in vitro. Thus it is possible that some differences in the amino acid sequence of *ALSTER* between the scented and non scented genotypes, lead this protein to either produce or not produce myrcene. A previous study (Lee and Chapel, 2008) found minor differences in the amino acid sequences of two Magnolia TPS that leaded one of them to be an inactive form because of the presence of a stop codon in its sequence. Therefore further experiments including the full length sequencing of *ALSTER* from non scented genotypes, and its subsequent alignment with the *ALSTER* already sequenced in the scented *A.* cv. 'Sweet Laura', could demonstrate the existence of differences in their sequences that probably explain the presence or absence of the scent character.

9.2. *ALSTER* FOR BREEDING PURPOSES

The identification and isolation of *ALSTER* as a specific gene present in Alstroemeria and involved in the synthesis of the floral volatile compound myrcene, opens possibilities of using this gene for breeding purposes. Moreover, considering that almost all the new cultivars of Alstroemeria are non scented, breeding aimed at incorporating this trait into new cultivars could be an interesting opportunity to renovate the market.

9.2.1. Marker assisted selection (MAS)

The use of specific regions of DNA as molecular markers for marker assisted selection (MAS) has been widely studied in crop plants to increase the effectiveness of selection in breeding and to shorten the development time of new cultivars significantly (Ribaut and Hoisington, 1998; Young, 1999). However, to be useful to plant breeders, gains made from MAS must be more cost-effective than gains made through traditional breeding based on phenotypic assays (Stromberg *et al.*, 1994). Thus in a hypothetical breeding program for Alstroemeria aimed at including the scent character in new varieties, the progeny could be evaluated at early stages of development by using specific primers to check whether *ALSTER* is present in their genome. However, before the use of *ALSTER* as a molecular marker, possible differences in the amino acid sequence of *ALSTER* in scented and non scented genotypes, as previously discussed in section 9.1.4., should be demonstrated. If such divergent regions are found, primers could be designed to amplify starting in these divergent regions that allow discrimination between scented and non scented genotypes.

9.2.2. Transformation

The insertion of a gene of interest into plants by means of genetic engineering has been successfully used in plant breeding (De Block, 1993; Dale *et al.*, 1993; Birch, 1997). Particularly for TPS, strategies for its transgenic manipulation in plants have been studied (Mahmoud and Croteau, 2002) and some TPS have been successfully inserted in tobacco using *Agrobacterium tumefaciens* (Lee and Chapel, 2008; Nieuwenhuizen *et al.*, 2009) for reporter protein localization studies. Furthermore, overexpression of a lemon basil α-zingiberene synthase gene inserted in tomato, has triggered an increase in terpenoid contents in the fruits (Davidovich-Rikanati, 2008).

Although transformation of Alstroemeria is not routine, Akutsu *et al.* (2004) have described a procedure for the transformation of Alstroemeria by *A. tumefaciens* via callus regeneration. Although more studies are necessary, this offers the possibilities of inserting *ALSTER* in the Alstroemeria genome and thus avoid all the problems that the Alstroemeria breeders have reported when trying to obtain scented flowers by traditional breeding (Kuipers, 2008; Meijles, 2008; Bridgen, 2008).

9.3. ECOLOGICAL ROLE OF FLORAL SCENT

It is clear that the main function of floral scent is to attract and guide pollinators (Ando *et al.*, 2001; Reinhard *et al.*, 2004; Dudareva *et al.*, 2004; Jürgens *et al.*, 2003) and the results of this study supported this role of floral scent, as the maximum scent output and the maximum expression of *ALSTER* were found during the maturation of reproductive organs, ready to be pollinated. Wild species of Alstroemeria have been described as naturally cross pollinated by Hymenopteran insects, however these studies have been performed only in the non scented Chilean species *A. aurea* (Aizen and Basilio, 1998), *A. pallida* (Cavieres *et al.*, 1998) and *A. ligtu* (Botto-Mahan and Ojeda-Camacho, 2000). No information is available on pollinators of the Brazilian scented *A. caryophyllaea*, hence an interesting study to perform would be to investigate whether specific pollinators are attracted because of *A. caryophyllaea* scent as opposed to its morphology, which is fairly similar to the Chilean species. This could provide an explanation for the scent character present in *A. caryophyllaea*, considering that it belongs to a genus where all the rest of the species are non scented. Different methodologies are available to conduct these studies, for example by simply recording the floral visitors in terms of number, species and quantification of pollen loads (Shuttleworth and Johnson, 2009), or by using sophisticated electrophysiological

techniques, in particular gas chromatography (GC) coupled directly to neuronal or single-cell recording (SCR) from the olfactory organs of insects (Birkett *et al.*, 2000; Ulland, 2006).

Based on previous investigations related to environmental effect on pollination ecology (Holtsford and Ellstrand, 1992; Fenster *et al.*, 2004) an alternative study to find out why *A. caryophyllaea* is scented and the other ~59 wild species of this genus are not, could be by characterizing the environment where it naturally grows and try to establish ecological relationships and interactions that perhaps drive *A. caryophyllaea* to produce scent.

9.4. GC-MS VERSUS SENSORIAL ANALYSIS

Although completely different techniques were employed, the evaluations performed using GC-MS and sensorial analysis were both aimed at characterizing the fragrance of Alstroemeria flowers. While through the GC-MS specific volatile compounds were identified, the sensorial analysis allowed an evaluation of the fragrance as a whole bouquet. Both advantages and disadvantages have been previously described for these methodologies and in the present study we can recognize them clearly. Although it has been proven that our olfactory system can be more sensitive than analytical tools (Hinterholzer and Schieberle, 1998), we are unable to identify these compounds independently (Burdock, 1995). Hence even though through the sensorial analysis people were able perhaps to detect a more accurate reading of the floral scent, only by the GC-MS was possible to identify each volatile compound by its name and chemical structure. Comments like 'sweet', 'floral', 'smells like (...)' or 'reminds me (...)' were observed during the sensorial analysis (Appendix H),

but of course none of the participants were able to name any of the volatile compounds. This subject would be better studied by training a panel of evaluators, giving them a certain correlation between fragrances with specific volatile compounds, for example in *A. caryophyllaea* – ocimene. However, as we are not able to discriminate single volatile compounds, further experiments using a trained panel, may still fail in the identification of ocimene in *A.* cv. 'Sweet Laura' fragrance, as this compound is part of a mixture in this flower, including many other compounds.

A review by Vainstein *et al.* (2001) suggested that the evaluation of fragrance is highly complex and linkage between our olfactory response and analyses performed by instruments is one of the main topics to be addressed. An attempt to deal with this problem has been the development of an electronic nose, an instrument that has three elements: an odour-sensor array, a data pre-processor, and a pattern-recognition engine (Craven *et al.*, 1996). A study aimed at correlating human perception of odour quality with responses of an electronic nose was performed, finding high correlation only for some specific descriptors but not for all of them (Burl *et al.*, 2001), thus more studies should be performed aimed at trying to correlate our olfactory system with electronic instruments.

If the main goal is to satisfy consumer demand by obtaining new scented cultivars of Alstroemeria, then definitely the best methodology is sensorial analysis as hedonic evaluation is exclusive for human beings. Maybe one of the big challenges in this sort of experiment is try to objectivize the evaluation as differences in odour perception among ethnic groups (Distel *et al.*, 1999), sex (Larsson *et al.*, 2004; Barber; 1997; Ship *et al.*, 1996; this study in Chapter VII, section 7.4) and age (Fusari *et al.*, 2008) have been described.

CHAPTER X.

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X. REFERENCES

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APPENDIX A

Raw data obtained from the analysis of volatile compounds through GC-MS in *A.* cvs. 'Sweet Laura' and 'Ajax'

Table A.1. Compounds detected in *A.* cv. 'Sweet Laura' flowers through GC-MS analysis using the red fibre, showing the raw value and the relative % of areas under the peak obtained during the morning and afternoon.

SWEET LAURA

		Rep 1 (1 h enclosed)		Rep 2 (2 h enclosed)	
		Morning	**Afternoon**	**Morning**	**Afternoon**
		10 to 11 am	**15 to 16 pm**	**11 to 12 am**	**17 to 18 pm**
Isocaryophyllene	Raw	1029051	1926132	1438502	3517041
	%	53,43	100,00	40,90	100,00
Ocimene	Raw	947241	4120519	1519555	3279807
	%	22,99	100,00	46,33	100,00

Table A.2. Isocaryophyllene detected in *A.* cv. 'Ajax' flowers through GC-MS analysis using the red fibre, showing the raw value and the relative % of areas under the peak obtained during the morning, afternoon and night.

AJAX

	Rep 1 (1 h enclosed)			Rep 2 (2 h enclosed)		
	Morning	**Afternoon**	**Night**	**Morning**	**Afternoon**	**Night**
	09 to 10 am	**17 to 18 pm**	**01 to 02 am**	**10 to 11 am**	**18 to 19 pm**	**02 to 03 am**
Raw	17075	29204	33809	99508	121083	65677
%	50,50	86,38	100,00	82,18	100,00	54,24

APPENDIX B

Identification of a putative Alstroemeria sesquiterpene and diterpene synthase

METHODOLOGY

Design of degenerate primers to amplify a putative Alstroemeria sesquiterpene synthase

Amino acid sequences of enzymes related to the biosynthesis of volatile compounds were searched in NCBI (http://www.ncbi.nlm.nih.gov/) and those closer both taxonomically and functionally to Alstroemeria were selected.

Amino acid sequences of enzymes related to beta caryophyllene and sesquiterpene synthases from different species were selected in order to align them and obtain conserved amino acid sequences putatively expressed also in Alstroemeria.

Sequences were aligned using BioEdit (v. 7.0.5.3) (Hall, 1999) and those segments with at least 6 consecutive highly conserved amino acids were chosen for primer design. The 3' end of the primer is the most important as it is from here that the newly synthesized DNA strand extends. Therefore the terminal 2 amino acids were selected such that they were conserved across all the genes and M and W were preferred and R, L and S were avoided when possible as they have the greater codon degeneracy. Corresponding DNA sequences were aligned and when discrepancies were found a 'wobble' was introduced, i.e. more than one alternative base was assigned to the same position. Wobbles were kept to a minimum. The proposed

forward and reverse primers were matched up for Tm (max and min), product size and complementarity.

Design of specific primers (GRNYL) to amplify a putative Alstroemeria diterpene synthase

Geranylgeranyl diphosphate, (GGDP) is the main precursor of diterpenes during the third biosynthesis phase of this compound (Trapp and Croteau, 2001; Douglas *et al*, 1995). Considering this, a Geranylgeranylated protein (GGprotein) was identified from a cDNA library from *A.* cv. 'Rebecca' (Wagstaff *et al*, 2010) and primers were designed for its amplification.

The primers designed for *GRNYL* amplification were the forward *GRNYLF* (5'-AGAATGGGAGGGAGTAATTT-3') and the reverse *GRNYLR* (5'-CTTTGTATTGCAGCCTTTTT-3'), flanking a nucleotide sequence of 356 bp.

Amplification and cloning of sequences

RNA from flowers of *A.* cv. 'Sweet Laura' and *A.* cv. 'Samora' at stage 4 was extracted following protocol described in Chapter II, section 2.2.1. Following cDNA synthesis, PCR, fragment purification, ligation, transformation and sequencing were performed as previously described in Chapter II, section 2.2.

RESULTS

Design of degenerate primers

From the alignment of two beta caryophyllene synthases and seven sesquiterpene synthases, sequences relatively well conserved among the 9 species analyzed and composed by 7 amino acids each one, were selected for the design of degenerate primers (Figure B.1).

The degenerate primers designed for beta caryophyllene synthase (*BCARYO*) were taken from the amino acid sequences -XRDRXVE- and -TXAIXRW- resulting in the forward *BCARYOF* (5'-GCNMGNGAYMGNATGGTNGA-3') and the reverse *BCARYOR* (5'-CCANCKYTSDATNGCYTCNGT-3'), flanking a nucleotide sequence of 141 bp.

For sesquiterpene synthase (*SESQUI*), the amino acid sequences -XXAKXDF- and -TXEAXXW- were selected for forward and reverse primer design, resulting in the primers *SESQUIF* (5'-RANYTNGCNAARBTNGAYTT-3') and *SESQUIR* (5'-CCANTYRAANGCYTCYTTNGT-3') respectively which amplified a nucleotide sequence of about 500 bp.

Figure B.1. Aminoacid sequence alignment of beta caryophyllene synthase: *Artemisia annua* (AAL79181) and *Oryza sativa* (ABJ16553) and sesquiterpene synthase: *Zingiber officinale* (AAX40664), *Artemisia absinthium* (AAX40670), *A. annua* (AAD39832), *A. dracunculus* (AAX40669), *Elaeis oleifera* (AAC31570), *Fabiana imbricate* AAX40666 and *Myrica gale* AAX40671).

(*) Aminoacid sequences selected for primer design of beta caryophyllene synthase (*BCARYO*) amplification.

(**) Aminoacid sequences selected for primer design of sesquiterpene synthase (*SESQUI*) amplification.

Amplification of *BCARYO* and *SESQUI* primers

A number of assays were performed for amplification trying different PCR conditions changing annealing temperature, primer and $MgCl_2$ concentrations (Table B.1), resulting in a lack of amplification in most cases.

Annealing T°	Primer concentration	$MgCl_2$ concentration	Amplification
58 °C	10 µM	1.5 mM	No
58°C	100 µM	1.5 mM	No
55 °C	100 µM	1.5 mM	No
55 °C	100 µM	2.5 mM	No
60 °C	100 µM	1.5 mM	No
60 °C	100 µM	2.5 mM	No
50 °C	100 µM	2.5 mM	Poor

Table B.1. Assays performed for *BCARYO* and *SESQUI* amplification.

The conditions in which some amplification (poor) was observed for both *BCARYO* and *SESQUI* were found with 40 cycles, 50 °C annealing temperature, 100 µM (10 X) of primer concentration and 2.5 mM $MgCl_2$ concentration. Two bands resulting from the amplification of the primer combinations (SESQUI F + SESQUI R) and (SESQUI F + BCARYO R) were selected for both *A.* cv 'Sweet Laura' and *A.* cv. 'Samora' for further work (Figure B.2).

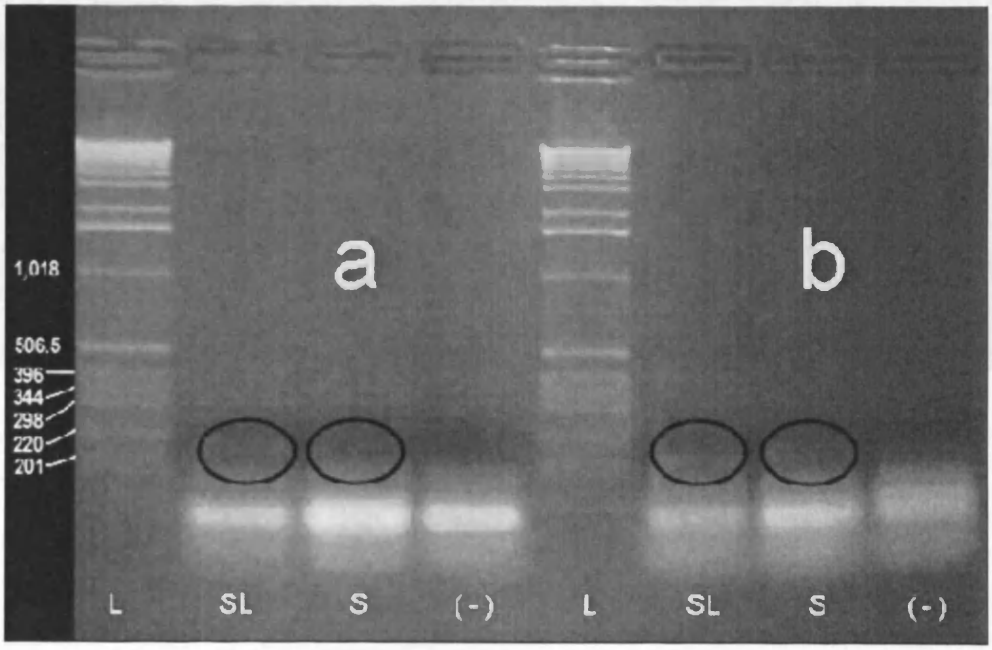


Figure B.2. Amplification of *Alstroemeria* cv. 'Sweet Laura' (SL) and cv. 'Samora' (S) cDNA through primer combinations: a) SESQUI F + SESQUI R and b) SESQUI F + BCARYO R.

Amplification of GRNYL primers

After the evaluation of three different Tm (58 °C, 55 °C and 50 °C), three different primer concentration (10, 20 and 100 µM) and two different concentrations of $MgCl_2$ (1.5 and 2.5 mM) no amplification was achieved for *GRNYL*.

Transformation of putative Alstroemeria sesquiterpene synthase

From the amplification using the primer combination (SESQUI F + SESQUI R and SESQUI F + BCARYO R), a total of 15 colonies were obtained from 'Samora' (Figure B.3) and 9 colonies from 'Sweet Laura' (Figure B.4).

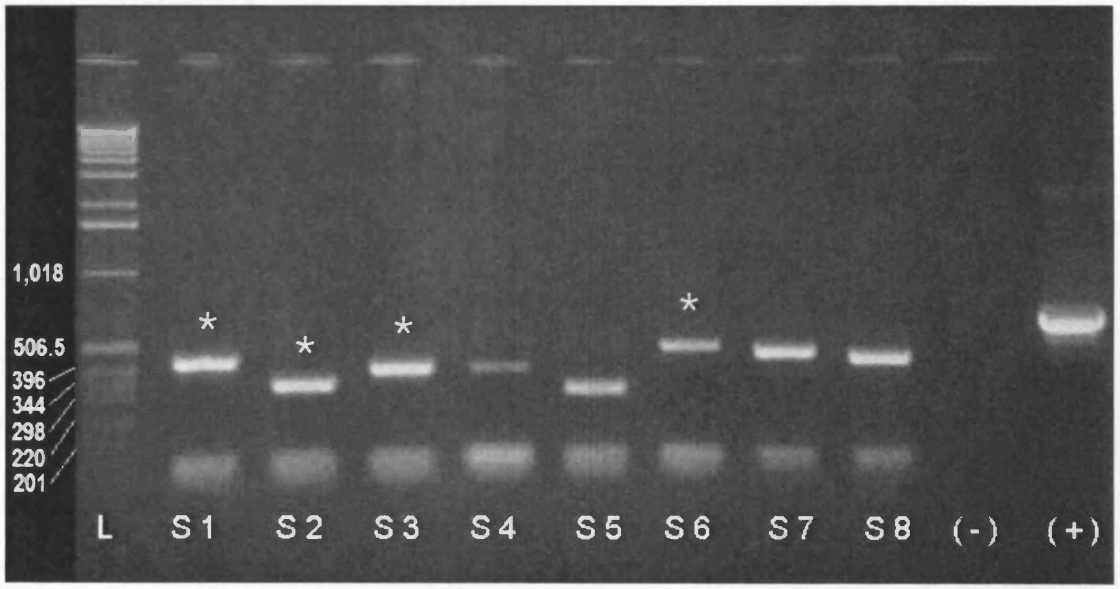


Figure B.3. Amplification of *A.* cv. 'Samora' (S) clones by M13 primers on agarose gel (1.5 %) electrophoresis. (*) Clones selected for further sequencing.

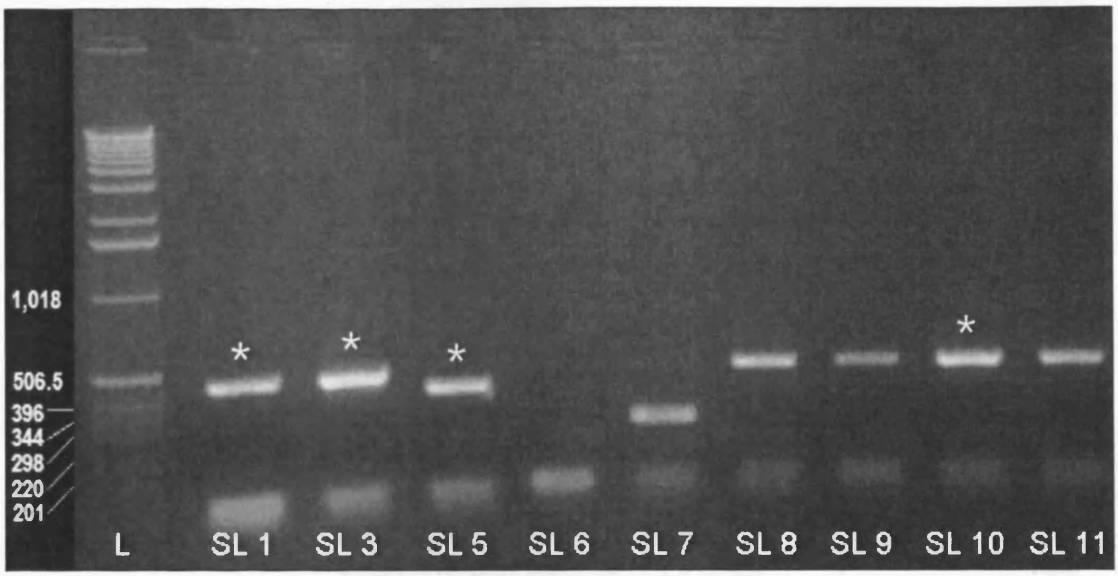


Figure B.4. Amplification of *A.* cv. 'Sweet Laura' (SL) clones by M13 primers on agarose gel (1.5 %) electrophoresis. (*) Clones selected for further sequencing.

Considering size and intensity of the bands, 4 clones of cv 'Samora' (S1, S2, S3 and S6) (Figure B.3) and 4 clones of cv. 'Sweet Laura' (SL1, SL3, SL5 and SL10) (Figure B.4) were selected for sequencing.

Sequencing of a putative Alstroemeria sesquiterpene synthases

After analysis of the sequences using BLAST (See Chapter II, section 2.2.12), no significant information was obtained related to any gene involved in the biosynthesis of sesquiterpene or beta caryophyllene, or any other volatile compound (Table B.2).

Sample	Sequence lenght	Blast	E - value
S1	167 bp	No significant similarity found	
S2	67 bp	Vitis vinifera contig VV78X174602.3	9.2
S3	126 bp	Oryza sativa Japonica Group cultivar Wuyujing 3 chloroplast 30S ribosomal protein S13 precursor, mRNA, complete cds; nuclear gene for chloroplast product.	7 e-5
		Arabidopsis thaliana 30S ribosomal protein S13, chloroplast (CS13) (AT5G14320) mRNA, complete cds.	1 e-4
		Zea mays clone 9724 mRNA sequence.	1 e-4
S6	211 bp	C. reinhardtii chloroplast ATP synthase gamma subunit mRNA	7 e-5
		Vitis vinifera contig VV78X055776.1	2 e-4
		Arabidopsis thaliana ATPC1 (ATP synthase)	0.001
SL3	196 bp	Vigna angularis mRNA for 14-3-3 protein	3 e-11
		Pisum sativum 14-3-3-like protein Mrna	1 e-10
		Nicotiana tabacum mRNA for 14-3-3 protein	3 e-8
SL5	139 bp	No significant similarity found	
SL10	199 bp	Vigna angularis mRNA for 14-3-3 protein	3 e-11
		Pisum sativum 14-3-3-like protein Mrna	1 e-10
		Nicotiana tabacum mRNA for 14-3-3 protein	4 e-8

Table B.2. Results from the sequencing and posterior blasting of 4 'Samora' clones (S1, S2, S3 and S6) and 3 'Sweet Laura' clones (SL3, SL5 and SL10). Vector (pGEM-T, Promega) was subtracted from the sequences.

DISCUSSION

Sequencing results did not show expected matching with any gene related to biosynthesis of sesquiterpene or beta caryophyllene, or any other volatile compound. Furthermore no amplification was found when GRNYL primers were used.

In the case of the GRNYL primers, the most likely answer for this lack of amplification could be the lack of this gene in the Alstroemeria genome. Looking at the GC-MS results previously described in this study, the chromatograms did not show any diterpene compound. Thus probably the GGprotein identification from the *A.* cv. 'Rebecca' cDNA library was not precise enough.

On the other hand, sesquiterpene compounds were observed in the chromatograms previously described in Chapter III, using the GC-MS. Thus some alternative explanations need to be suggested to explain the incorrect amplification of the selected clones:

i) Incorrect design of the degenerate primers in terms of lack of specificity. This is likely as design of these kinds of primers is often difficult. *BCARYO* primers showed 2048 and 3072 possible combinations for forward and reverse respectively. For *SESQUI* primers 12288 and 1024 possible combinations were calculated for forward and reverse respectively (Table B.3).

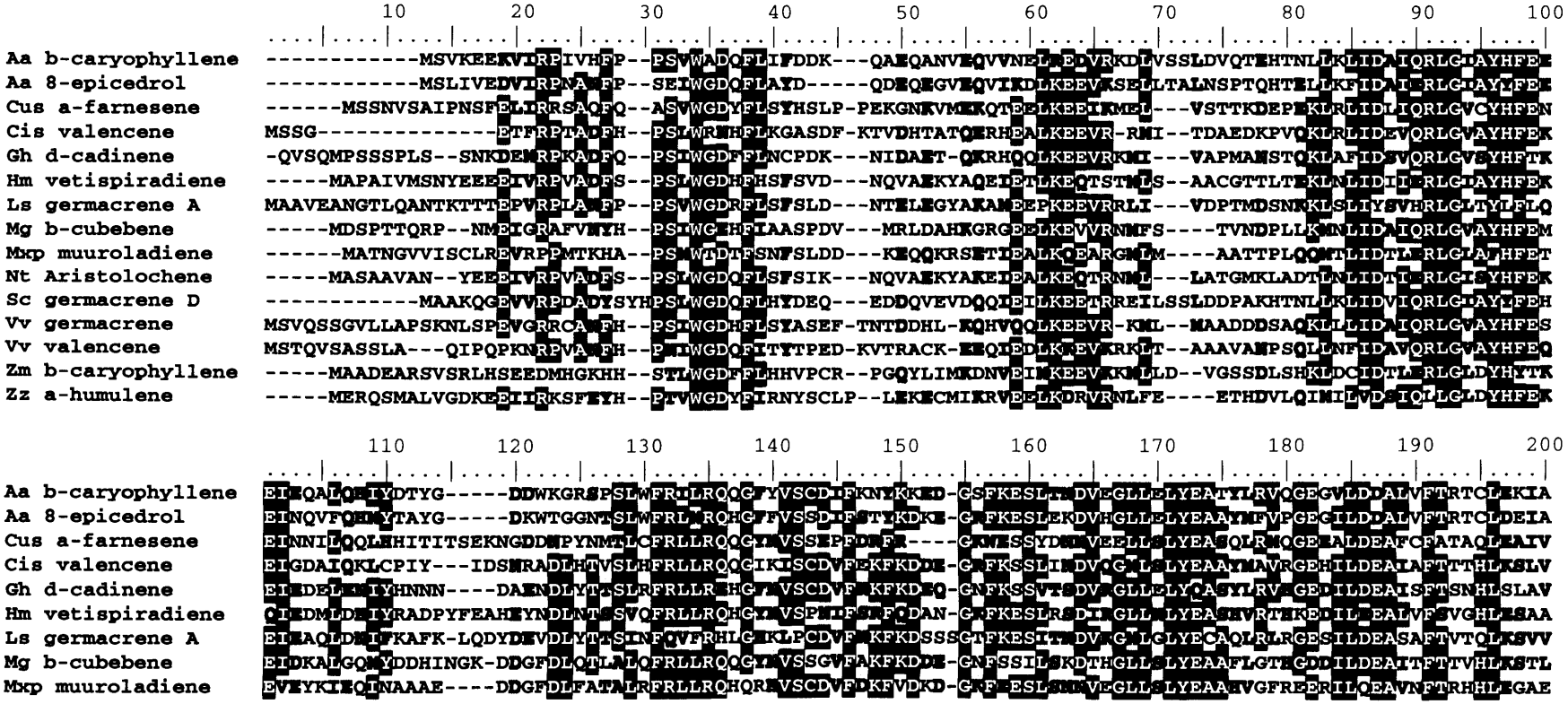
Primer	Sequence (5' - 3')	N° combinations
BCARYOF	GCN(CA)GNGA(TC)(CA)GNATGGTNGA	(4x2x4x2x2x4x4) = 2048
BCARYOR	CCANC(TG)(TC)T(CG)(AGT)ATNGC(TC)TCNGT	(4x2x2x2x3x4x2x4) = 3072
SESQUIF	(AG)AN(TC)TNGCNAA(AG)(CTG)TNGA(TC)TT	(2x4x2x4x4x2x3x4x2) = 12288
SESQUIR	CCANT(TC)(AG)AANGC(TC)TC(TC)TTNGT	(4x2x2x4x2x2x4) =1024

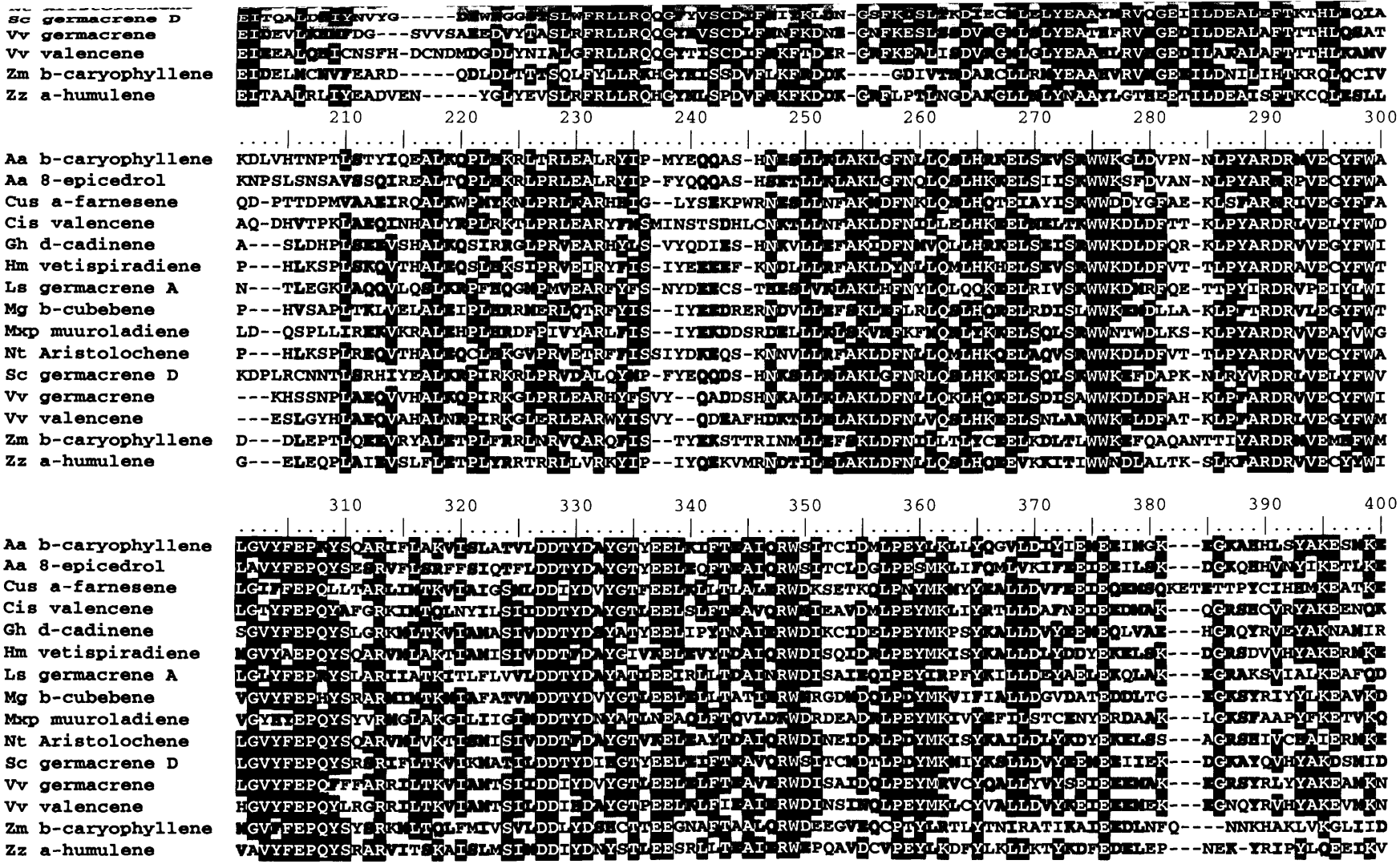
Table B.3. Degenerate primers (*BCARYOF*, *BCARYOR*, *SESQUIF* and *SESQUIR*) and their possible combinations. Highlighted are letters which represent more than one alternative of a nucleotide.

This large number of combinations promotes a competition between the correct primer versus partially correct primers which can bind but not amplify. Probably for this reason the quantity of PCR product obtained from the amplification with these primers was very low as seen in the gel electrophoresis (Figure B.2), even when 10X of primer concentration was used.

ii) Despite the fact that many attempts were made using different $MgCl_2$ and primer concentrations and different number of cycles and Tm temperatures, it is possible that the correct PCR conditions were not found.

ALIGNMENT SUBFAMILY TPS-a

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Vv germacrene
Vv valencene
Zm b-caryophyllene
Zz a-humulene
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Aa b-caryophyllene
Aa 8-epicedrol
Cus a-farnesene
Cis valencene
Gh d-cadinene
Hm vetispiradiene
Ls germacrene A
Mg b-cubebene
Mxp muuroladiene
Nt Aristolochene
Sc germacrene D
Vv germacrene
Vv valencene
Zm b-caryophyllene
Zz a-humulene
310 320 330 340 350 360 370 380 390 400
Aa b-caryophyllene
Aa 8-epicedrol
Cus a-farnesene
Cis valencene
Gh d-cadinene
Hm vetispiradiene
Ls germacrene A
Mg b-cubebene
Mxp muuroladiene
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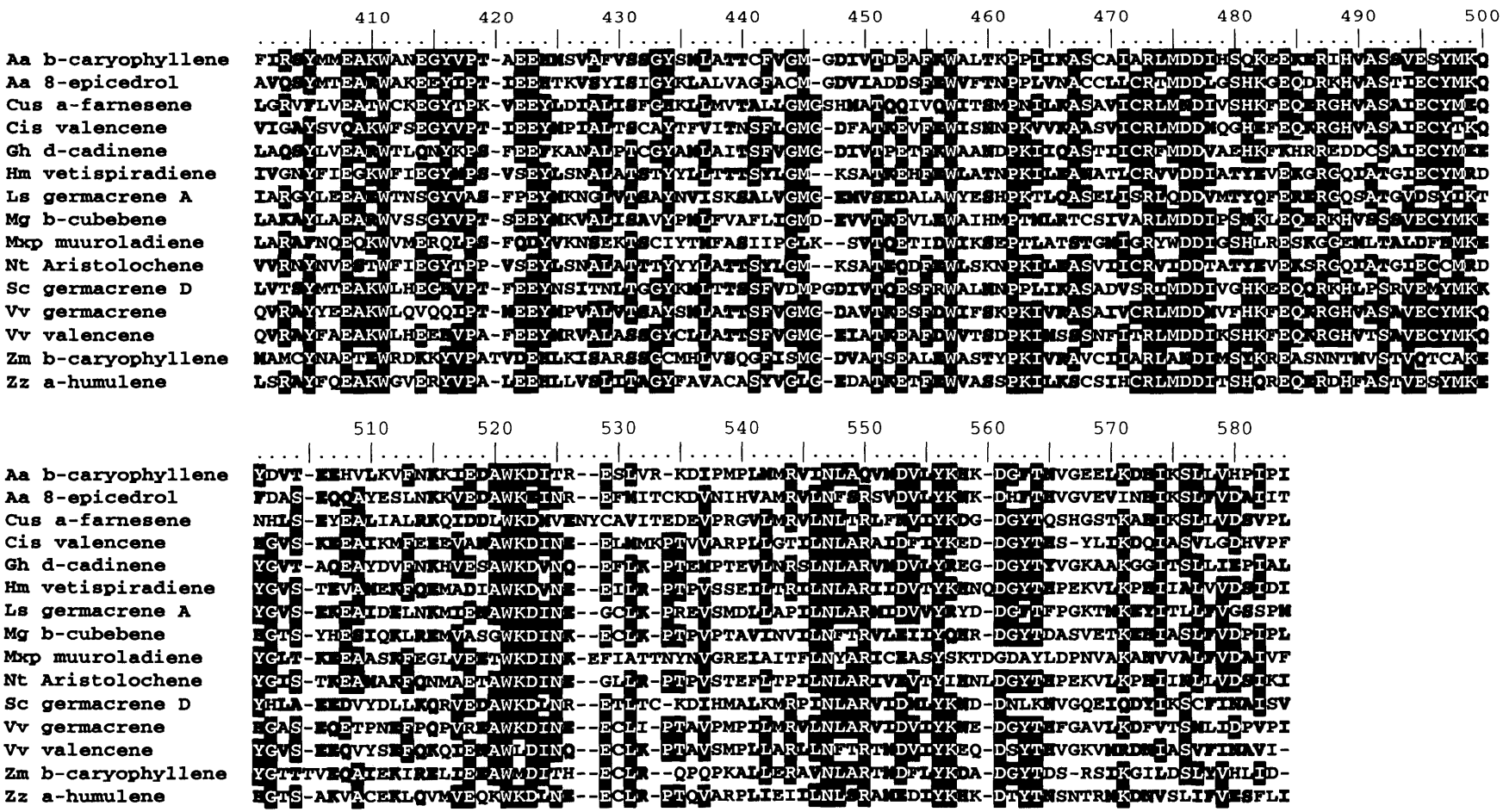
Vv valencene
Zm b-caryophyllene
Zz a-humulene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Aa 8-epicedrol
Cus a-farnesene
Cis valencene
Gh d-cadinene
Hm vetispiradiene
Ls germacrene A
Mg b-cubebene
Mxp muuroladiene
Nt Aristolochene
Sc germacrene D
Vv germacrene
Vv valencene
Zm b-caryophyllene
Zz a-humulene
510 520 530 540 550 560 570 580
Aa b-caryophyllene
Aa 8-epicedrol
Cus a-farnesene
Cis valencene
Gh d-cadinene
Hm vetispiradiene
Ls germacrene A
Mg b-cubebene
Mxp muuroladiene
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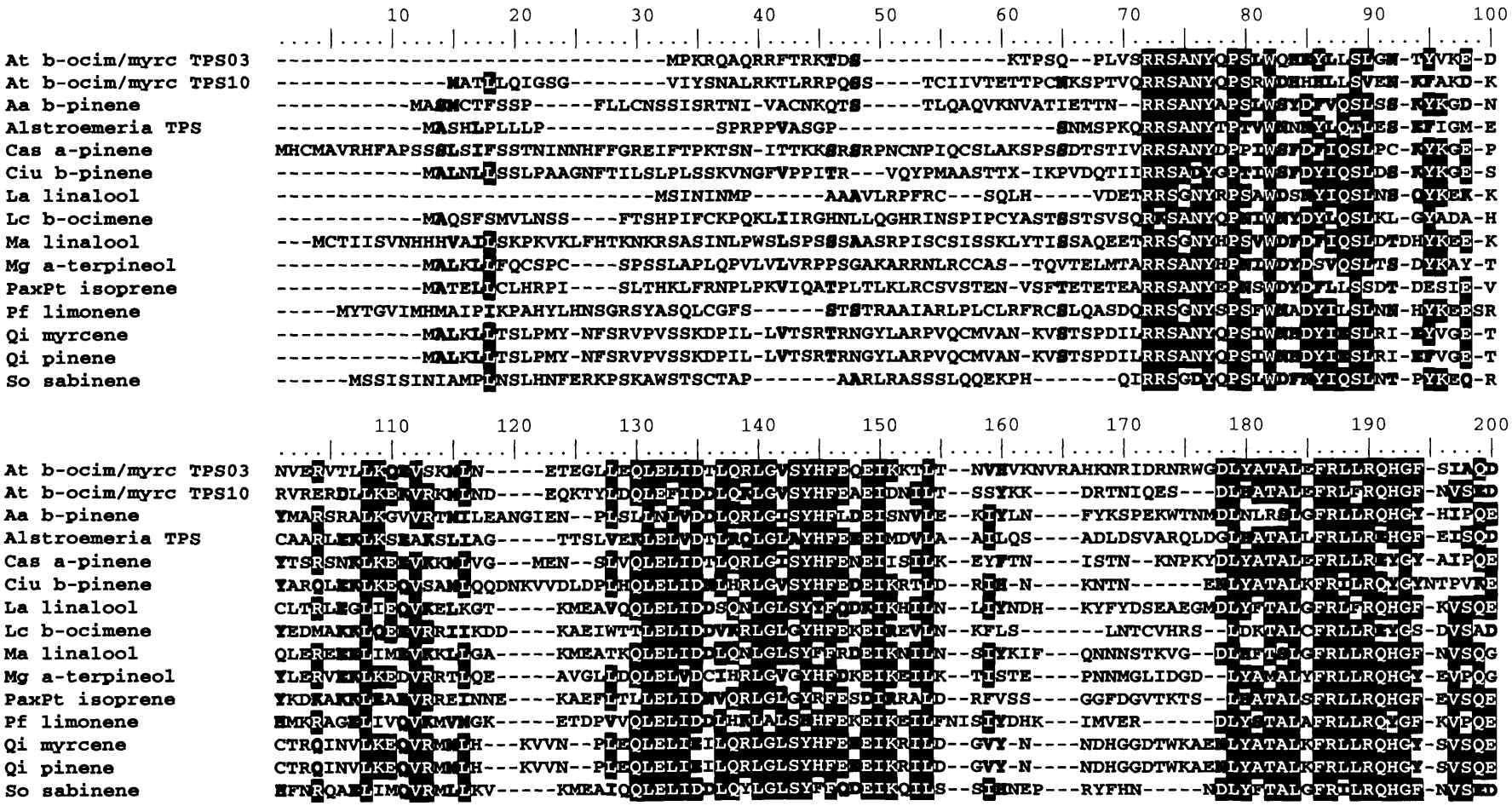
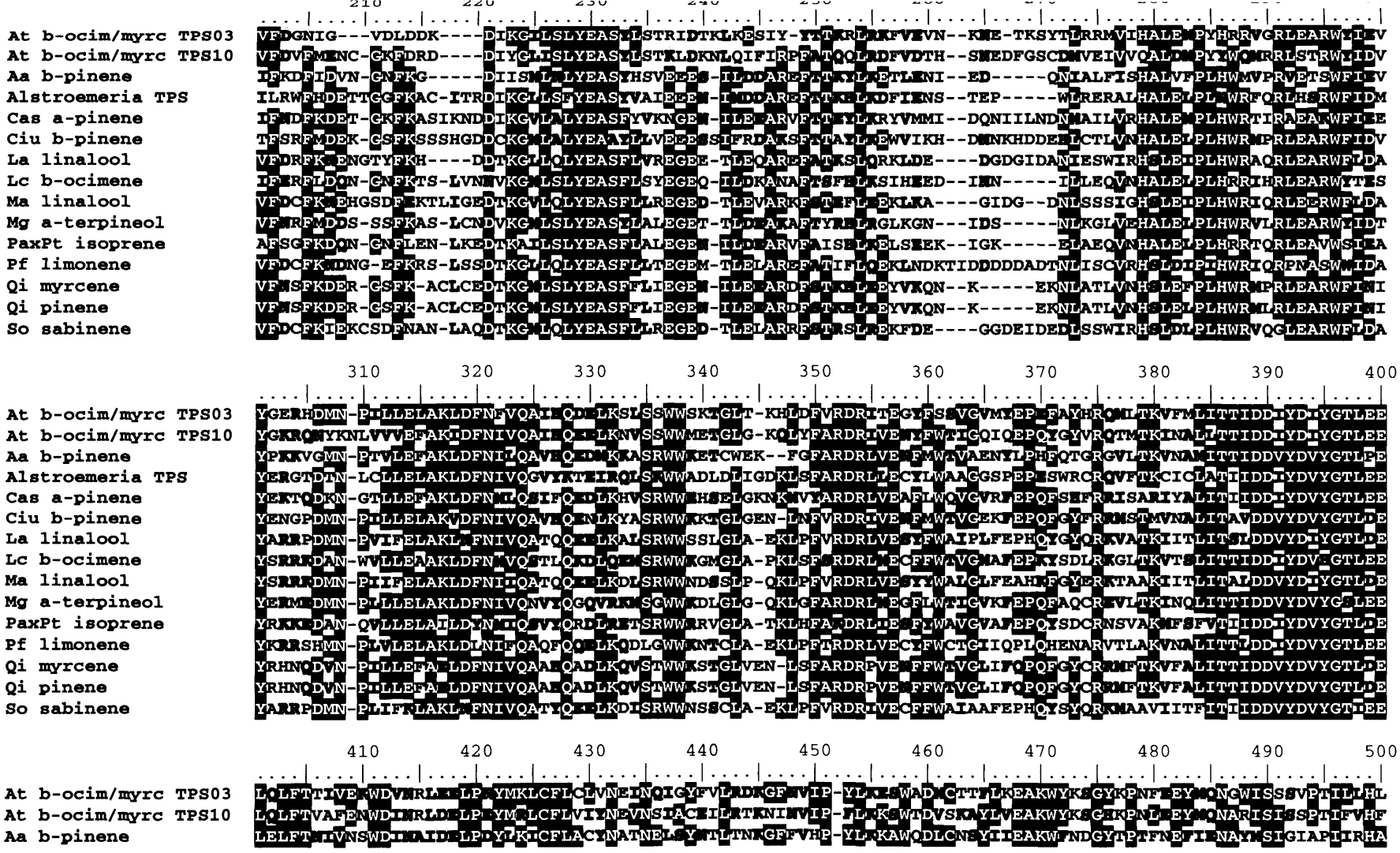
Vv valencene
Zm b-caryophyllene
Zz a-humulene

ALIGNMENT SUBFAMILY TPS-b

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At b-ocim/myrc TPS10
Aa b-pinene
Alstroemeria TPS
Cas a-pinene
Ciu b-pinene
La linalool
Lc b-ocimene
Ma linalool
Mg a-terpineol
PaxPt isoprene
Pf limonene
Qi myrcene
Qi pinene
So sabinene
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340
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360
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380
390
400
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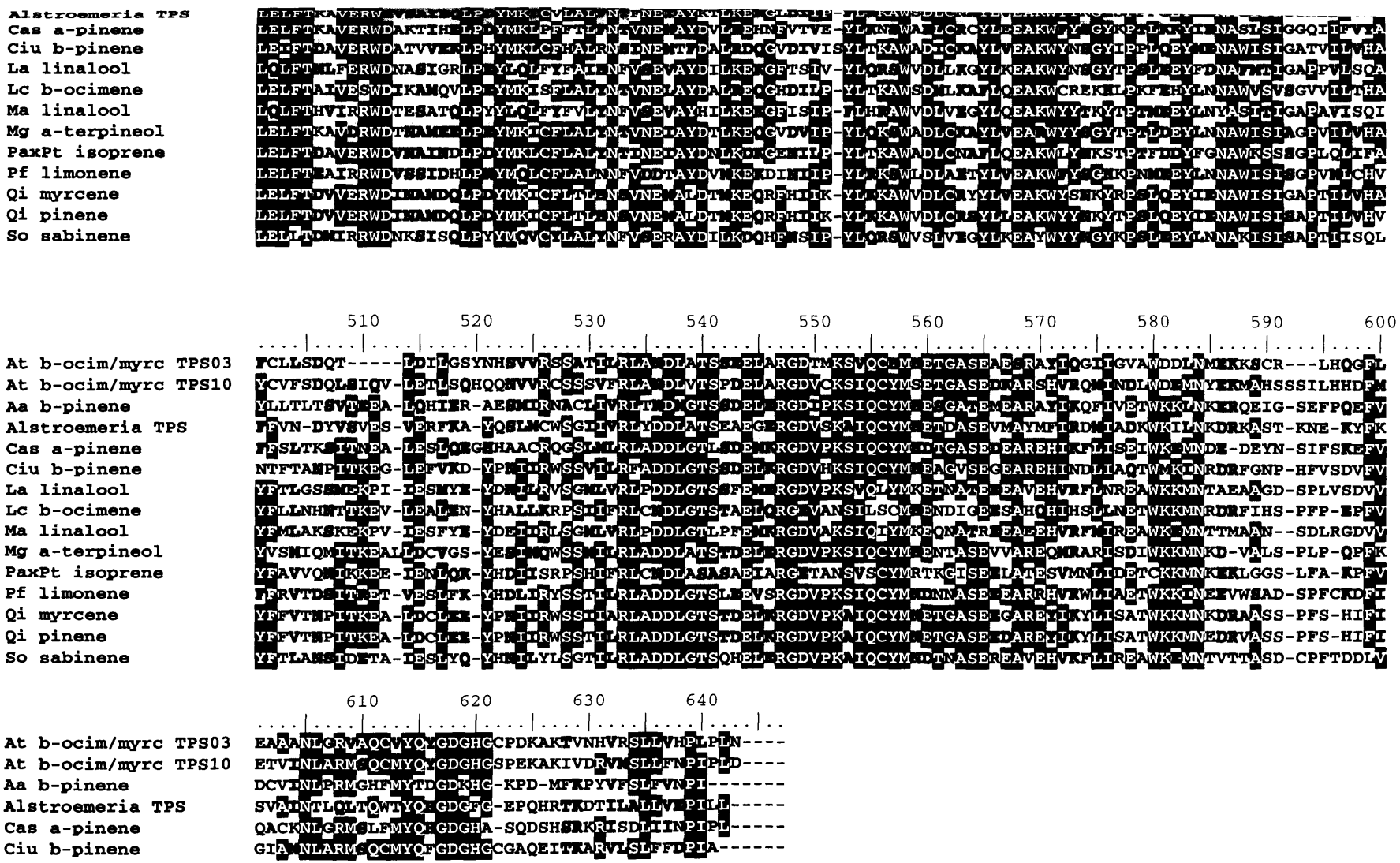
420
430
440
450
460
470
480
490
500


Alstroemeria TPS
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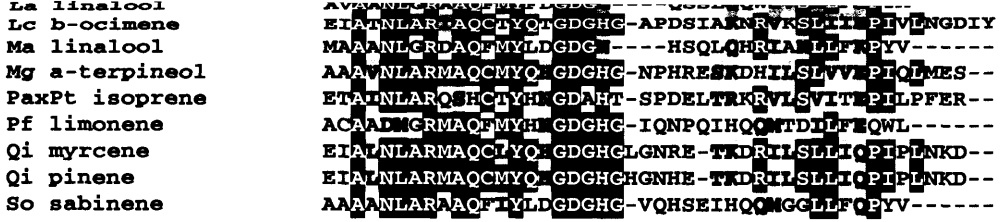
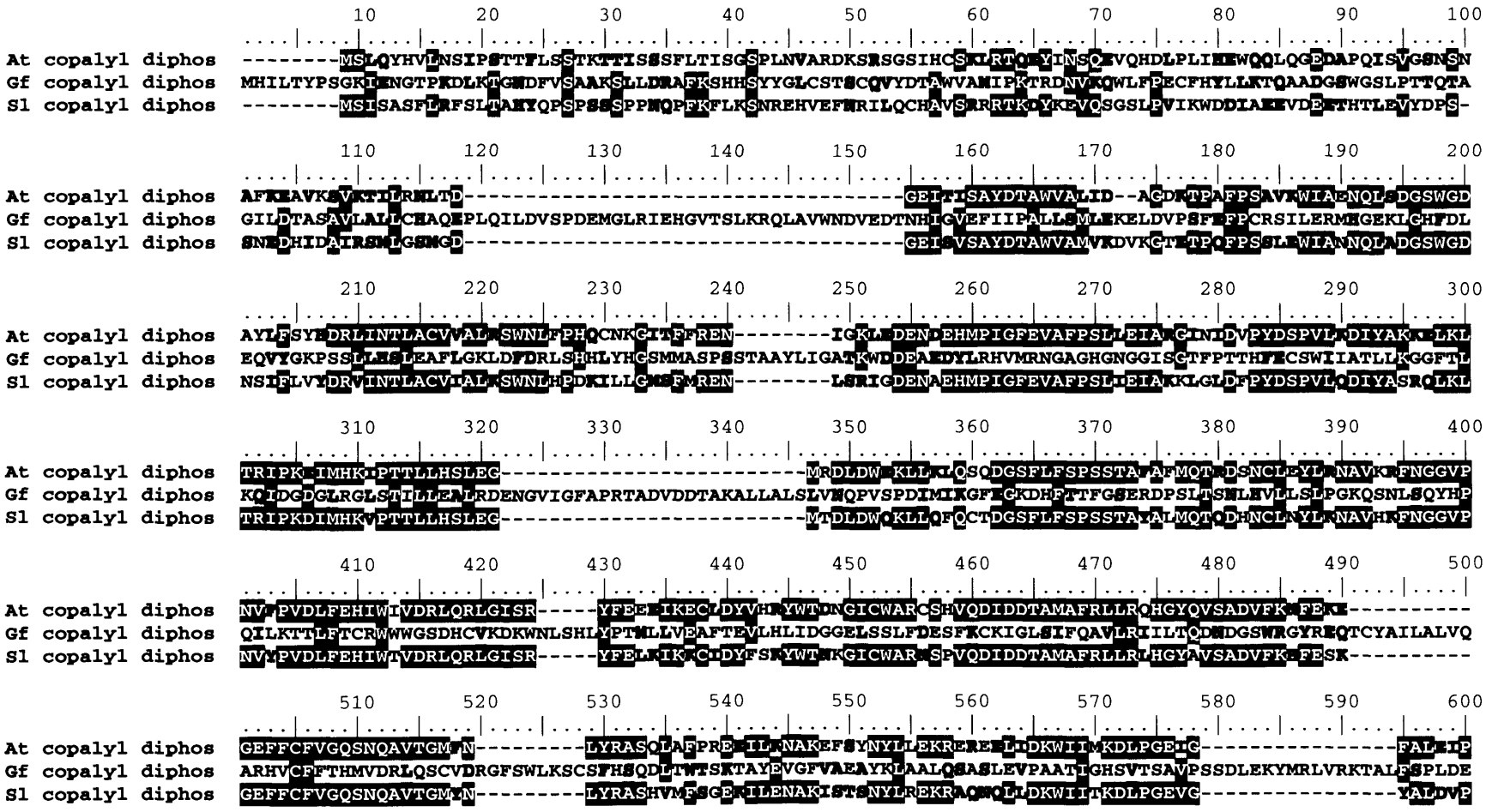
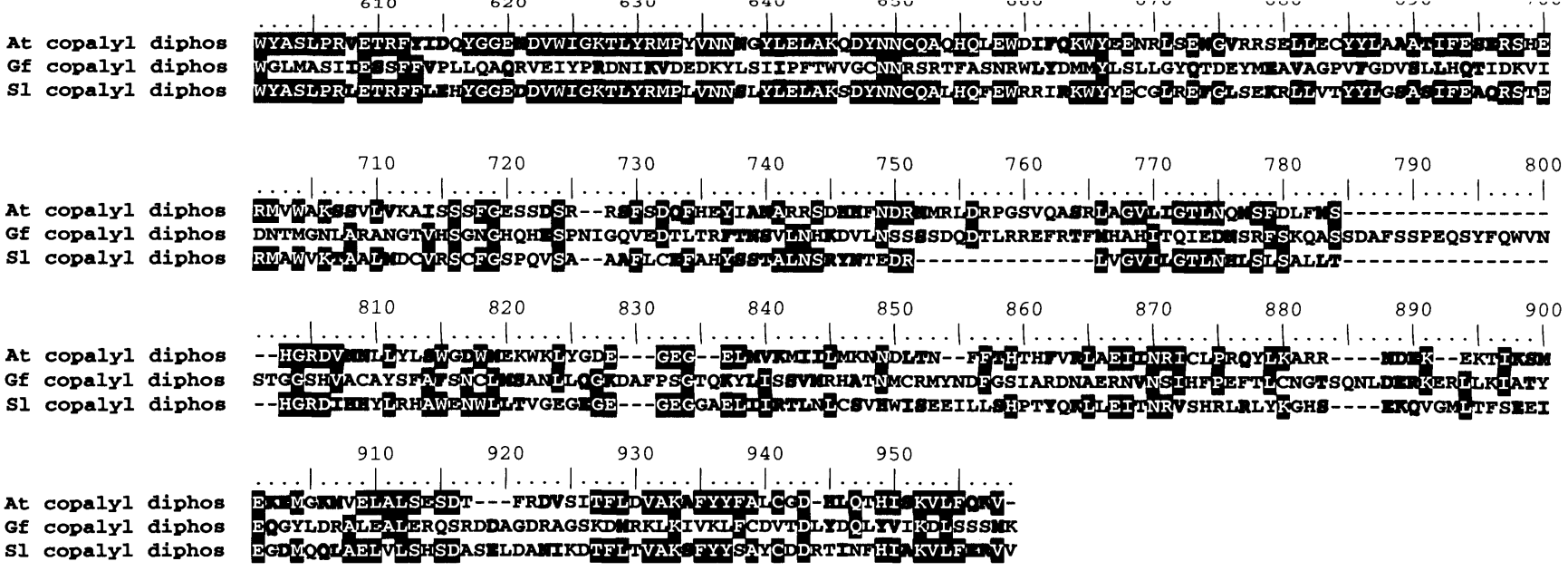
Ciu b-pinene
La linalool
Lc b-ocimene
Ma linalool
Mg a-terpineol
PaxPt isoprene
Pf limonene
Qi myrcene
Qi pinene
So sabinene
510
520
530
540
550
560
570
580
590
600
At b-ocim/myrc TPS03
At b-ocim/myrc TPS10
Aa b-pinene
Alstroemeria TPS
Cas a-pinene
Ciu b-pinene
La linalool
Lc b-ocimene
Ma linalool
Mg a-terpineol
PaxPt isoprene
Pf limonene
Qi myrcene
Qi pinene
So sabinene
610
620
630
640
At b-ocim/myrc TPS03
At b-ocim/myrc TPS10
Aa b-pinene
Alstroemeria TPS
Cas a-pinene
Ciu b-pinene


La linalool
Lc b-ocimene
Ma linalool
Mg a-terpineol
PaxPt isoprene
Pf limonene
Qi myrcene
Qi pinene
So sabinene

ALIGNMENT SUBFAMILY TPS-c

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Gf copalyl diphos
Sl copalyl diphos
710 720 730 740 750 760 770 780 790 800
At copalyl diphos
Gf copalyl diphos
Sl copalyl diphos
810 820 830 840 850 860 870 880 890 900
At copalyl diphos
Gf copalyl diphos
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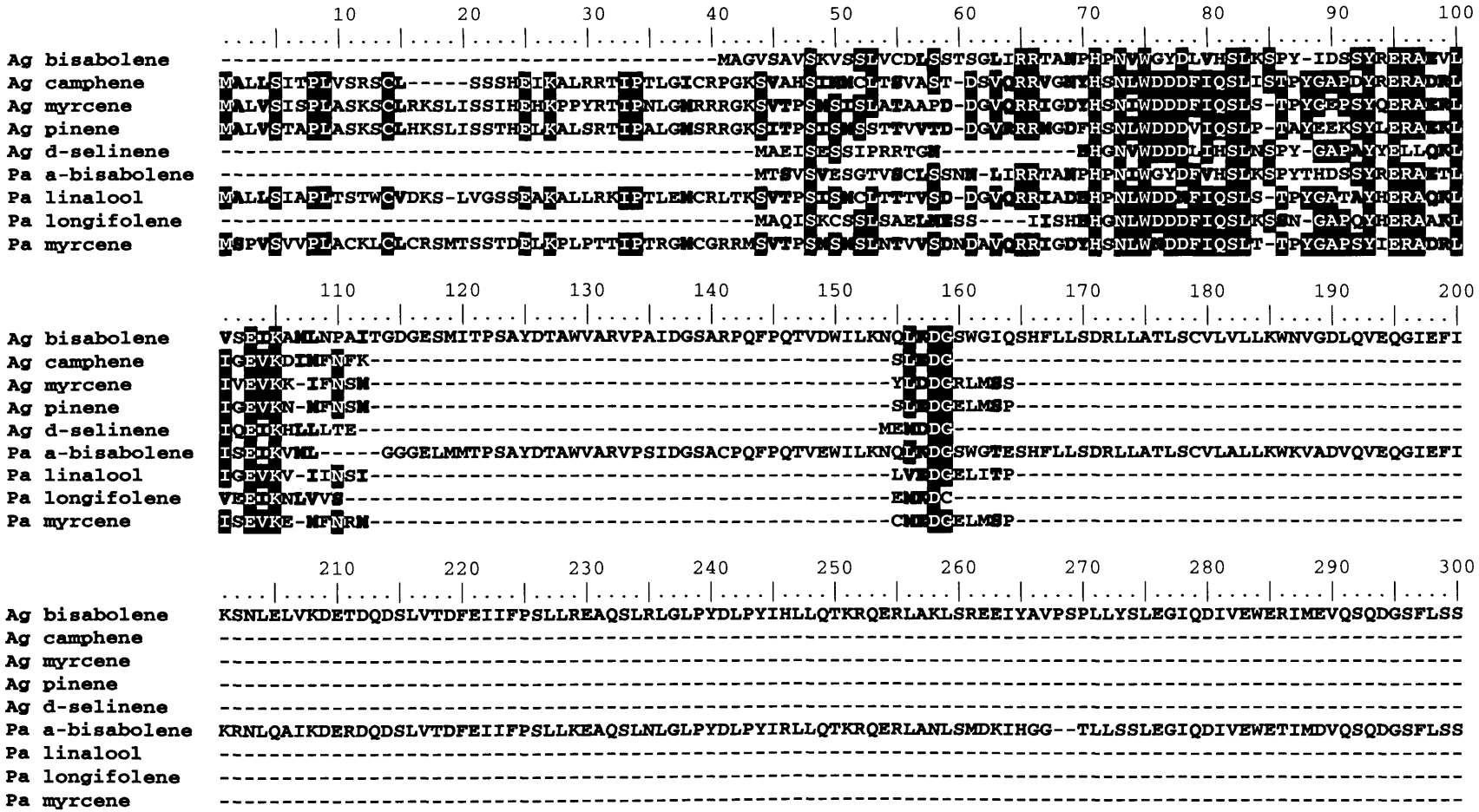
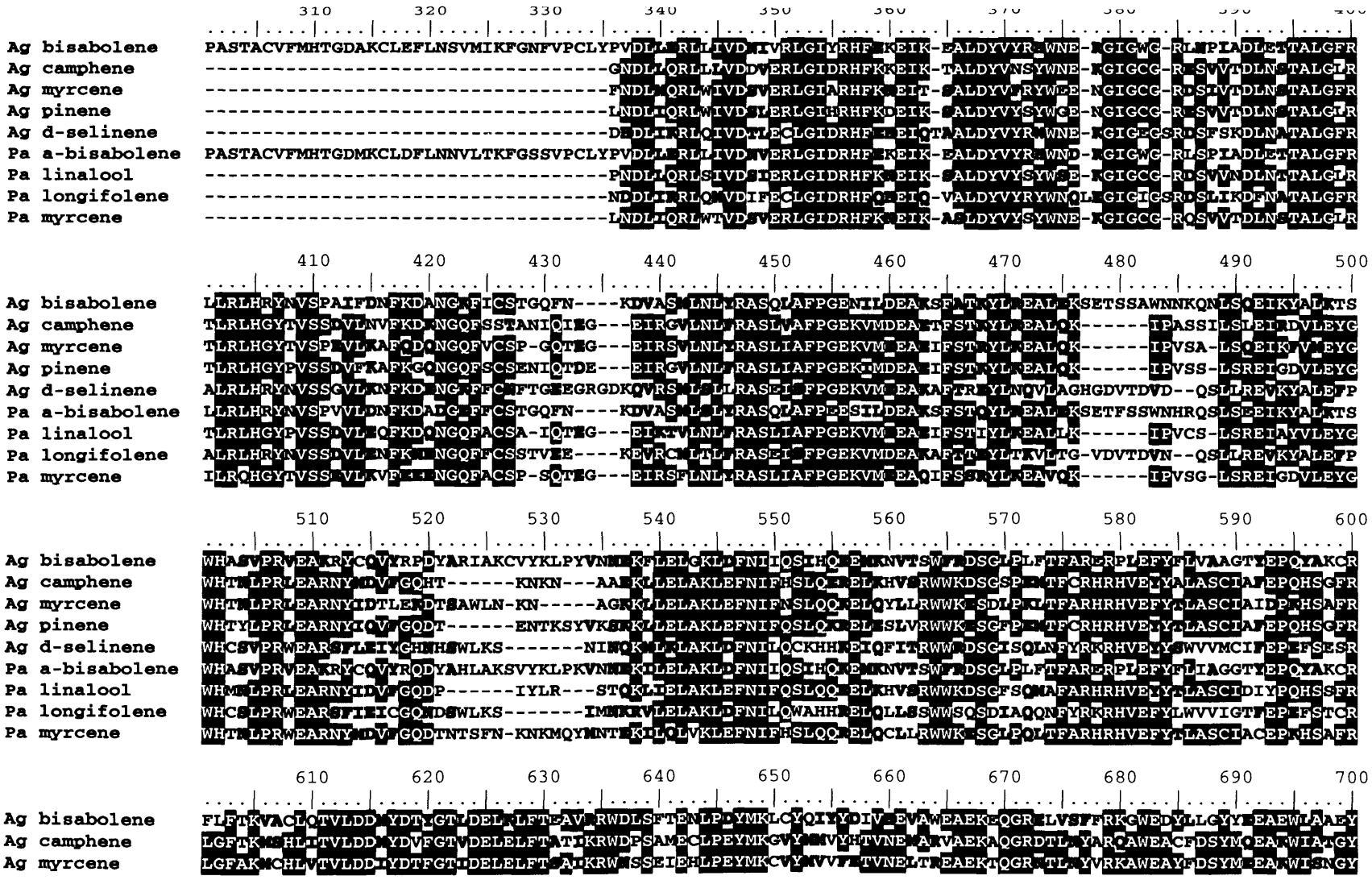
Gf copalyl diphos
Sl copalyl diphos

ALIGNMENT SUBFAMILY TPS-d

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Ag camphene
Ag myrcene
Ag pinene
Ag d-selinene
Pa a-bisabolene
Pa linalool
Pa longifolene
Pa myrcene
410 420 430 440 450 460 470 480 490 500
Ag bisabolene
Ag camphene
Ag myrcene
Ag pinene
Ag d-selinene
Pa a-bisabolene
Pa linalool
Pa longifolene
Pa myrcene
510 520 530 540 550 560 570 580 590 600
Ag bisabolene
Ag camphene
Ag myrcene
Ag pinene
Ag d-selinene
Pa a-bisabolene
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Pa longifolene
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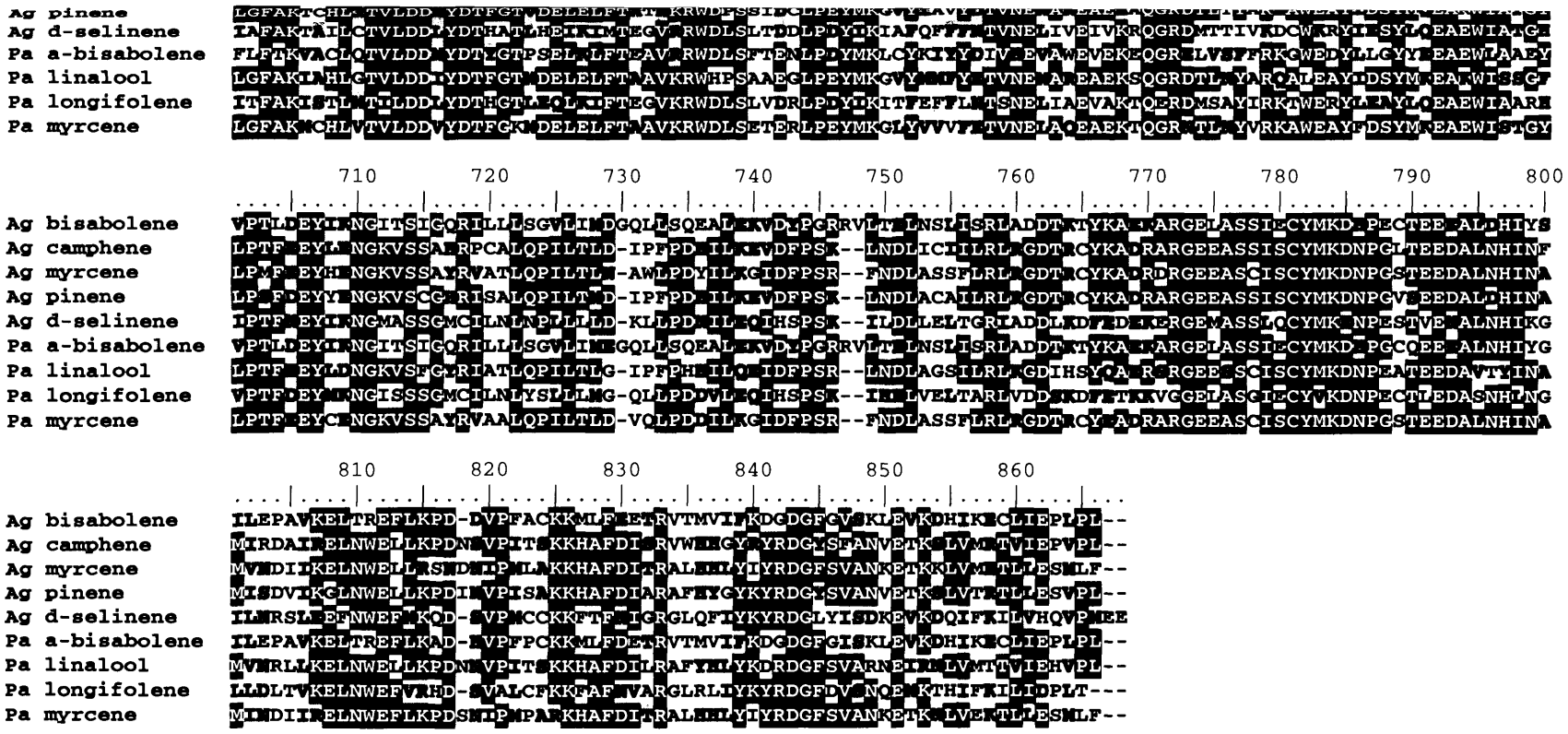
Ag camphene
Ag myrcene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Ag d-selinene
Pa a-bisabolene
Pa linalool
Pa longifolene
Pa myrcene
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720
730
740
750
760
770
780
790
800
Ag bisabolene
Ag camphene
Ag myrcene
Ag pinene
Ag d-selinene
Pa a-bisabolene
Pa linalool
Pa longifolene
Pa myrcene
810
820
830
840
850
860
Ag bisabolene
Ag camphene
Ag myrcene
Ag pinene
Ag d-selinene
Pa a-bisabolene
Pa linalool
Pa longifolene
Pa myrcene

```
                10        20        30        40        50        60        70        80        90       100
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Sr kaurene      MNLSLCIASPLLTKSNRPAALSAIHTASTSHGGQTNPTNLIIDTTKERIQKQFKNVEISVSSYDTAWVAMVPSPNSPKSPCFPECLNWLINNQLNDGSWG

               110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sr kaurene      LVNHTHNHNHPLLKDSLSSTLACIVALKRWNVGEDQINKGLSFIESNLASATEKSQPSPIGFDIIFPGLLEYAKNLDINLLSKQTDFSLMLHKRELEQKR

               210       220       230       240       250       260       270       280       290       300
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Sr kaurene      CHSNEMDGYLAYISEGLGNLYDWNMVKKYQMKNGSVFNSPSATAAAFINHQNPGCLNYLNSLLDKFGNAVPTVYPHDLFIRLSMVDTIERLGISHHFRVE

               310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sr kaurene      IKNVLDETYRCWVERDEQIFMDVVTCALAFRLLRINGYEVSPDPLAEITNELALKDEYAALETYHASHILYQEDLSSGKQILKSADFLKEIISTDSNRLS

               410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sr kaurene      KLIHKEVENALKFPINTGLERINTRRNIQLYNVDNTRILKTTYHSSNISNTDYLRLAVEDFYTCQSIYREELKGLERWVVENKLDQLKFARQKTAYCYFS

               510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Sr kaurene      VAATLSSPELSDARISWAKNGILTTVVDDFFDIGGTIDELTNLIQCVEKWNVDVDKDCCSEHVRILFLALKDAICWIGDEAFKWQARDVTSHVIQTWLEL

               610       620       630       640       650       660       670       680       690       700
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Sr kaurene      MNSMLREAIWTRDAYVPTLNEYMENAYVSFALGPIVKPAIYFVGPKLSEEIVESSEYHNLFKLMSTQGRLLNDIHSFKREFKEGKLNAVALHLSNGESGK

               710       720       730       740       750       760       770       780
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
Sr kaurene      VEEEVVEEMMMMIKNKRKELMKLIFEENGSIVPRACKDAFWNMCHVLNFFYANDDGFTGNTILDTVKDIIYNPLVLVNENEEQR
```

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                10        20        30        40        50        60        70        80        90        100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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                110       120       130       140       150       160       170       180       190       200
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Cb linalool     LLSTLACLVALKRWQLAPDMIHKGLEFVNRNTERLVMKQKPSDVPRWFTIMFPAMLELAGASSLRVDFSENLNRILVELSQNRDDILTREEVDEKKQYSP

                210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cb linalool     LLLFLEALPAQSYDNDVLKQIIDKNLSNDGSLLQSPSATARAYMITGNTRCLSYLHSLTNSCSNGGVPSFYPVDDDLHDLVMVNQLTRSGLTEHLIPEID

                310       320       330       340       350       360       370       380       390       400
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                410       420       430       440       450       460       470       480       490       500
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Cb linalool     LEKILATGNIHKTNADISSSLHKMIEHELRVPWTARMDHVENRIWIEEIASSALWFGKSSYLRLSCFHKMSLQQLAVKNYTLRQLVYRDELAEVERWSKE

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Cb linalool     RGLCDMGFCREKTGYCYYAFAASTCLPWSSDVRLVLTKAAVVITVADDFFDVEGSMVDLEKLTDAVRRWDAEGLGSHSKTIFEALDDLVNEVRLKCFQQN

                610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cb linalool     GQDIKNNLQQLWYETFHSWLMEAKWGKGLTSKPSVDVYLGNAMTSIAAHTMVLTASCLLGPGFPVHQLWSQRRHQDITSLLMVLTRLLNDIQSYLKEEDE

                710       720       730       740       750       760       770       780       790       800
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Cb linalool     GKINYVWMYMIENNQASIDDSVRHVQTIINVKKQEFIQRVLSDQHCNLPKSFKQLHFSCLKVFNMFFNSSNIFDTDTDLLLDIHEAFVSPPQVPKFKPHI

                810       820       830       840       850       860       870
                ....|....|....|....|....|....|....|....|....|....|....|....|
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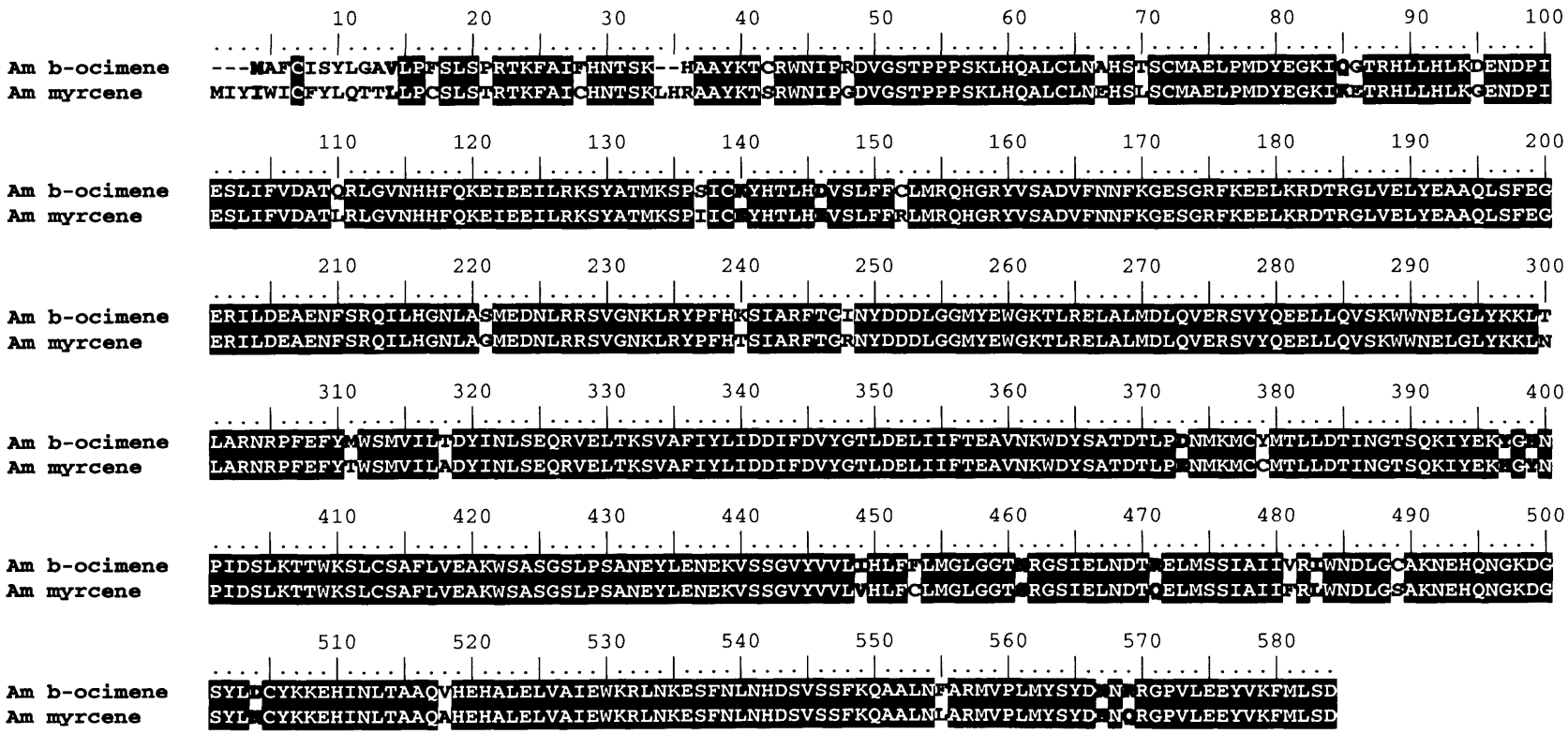
ALIGNMENT SUBFAMILY TPS-g



APPENDIX D

Calibration curves and amplification efficiencies

For 18S rRNA primers, between 18 and 24 cycles, was found to be in the middle of the exponential phase of amplification. The optimum number of cycles for *ALSTER* expression was always found to be higher than for 18S rRNA and ranged from 34 in *A.* cv. 'Samora' to 40 cycles in *A. caryophyllaea* (Table D.1).

Table D.1. Results of the optimization in number of cycles for PCR reactions using 18S rRNA and *ALSTER* primers on four different Alstroemeria genotypes for both stages of development and different organs.

Genotype	Number of cycles	
	18S rRNA	*ALSTER*
A. cv 'Rebecca' (stages of development)	22	36
A. cv 'Samora' (stages of development)	18	34
A. cv. 'Sweet Laura' (stages of development)	24	34
A. cv. 'Sweet Laura' (different organs)	24	38
A. caryophyllea (stages of development)	20	40
A. caryophyllea (different organs)	18	40

Calibration curves and amplification efficiencies

All the calibration curves obtained showed a high efficiency of the Real time qRT-PCRs performed, showing slope values ranging from -3.0603 (DFR 142) to -3.6561 (18S rRNA). Furthermore correlations close to 1 were found for all the linear regressions performed (Figure D.1). Efficiency values calculated were 0.88, 0.94 and 1.12 for 18S rRNA, *ALSTER* and DFR142 respectively.

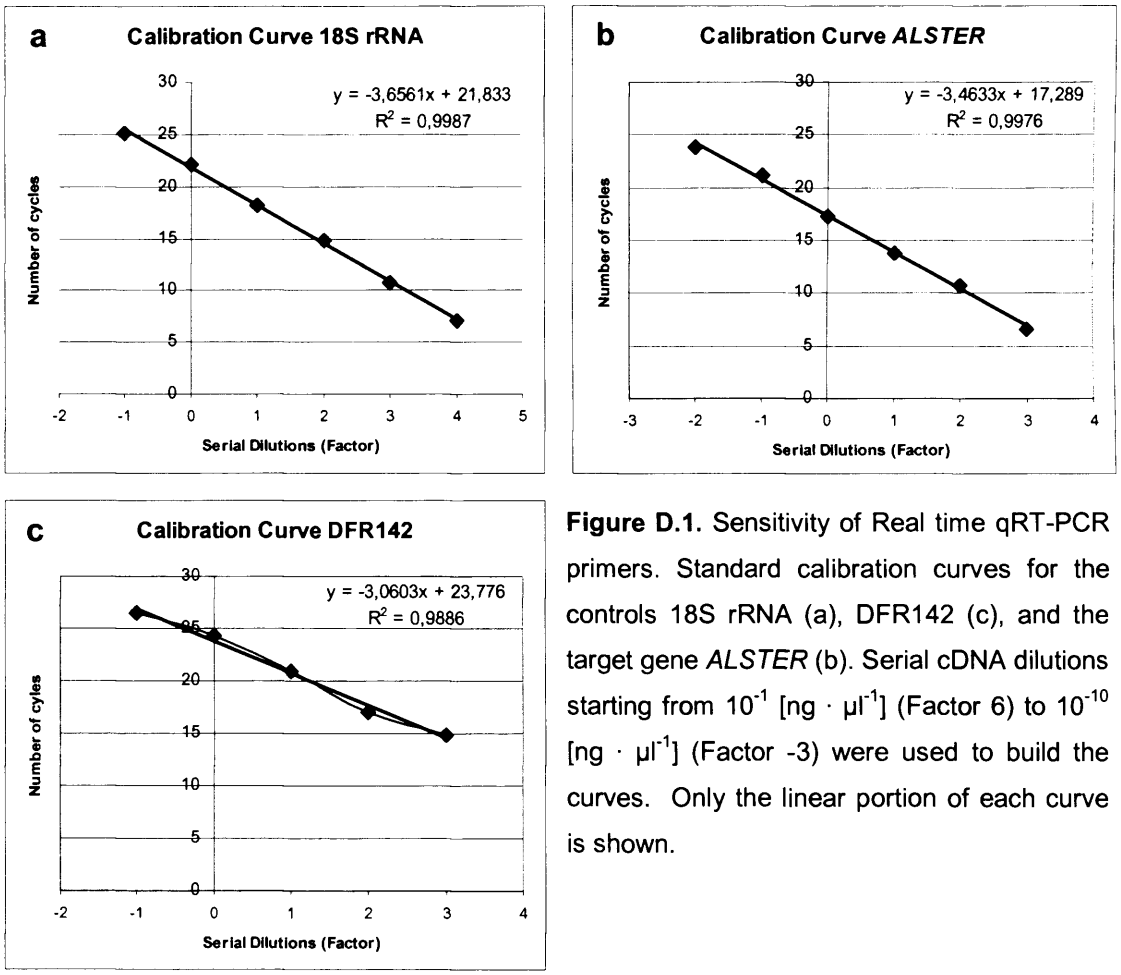


Figure D.1. Sensitivity of Real time qRT-PCR primers. Standard calibration curves for the controls 18S rRNA (a), DFR142 (c), and the target gene *ALSTER* (b). Serial cDNA dilutions starting from 10^{-1} [ng · μl^{-1}] (Factor 6) to 10^{-10} [ng · μl^{-1}] (Factor -3) were used to build the curves. Only the linear portion of each curve is shown.

APPENDIX E

Statistical analysis: Semi qRT-PCR

Table E.1. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in 8 different floral stages of development in *A. caryophyllaea*

Caryophyllea - ALSTER expression - Semi qPCR				
Tukey HSD[a]				
Stages of development	N	Subset for alpha = 0.05		
		1	2	3
0	4	35,8975		
1	4	41,7025	41,7025	
2	4	44,8350	44,8350	
3	4	47,5175	47,5175	
5	4	50,5825	50,5825	
7	4	60,4750	60,4750	
6	4		79,1875	79,1875
4	4			100,0000
Sig.		,421	,056	,620

Table E.2. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in 8 different floral stages of development in *A.* cv. 'Sweet Laura'.

Sweet Laura - ALSTER expression - Semi qPCR					
Tukey HSD[a]					
Stages of development	N	Subset for alpha = 0.05			
		1	2	3	4
0	3	17,0833			
7	3	28,9633			
6	3		50,0167		
2	3			76,3267	
5	3			92,6800	92,6800
4	3			93,4367	93,4367
1	3			93,9867	93,9867
3	3				98,7200
Sig.		,486	1,000	,108	,961

Table E.3. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in 8 different floral stages of development in *A.* cv. 'Rebecca'.

Rebecca - ALSTER expression - Semi qPCR

Tukey HSD[a]

Stages of development	N	Subset for alpha = 0.05			
		1	2	3	4
3	4	29,1075			
7	4	32,5275			
5	4	36,0475			
4	4		59,9850		
6	4		76,6925		
2	4		77,7575	77,7575	
1	4			97,9725	97,9725
0	4				98,6950
Sig.		,951	,138	,064	1,000

Table E.4. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in 8 different floral stages of development in *A.* cv. 'Samora'.

Samora - ALSTER expression - Semi qPCR

Tukey HSD[a]

Stages of development	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
7	3	9,0467					
6	3		41,1867				
5	3			64,9267			
3	3				78,5133		
4	3				86,2067	86,2067	
2	3					88,2767	
0	3					88,7600	
1	3						100,000
Sig.		1,000	1,000	1,000	,129	,972	1,000

Table E.5. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in different floral organs in *A.* cv. 'Sweet Laura'.

Caryophyllea - ALSTER expression - Semi qPCR					
Tukey HSD[a]					
Organ	N	Subset for alpha = 0.05			
		1	2	3	4
Leaves	3	1,7733			
Stamens	3		29,5333		
Inner Tepals	3			53,6767	
Outer Tepals	3				88,9767
Pistil	3				100,0000
Sig.		1,000	1,000	1,000	,298

Table E.6. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in different floral organs in *A. caryophyllaea*.

Sweet Laura - ALSTER expression - Semi qPCR			
Tukey HSD[a]			
Organ	N	Subset for alpha = 0.05	
		1	2
Leaves	3	26,8200	
Stamens	3	36,0400	
Outer Tepals	3		74,0567
Pistil	3		86,5033
Inner Tepals	3		100,0000
Sig.		,873	,131

Table E.7. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Semi qRT-PCR in different genotypes of Alstroemeria.

ALSTER expression - Semi qPCR			
Tukey HSD[a]			
Genotype	N	Subset for alpha = 0.05	
		2	1
Caryophyllea	3	35,6834	
Rebecca	3	62,7049	62,7049
Sweet Laura	3	72,3707	72,3707
Samora	3		96,37
Sig.		0,076	0,107

Statistical analysis: Real time qRT-PCR

Table E.8. ANOVA analysis (Tukey 95 %) for DFR 142 expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A.* cv. 'Ajax'.

Ajax - DFR 142 expression - Real Time			
Tukey HSD[a]			
Stages of development	N	Subset for alpha = 0.05	
		1	2
7	12	0,01351988	
5	12	0,02072451	
6	12	0,05721572	
4	12	0,05876728	
0	12		2,6161983
3	12		3,4975128
1	12		3,7843089
2	12		4,0497148
Sig.		1,00000000	0,1530318

Table E.9. ANOVA analysis (Tukey 95 %) for DFR 142 expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A. caryophyllaea*.

Caryophyllea - DFR 142 expression - Real Time			
Tukey HSD[a]			
Stages of development	N	Subset for alpha = 0.05	
		1	2
7	12	0,00026621	
6	12	0,00051622	
5	12	0,00099904	
4	12	0,00828932	
0	12	0,04824795	
3	12	0,09241392	0,09241392
1	12	0,35079778	0,35079778
2	12		0,48366053
Sig.		0,13543343	0,06646996

Table E.10. ANOVA analysis (Tukey 95 %) for DFR 142 expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A.* cv. 'Rebecca'.

Rebecca - DFR 142 expression - Real Time			
Tukey HSD[a,,b]			
Stages of development	N	Subset for alpha = 0.05	
		1	2
5	12	0,00000400	
7	12	0,00003337	
6	12	0,00005028	
3	12	0,00006235	
4	12	0,00009580	
2	12	0,01700928	
0	12	0,03141391	
1	12		0,13644688
Sig.		0,96278078	1,00000000

Table E.11. ANOVA analysis (Tukey 95 %) for DFR 142 expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A.* cv. 'Sweet Laura'.

Sweet Laura - DFR 142 expression - Real Time			
Tukey HSD[a,b]			
Stages of development	N	Subset for alpha = 0.05	
		1	2
7	12	0,00154112	
6	12	0,00231469	
4	12	0,00355933	
5	12	0,01368165	
2	12	0,09345844	
3	12	0,12481587	
0	12	0,16179455	
1	12		0,47807704
Sig.		0,53507830	1,00000000

Table E.12. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A.* cv. 'Ajax'.

Ajax - *ALSTER* expression - Real Time				
Tukey HSD[a]				
Stages of development	N	Subset for alpha = 0.05		
		1	2	3
7	12	0,00000639		
5	12	0,00001130		
6	12	0,00001511		
4	12	0,00001871		
1	12	0,00042266	0,00042266	
3	12		0,00056143	
2	12		0,00061618	
0	12			0,00140715
Sig.		0,06262854	0,83198462	1,00000000

Table E.13. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A. caryophyllaea.*

Caryophyllea - *ALSTER* expression - Real Time		
Tukey HSD[a,b]		
Stages of development	N	Subset for alpha = 0.05
		1
0	12	0,00001222
6	12	0,00001538
4	12	0,00001829
5	12	0,00002111
3	12	0,00002755
1	12	0,00003028
7	12	0,00003209
2	12	0,00004511
Sig.		0,05889724

Table E.14. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A.* cv. 'Rebecca'.

Rebecca - *ALSTER* expression - Real Time			
Tukey HSD[a,b]			
Stages of development	N	Subset for alpha = 0.05	
		1	2
7	12	0,00000160	
6	12	0,00000441	
5	12	0,00000592	0,00000592
3	12	0,00000667	0,00000667
1	12	0,00000707	0,00000707
2	12	0,00000746	0,00000746
4	12	0,00001391	0,00001391
0	12		0,00002814
Sig.		0,63070075	0,05662641

Table E.15. ANOVA analysis (Tukey 95 %) for *ALSTER* expression evaluated through Real Time qRT-PCR in 8 different floral stages of development in *A.* cv. 'Sweet Laura'.

Sweet Laura - *ALSTER* expression - Real Time			
Tukey HSD[a,b]			
Stages of development	N	Subset for alpha = 0.05	
		1	2
7	12	0,00014448	
6	12	0,00020932	
0	12	0,00062114	
1	12	0,00095465	
5	12	0,00243064	
2	12	0,00300946	
4	12	0,00380189	
3	12		0,01475602
Sig.		0,44172109	1,00000000

APPENDIX F

Information sheet for the sensorial analysis of new lines of scented Alstroemerias.

INFORMATION SHEET

Dear evaluator:

This research is aimed at evaluating some new lines of Alstroemeria flowers through sensorial analysis considering the human perception of floral scent and aesthetic value (appearance) of the samples exhibited.

This sensorial analysis considers the general population's evaluation of scent and not an evaluation of an individual's perception.

You will be asked to answer an introductory survey (1) with some general questions about yourself (age, sex, etc) and about your perception of flowers.

Then another three surveys (2, 3 and 4) must be completed in order to evaluate your liking, in terms of scent and aesthetical value, for each of the samples presented.

Please note that your participation in this study is totally anonymised and voluntary. Furthermore, feel free to withdraw from the study or ask any questions at any time.

Thanks for your participation!

Information sheet for the sensorial analysis of different floral scents.

INFORMATION SHEET

Dear evaluator:

This research is aimed at finding out about human perception of different types of floral scent in order to correlate this information with previous analysis performed using other methodologies to evaluate floral scent.

This sensorial analysis considers the general population's evaluation of scent and not an evaluation of an individual's perception.

You will be asked to answer an introductory survey (1) with some general questions about yourself (age, sex, etc) and about your perception of flowers.

Then another three surveys (2, 3 and 4) must be completed in order to evaluate your liking, in terms of scent and aesthetical value, for each of the samples presented.

Please note that your participation in this study is totally anonymised and voluntary. Furthermore, feel free to withdraw from the study or ask any questions at any time.

Thanks for your participation!

Survey included to obtain general information about the volunteers.

SURVEY

How old are you?

☐ Years

Sex?

☐ Female

☐ Male

Are you a?

☐ Undergraduate student

☐ Postgraduate student

☐ Teaching staff

☐ Non teaching staff

☐ Unemployed / Other

How often do you buy flowers?

☐ Never

☐ Only for special occasions (Birthdays, St. Valentine's day, etc)

☐ Once per month

☐ Once per week

About your smoking habits, you are a

☐ Non smoker

☐ Occasional smoker

☐ Regular smoker

Survey included to assess the relative importance of floral scent as a character when volunteers buy or look at flowers.

SURVEY

When you buy/look at flowers the character you appreciate more is:
(Please answer all questions)

Flower size?

☐ Strongly agree ☐ Agree ☐ Neutral ☐ Disagree ☐ Strongly disagree

Stem length?

☐ Strongly agree ☐ Agree ☐ Neutral ☐ Disagree ☐ Strongly disagree

Flower colour?

☐ Strongly agree ☐ Agree ☐ Neutral ☐ Disagree ☐ Strongly disagree

Floral scent?

☐ Strongly agree ☐ Agree ☐ Neutral ☐ Disagree ☐ Strongly disagree

Flower longevity (Vase life) ?

☐ Strongly agree ☐ Agree ☐ Neutral ☐ Disagree ☐ Strongly disagree

Survey provided to volunteers for the evaluation of floral scents presented.

SURVEY

How do you rate your **overall liking** of the floral scent presented?

Consider only floral scent

(Please tick your answer)

- ☐ Like extremely
- ☐ Like very much
- ☐ Like moderately
- ☐ Like slightly
- ☐ Neither like nor dislike
- ☐ Dislike slightly
- ☐ Dislike moderately
- ☐ Dislike very much
- ☐ Dislike extremely

SAMPLE _____

Survey provided to volunteers for the evaluation of the intensity of the floral scents presented.

SURVEY

How do you rate **the intensity** of the floral scent presented?

(Please tick your answer)

- ☐ Extremely high
- ☐ Very high
- ☐ Moderately high
- ☐ Slightly high
- ☐ Neither high nor low
- ☐ Slightly low
- ☐ Moderately low
- ☐ Very low
- ☐ Extremely low

SAMPLE _____

Survey provided to volunteers for the evaluation of aesthetic value – appearance of the flowers presented.

SURVEY

How do you rate your **overall liking** of the flower presented?

Consider only aesthetic value – appearance

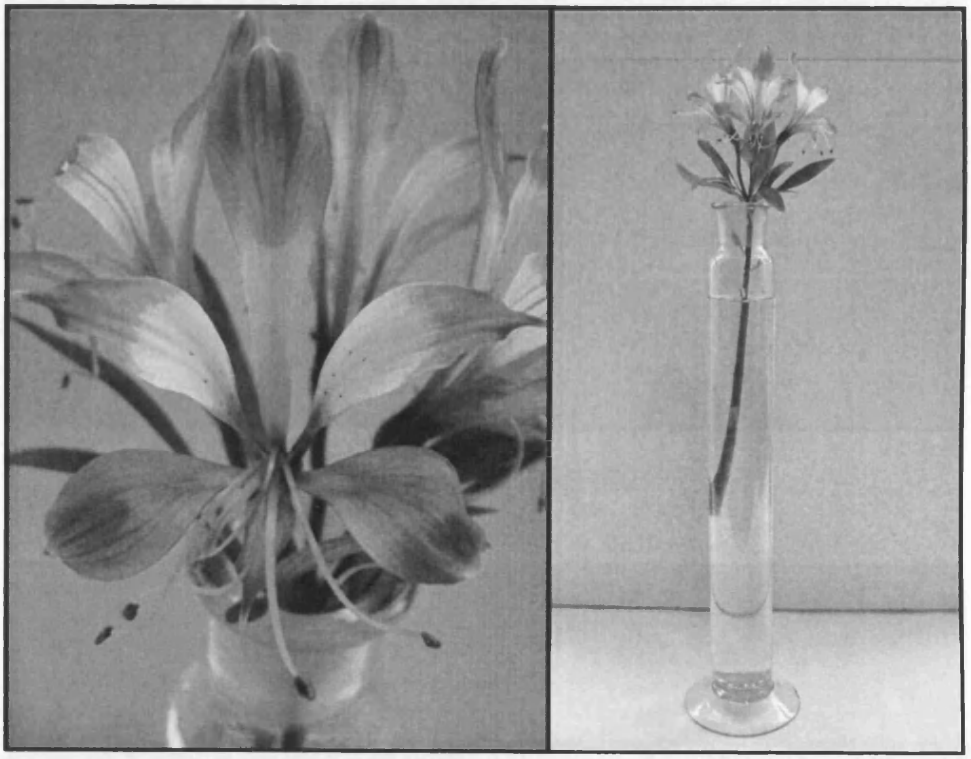
(Please tick your answer)

- ☐ Like extremely
- ☐ Like very much
- ☐ Like moderately
- ☐ Like slightly
- ☐ Neither like nor dislike
- ☐ Dislike slightly
- ☐ Dislike moderately
- ☐ Dislike very much
- ☐ Dislike extremely

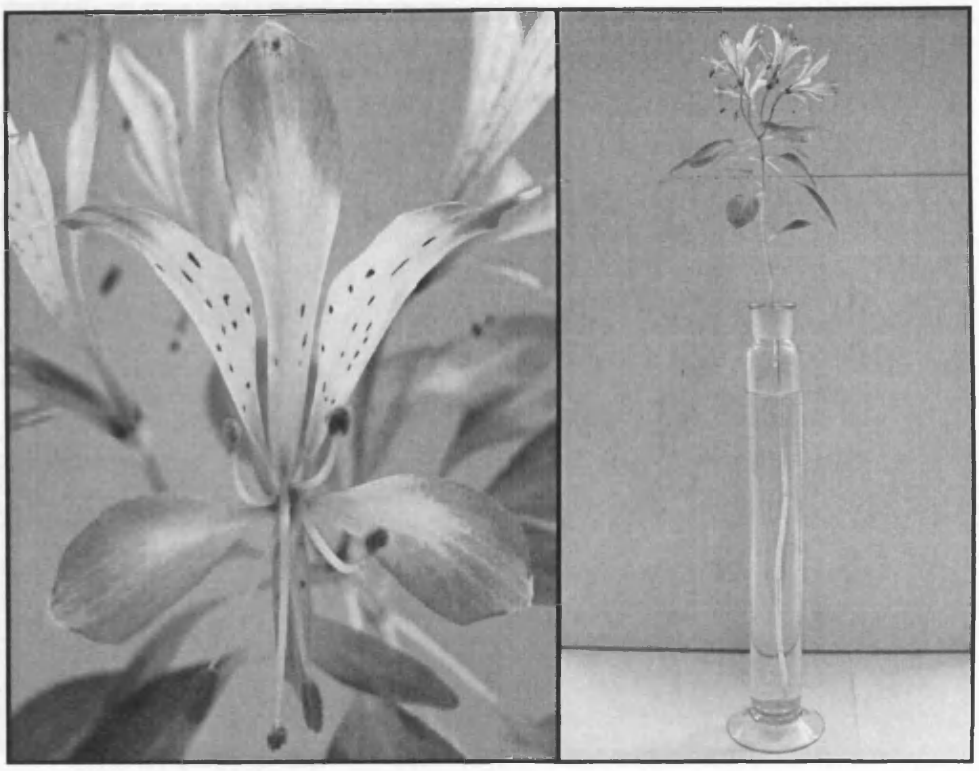
SAMPLE _____

APPENDIX G

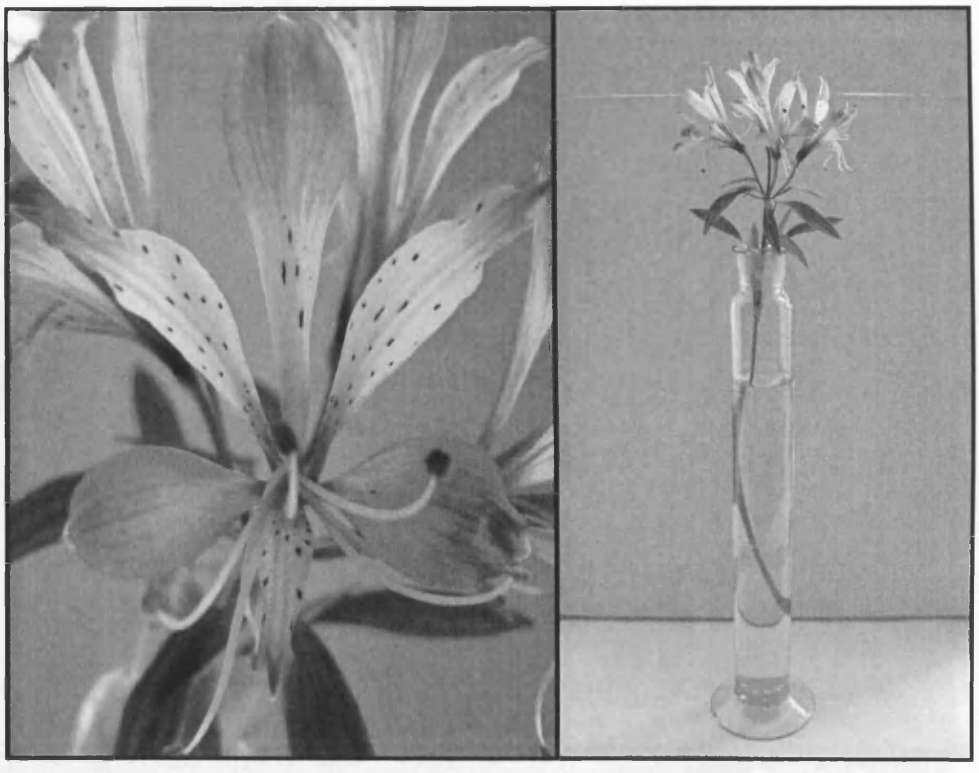
Pictures of the new lines of *A. caryophyllaea*



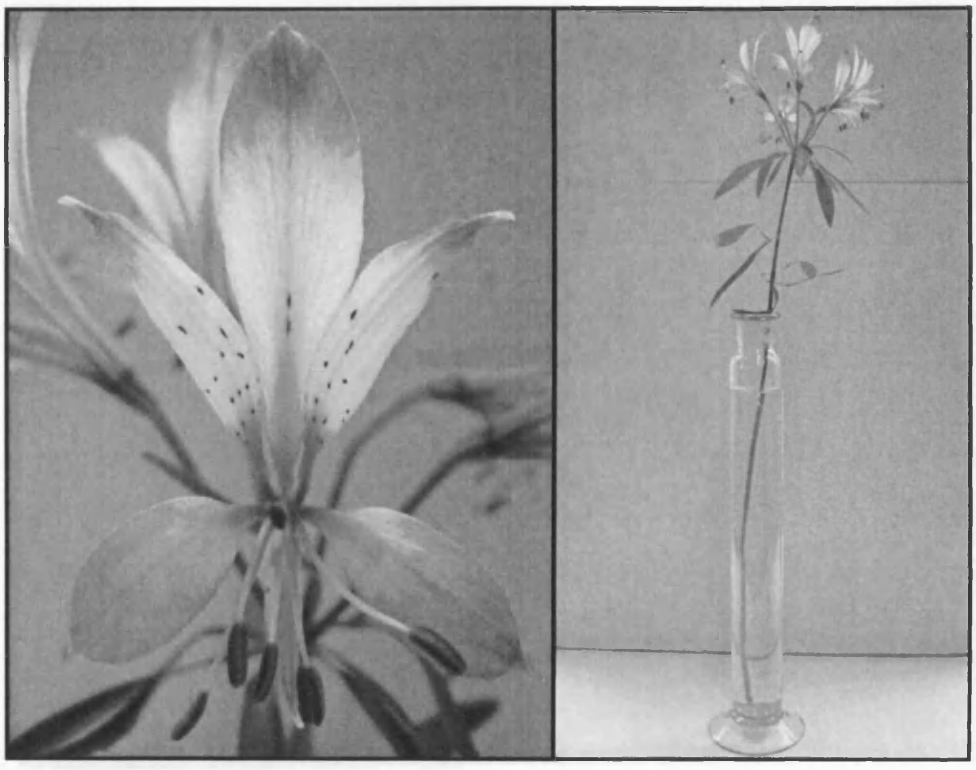
Flowers of DANCAR 001



Flowers of DANCAR 002



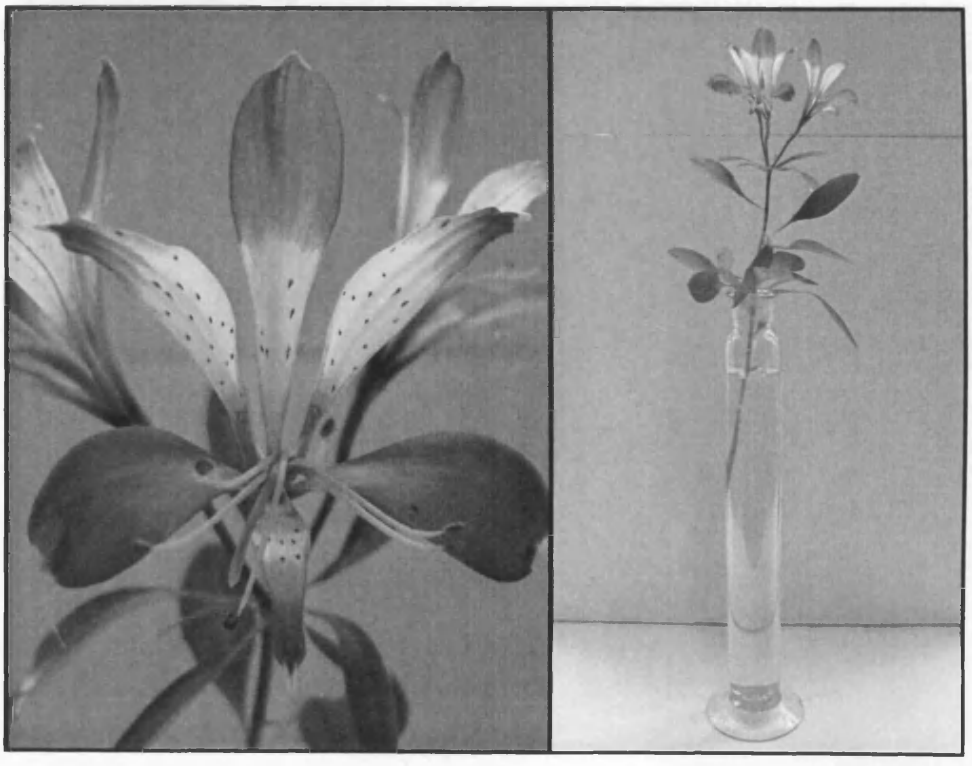
Flowers of DANCAR 003



Flowers of DANCAR 004

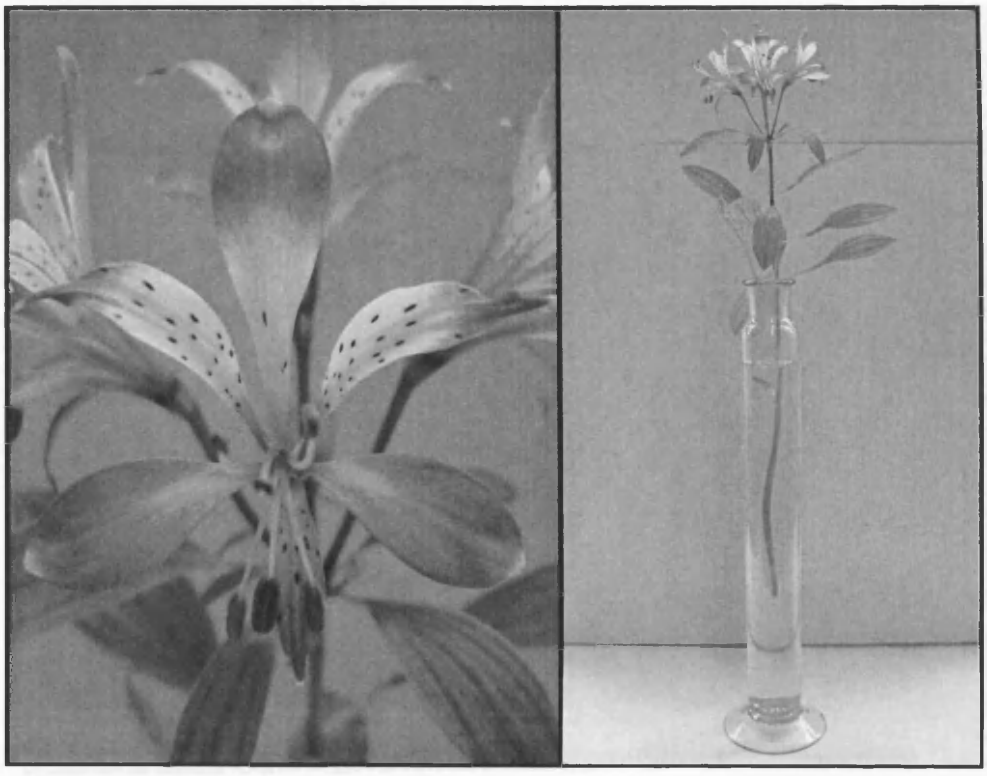
Flowers of DANCAR 005

Flowers of DANCAR 006

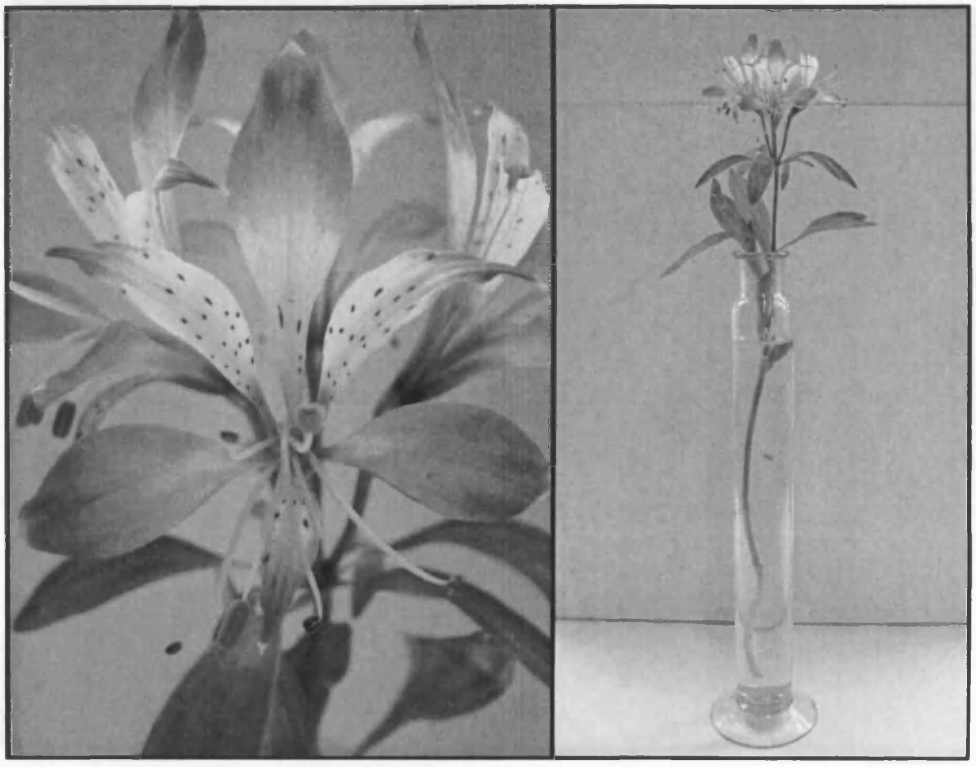


Flowers of DANCAR 008

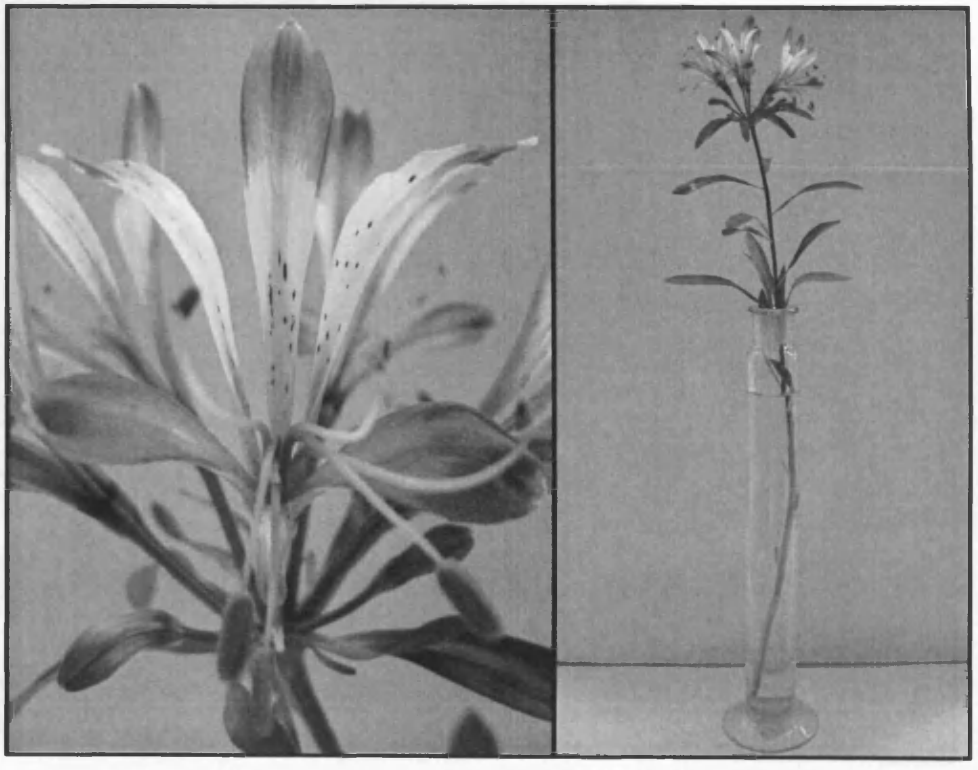
Flowers of DANCAR 009



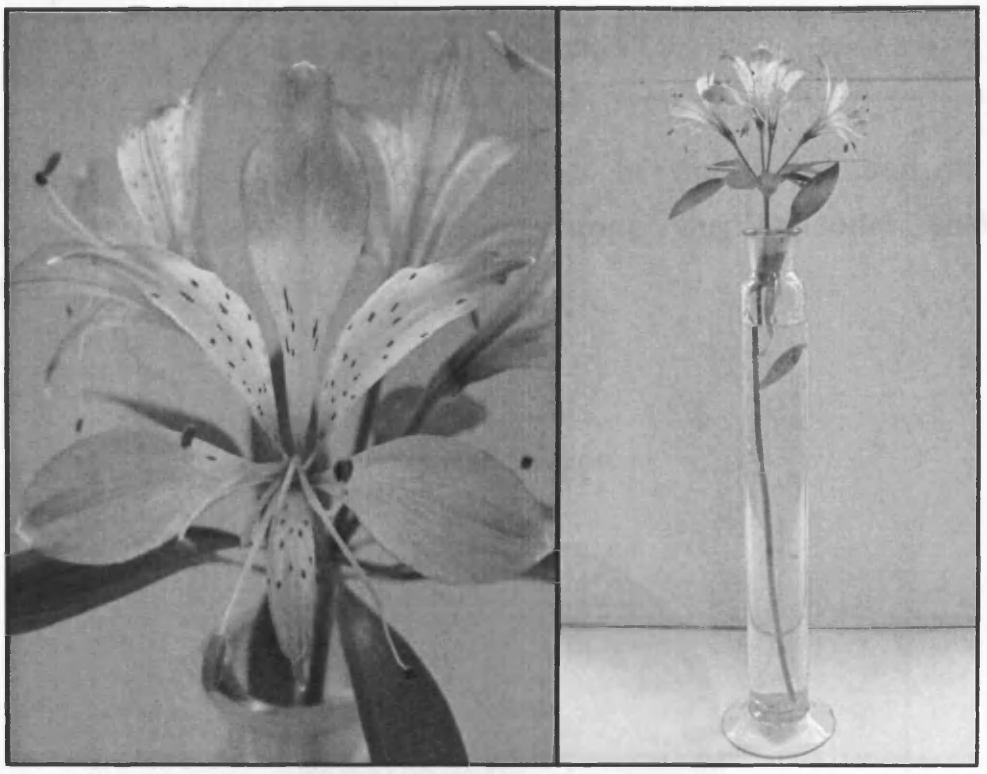
Flowers of DANCAR 010



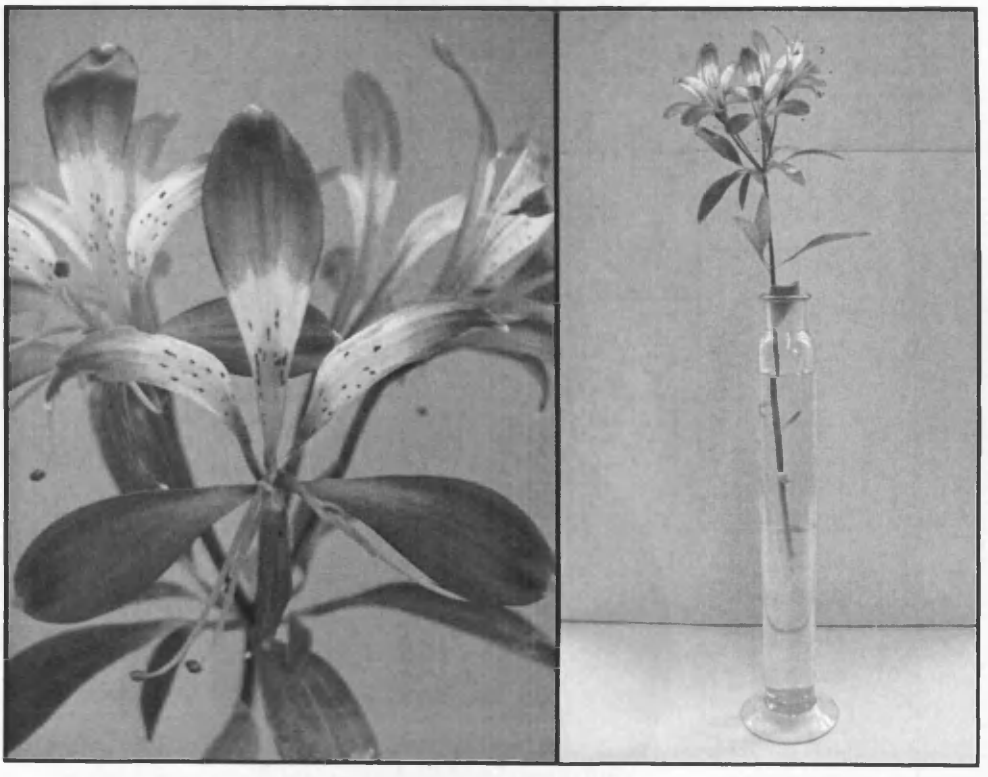
Flowers of DANCAR 013



Flowers of DANCAR 014



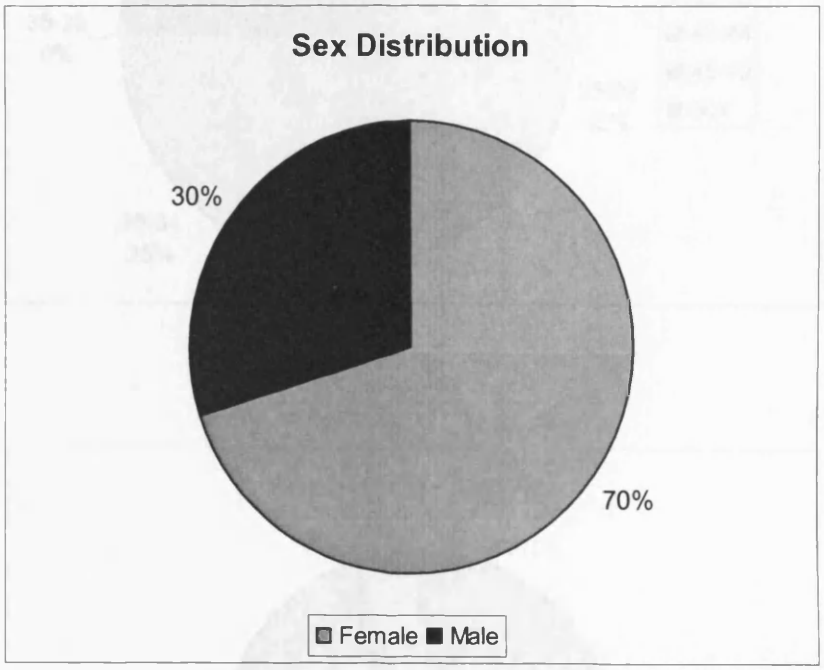
Flowers of DANCAR 016

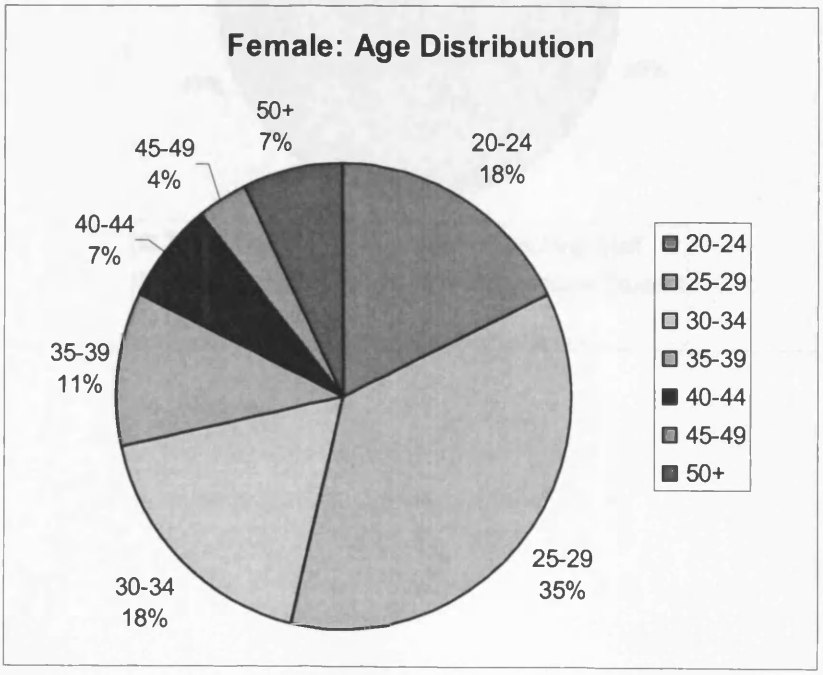


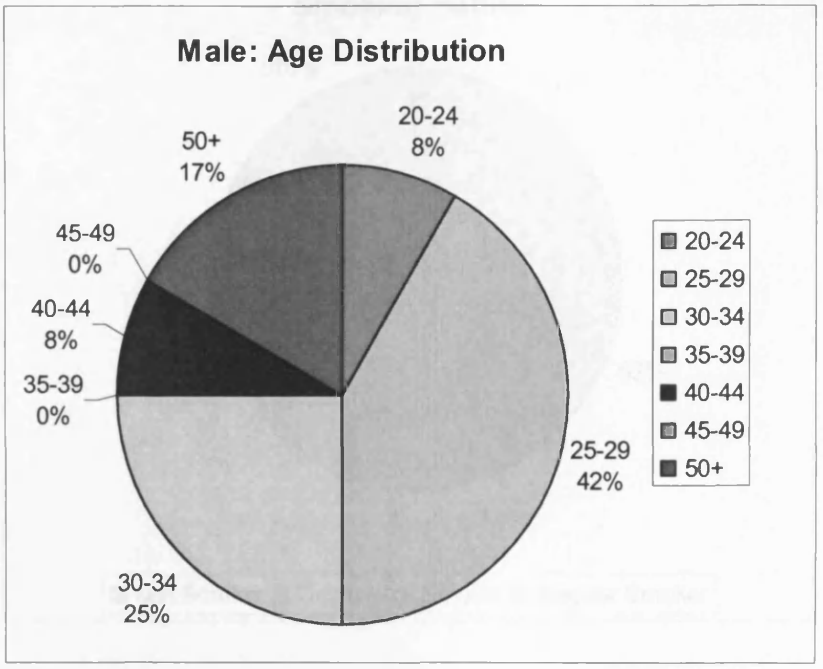
Flowers of DANCAR 017

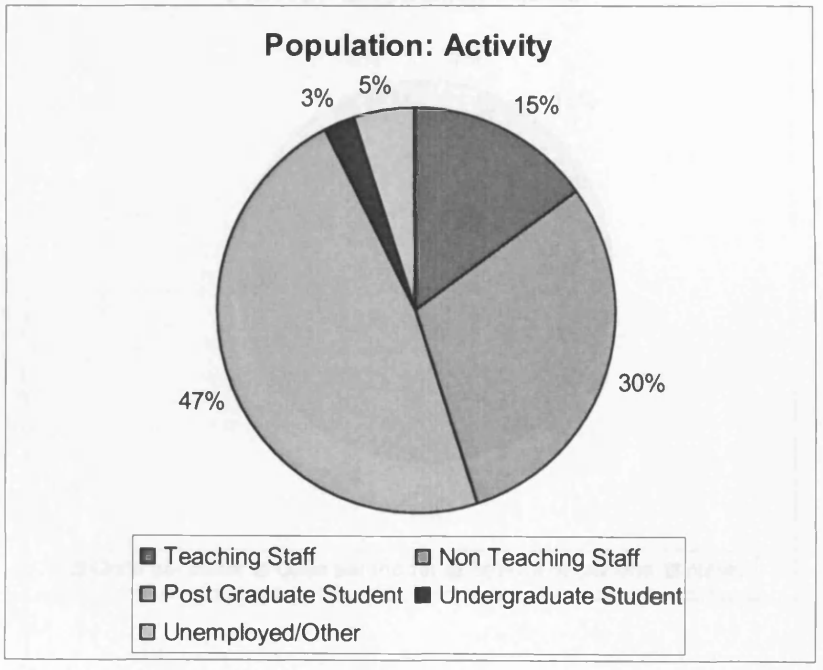
APPENDIX H

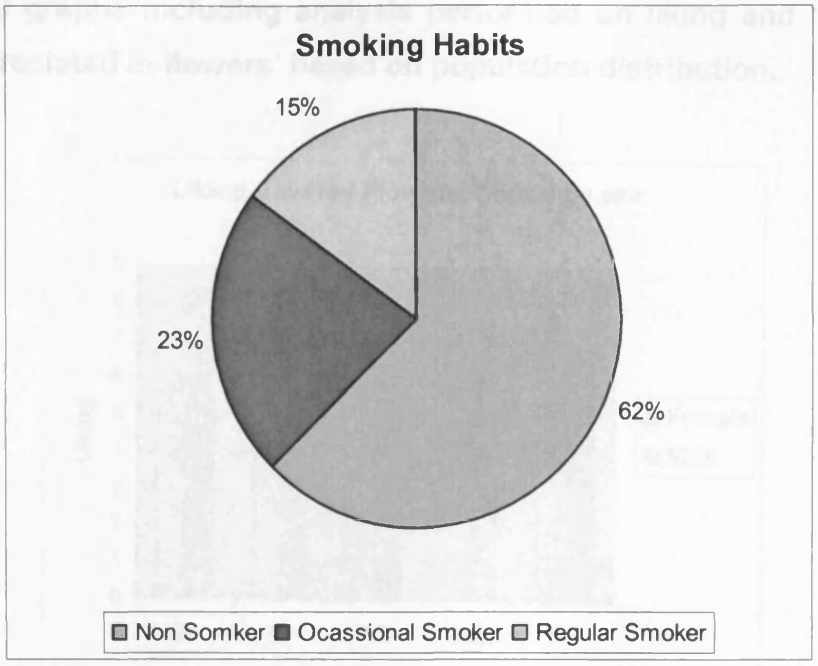
Additional graphs including information of the distribution of the population included as evaluators during the sensorial analysis performed on floral scent.

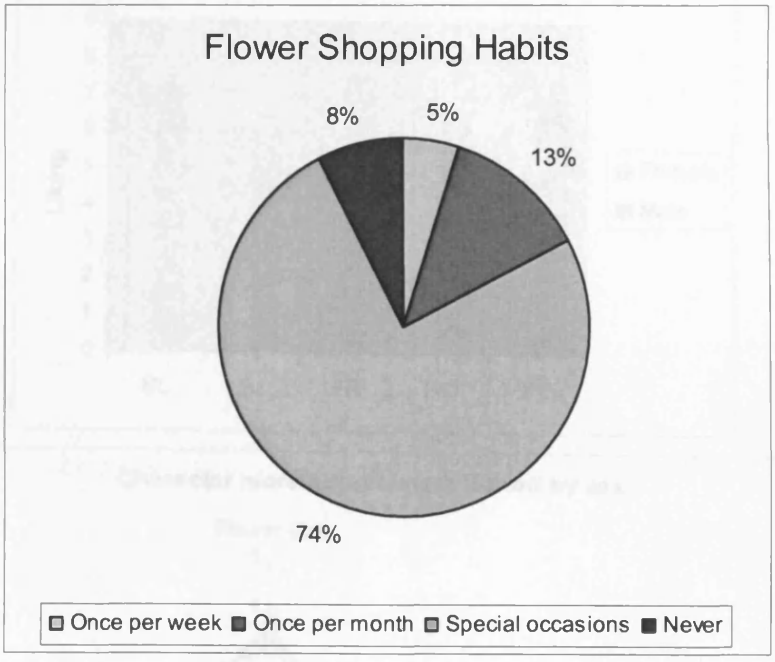




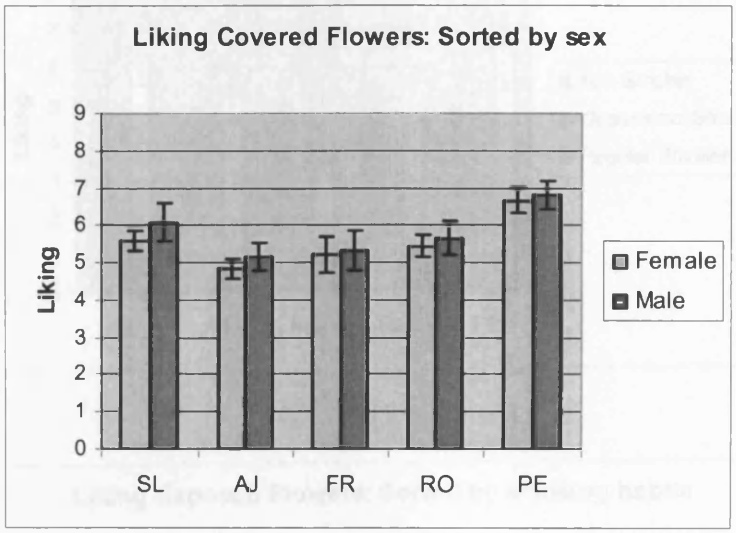

Male: Age Distribution
20-24
8%
50+
17%
45-49
0%
40-44
8%
35-39
0%
25-29
42%
30-34
25%
20-24
25-29
30-34
35-39
40-44
45-49
50+

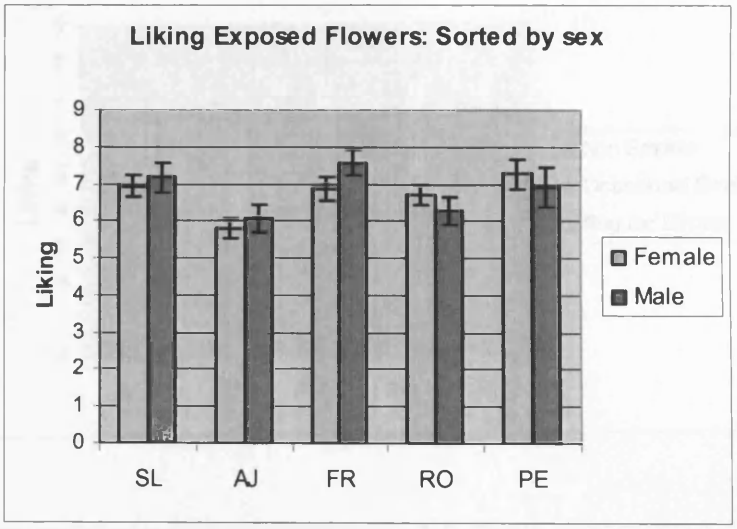

Population: Activity
3%
5%
15%
30%
47%
Teaching Staff
Non Teaching Staff
Post Graduate Student
Undergraduate Student
Unemployed/Other

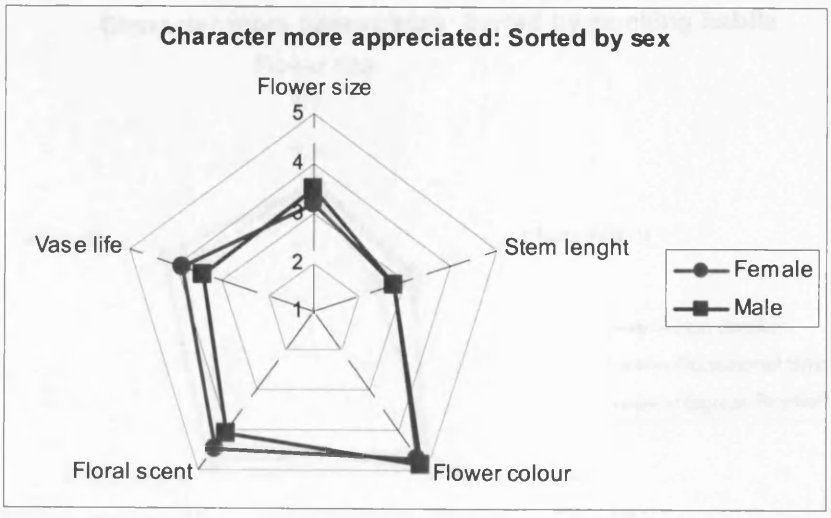

Smoking Habits
15%
23%
62%
Non Somker
Ocassional Smoker
Regular Smoker

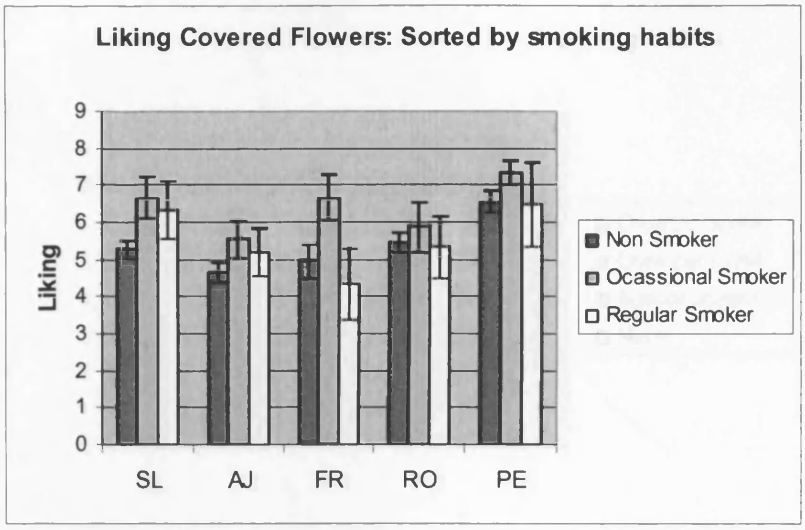

Flower Shopping Habits
8%
5%
13%
74%
Once per week
Once per month
Special occasions
Never

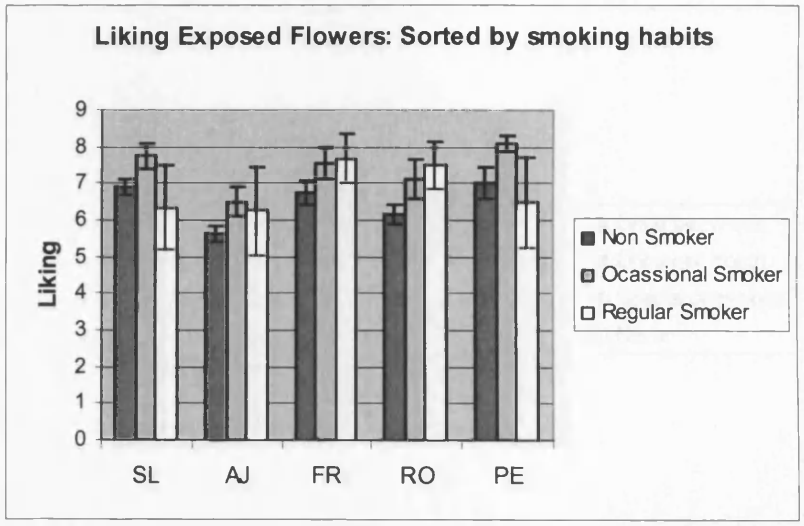
Additional graphs including analysis performed on liking and 'character more appreciated in flowers' based on population distribution.

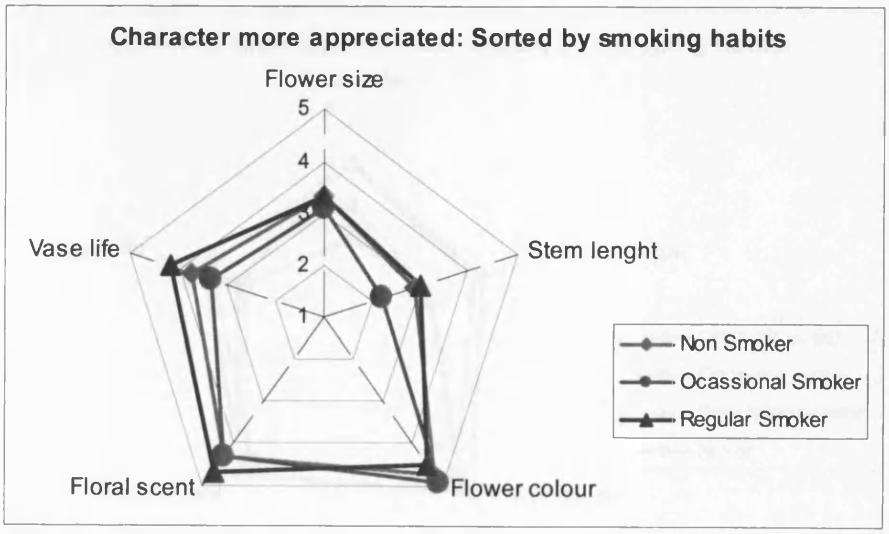


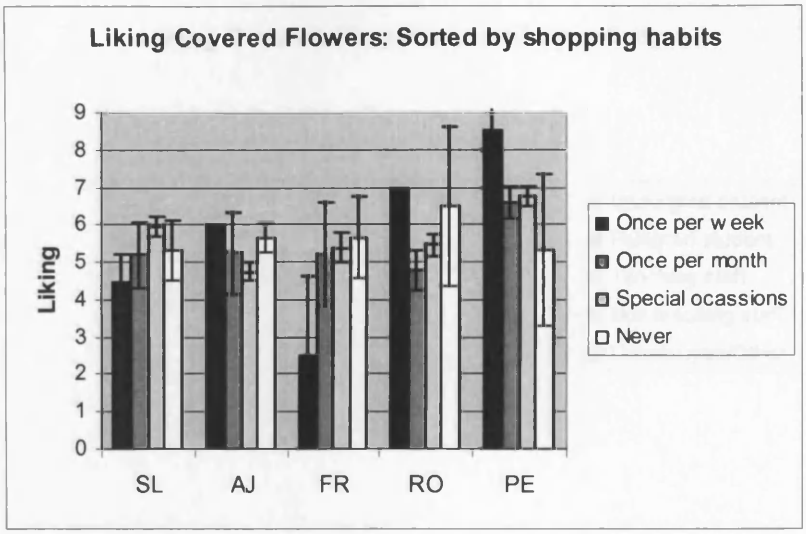


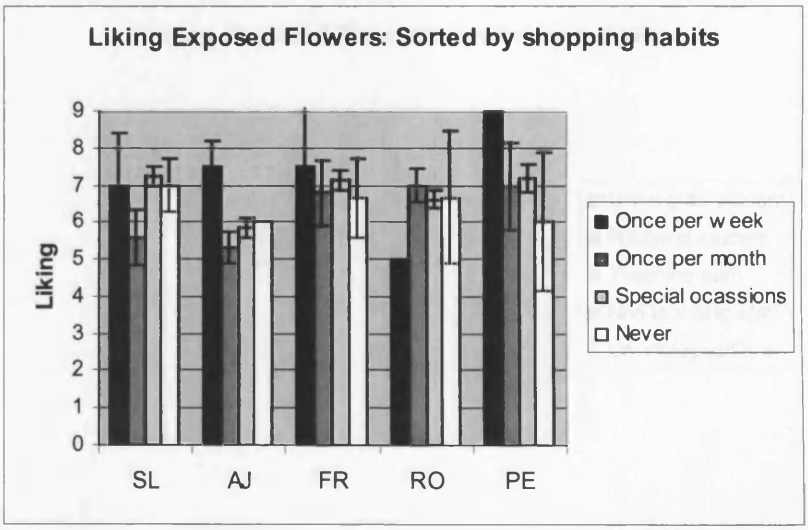


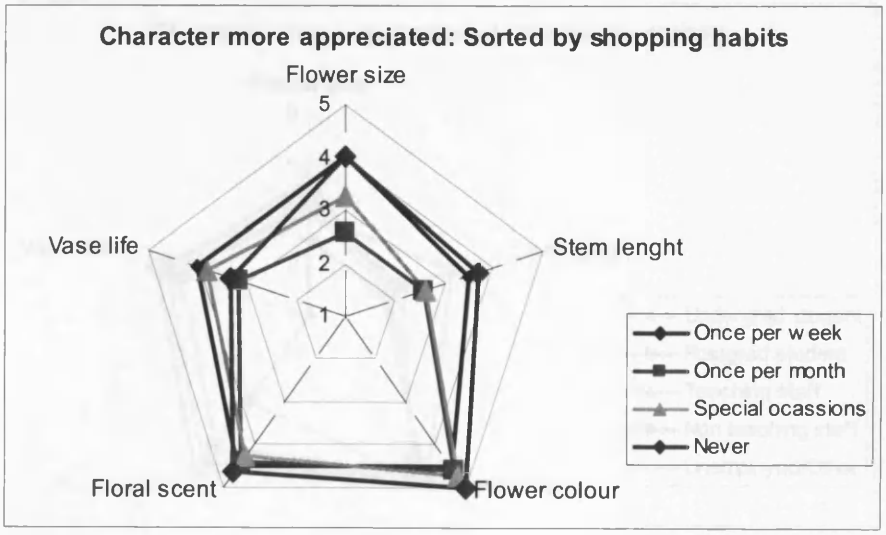

Liking Covered Flowers: Sorted by smoking habits
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
Non Smoker
Ocassional Smoker
Regular Smoker

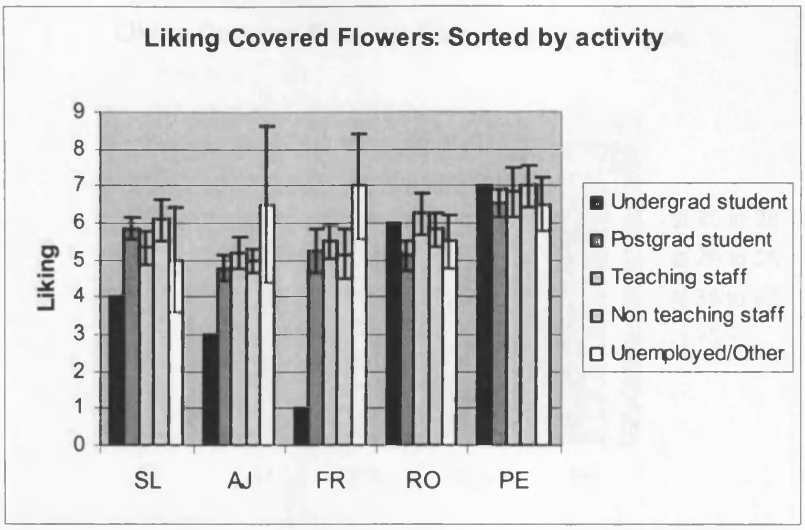

Liking Exposed Flowers: Sorted by smoking habits
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
Non Smoker
Ocassional Smoker
Regular Smoker

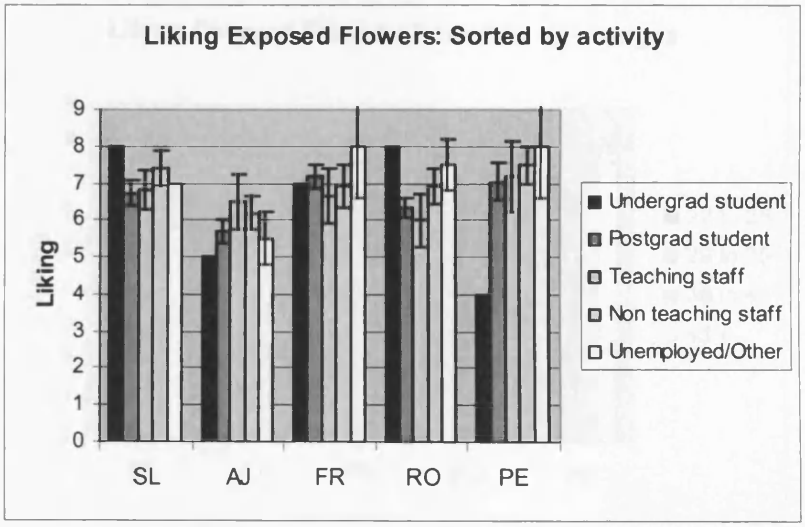

Character more appreciated: Sorted by smoking habits
Flower size
5
4
2
1
Stem lenght
Flower colour
Floral scent
Vase life
Non Smoker
Ocassional Smoker
Regular Smoker

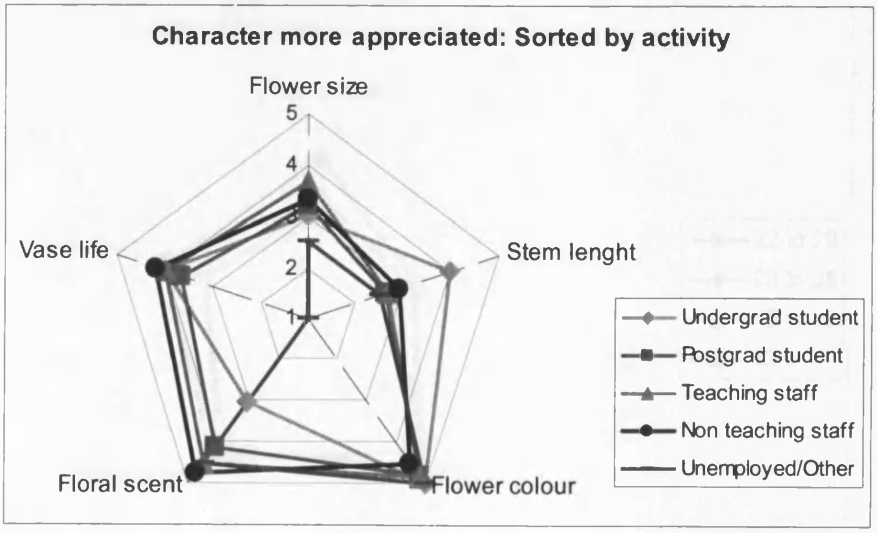

Liking Covered Flowers: Sorted by shopping habits
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
Once per w eek
Once per month
Special ocassions
Never

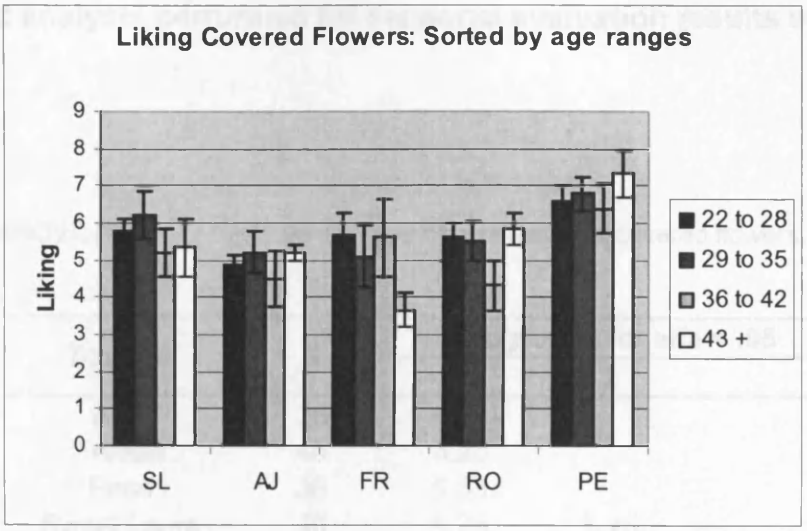

Liking Exposed Flowers: Sorted by shopping habits
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
Once per w eek
Once per month
Special ocassions
Never

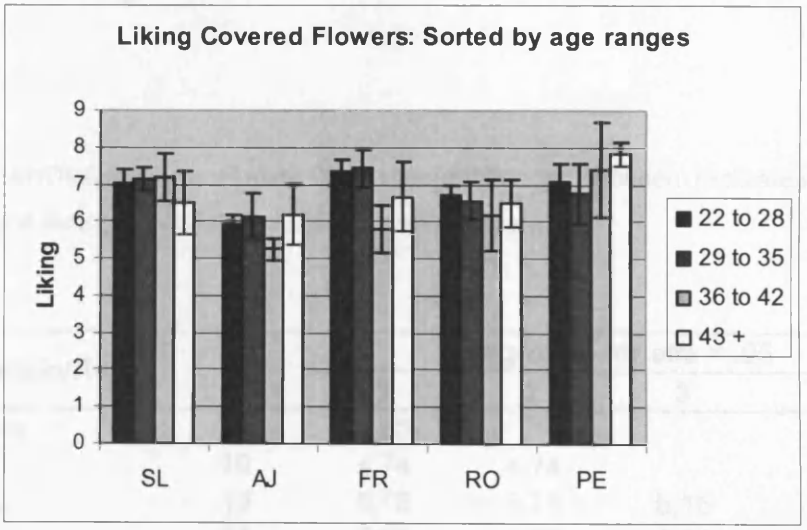

Character more appreciated: Sorted by shopping habits
Flower size
5
4
3
2
1
Stem lenght
Flower colour
Floral scent
Vase life
Once per w eek
Once per month
Special ocassions
Never

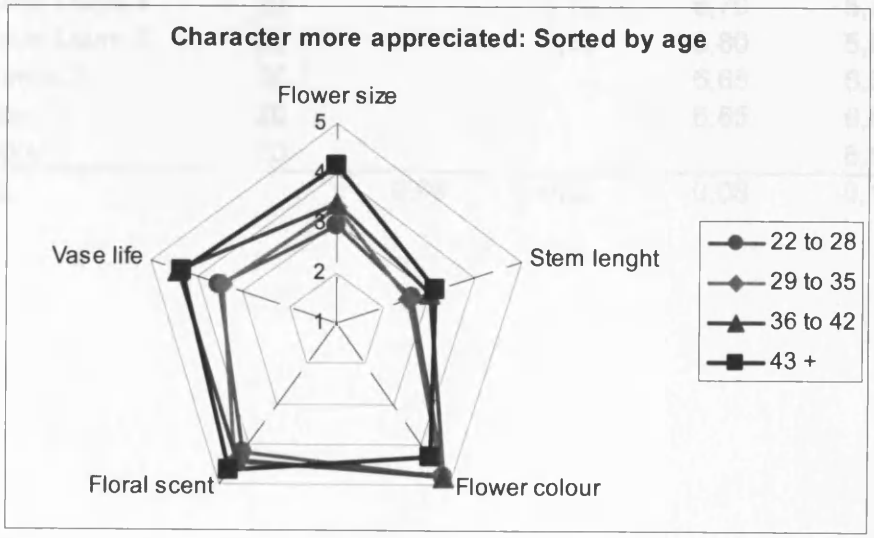

Liking Covered Flowers: Sorted by activity
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
Undergrad student
Postgrad student
Teaching staff
Non teaching staff
Unemployed/Other


Liking Exposed Flowers: Sorted by activity
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
Undergrad student
Postgrad student
Teaching staff
Non teaching staff
Unemployed/Other


Character more appreciated: Sorted by activity
Flower size
5
4
3
2
1
Stem lenght
Flower colour
Floral scent
Vase life
Undergrad student
Postgrad student
Teaching staff
Non teaching staff
Unemployed/Other


Liking Covered Flowers: Sorted by age ranges
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
22 to 28
29 to 35
36 to 42
43 +


Liking Covered Flowers: Sorted by age ranges
Liking
9
8
7
6
5
4
3
2
1
0
SL
AJ
FR
RO
PE
22 to 28
29 to 35
36 to 42
43 +


Character more appreciated: Sorted by age
Flower size
5
4
3
2
1
Stem lenght
Flower colour
Floral scent
Vase life
22 to 28
29 to 35
36 to 42
43 +

Statistical analysis performed for sensorial evaluation results using ANOVA.

Table H.1. ANOVA analysis (Tukey 95 %) for liking evaluation of covered flowers.

Sample	N	Subgroups for alfa = .05	
		1	2
Ajax	38	4,95	
Freesia	40	5,25	
Rose	36	5,53	
Sweet Laura	40	5,75	5,75
Peony	40		6,73
Sig.		0,21	0,08

Table H.2. ANOVA analysis (Tukey 95 %) for differences between replicates of the same samples found during sensorial analysis of covered flowers.

Sample/Rep	N	Subgroups for alfa = .05			
		1	2	3	4
Freesia 1	20	3,85			
Ajax 1	19	4,74	4,74		
Ajax 2	19	5,16	5,16	5,16	
Rose 2	17	5,35	5,35	5,35	5,35
Rose 1	19		5,68	5,68	5,68
Sweet Laura 1	20		5,70	5,70	5,70
Sweet Laura 2	20		5,80	5,80	5,80
Freesia 2	20			6,65	6,65
Peony 2	20			6,65	6,65
Peony 1	20				6,80
Sig.		0,08	0,50	0,08	0,11

Table H.3. ANOVA analysis (Tukey 95 %) for liking evaluation of exposed flowers.

Sample	N	Subgroups for alfa = .05	
		1	2
Ajax	34	5,91	
Rose	38	6,58	6,58
Sweet Laura	39		7,00
Freesia	40		7,08
Peony	40		7,18
Sig.		0,32	0,44

Table H.4. ANOVA analysis (Tukey 95 %) for evaluation of the character more appreciated in flowers.

Character	N	Subgroups para alfa = .05		
		1	2	3
Stem lenght	40	2,75		
Flower size	40	3,28	3,28	
Vase life	40		3,73	
Floral scent	40			4,35
Flower colour	40			4,73
Sig.		0,06	0,16	0,32

Raw data obtained from the sensorial analysis performed in floral scent.

SUBJECT	SURVEY 1 - Covered Flowers				
	Rep 1				
	SL	AJ	FR	RO	PE
	413	532	254	371	193
1	5	5	5	5	7
2	-	-	-	-	-
3	8	6	5	7	7
4	-	-	-	-	-
5	-	-	-	-	-
6	4	3	1	6	7
7	9	6	4	8	8
8	-	-	-	-	-
9	6	5	5	6	7
10	6	5	4	5	7
11	7	4	3	4	7
12	-	-	-	-	-
13	3	no	2	5	8
14	-	-	-	-	-
15	7	4	7	7	8
16	7	4	4	5	5
17	-	-	-	-	-
18	-	-	-	-	-
19	5	6	4	7	8
20	6	5	4	5	7
21	-	-	-	-	-
22	-	-	-	-	-
23	8	5	3	5	9
24	-	-	-	-	-
25	4	6	1	7	9
26	-	-	-	-	-
27	4	5	4	7	5
28	5	4	4	5	7
29	-	-	-	-	-
30	-	-	-	-	-
31	-	-	-	-	-
32	-	-	-	-	-
33	4	6	5	4	6
34	7	4	8	5	6
35	-	-	-	-	-
36	4	5	3	no	4
37	-	-	-	-	-
38	5	2	1	5	4
39	-	-	-	-	-
40	-	-	-	-	-

Averages	5,70	4,74	3,85	5,68	6,80
STEDV	1,66	1,10	1,81	1,16	1,44
SE	0,38	0,26	0,42	0,27	0,33

Code	Meaning
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely
no	No Smell

SUBJECT	SURVEY 1 - Covered Flowers				
	Rep 2				
	SL	AJ	FR	RO	PE
	316	638	759	175	892
1	-	-	-	-	-
2	6	6	8	6	5
3	-	-	-	-	-
4	4	2	8	6	6
5	4	4	9	5	7
6	-	-	-	-	-
7	-	-	-	-	-
8	4	8	8	6	6
9	-	-	-	-	-
10	-	-	-	-	-
11	-	-	-	-	-
12	5	7	5	6	5
13	-	-	-	-	-
14	5	6	6	7	8
15	-	-	-	-	-
16	-	-	-	-	-
17	7	5	8	3	9
18	7	3	5	3	7
19	-	-	-	-	-
20	-	-	-	-	-
21	6	6	7	8	2
22	7	4	3	3	6
23	-	-	-	-	-
24	6	5	8	no	8
25	-	-	-	-	-
26	6	3	8	9	9
27	-	-	-	-	-
28	-	-	-	-	-
29	8	6	9	7	8
30	5	4	7	4	7
31	8	5	7	5	8
32	6	6	7	4	8
33	-	-	-	-	-
34	-	-	-	-	-
35	5	no	6	no	4
36	-	-	-	-	-
37	4	6	4	no	7
38	-	-	-	-	-
39	7	7	4	4	6
40	6	5	6	5	7

Averages	**5,79**	**5,17**	**6,68**	**5,38**	**6,63**
STEDV	1,28	1,54	1,73	1,77	1,73
SE	0,29	0,36	0,40	0,44	0,40

Code	Meaning
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely
no	No Smell

SUBJECT	SURVEY 2 - General Info				
	Age	Sex	Are you a?	How often do you buy flowers?	Smoking habits
1	34	F	TS	1M	NS
2	23	M	PS	SO	NS
3	30	M	PS	SO	OS
4	25	M	PS	SO	NS
5	41	F	NTS	SO	NS
6	22	F	US	SO	NS
7	32	M	NTS	SO	OS
8	30	F	UO	1M	OS
9	43	M	TS	SO	NS
10	46	F	NTS	SO	NS
11	30	F	PS	SO	NS
12	27	F	PS	SO	NS
13	50	F	NTS	1M	RS
14	25	M	TS	SO	OS
15	24	F	TS	SO	RS
16	25	F	PS	SO	NS
17	25	F	PS	SO	NS
18	37	F	NTS	SO	OS
19	50	F	NTS	1W	NS
20	39	F	PS	SO	NS
21	26	F	NTS	NE	RS
22	28	M	PS	1M	RS
23	50	M	NTS	SO	RS
24	31	F	PS	SO	OS
25	23	F	PS	1W	NS
26	25	F	PS	SO	NS
27	51	M	NTS	SO	NS
28	23	F	NTS	SO	NS
29	25	F	NTS	SO	OS
30	24	F	PS	SO	NS
31	34	F	NTS	SO	NS
32	28	M	PS	SO	OS
33	30	M	PS	SO	NS
34	28	F	PS	1M	OS
35	38	F	TS	SO	NS
36	26	F	PS	SO	NS
37	42	F	TS	NE	NS
38	29	F	PS	SO	NS
39	28	F	PS	SO	RS
40	27	M	UO	NE	NS

Averages	31,85
STEDV	8,66
SE	2,04

Code	Meaning
F	Female
M	Male
US	Undergraduate Student
PS	Postgraduate Student
TS	Teaching Staff
NTS	Non Teaching Staff
UO	Unemployed/Other
NE	Never
SO	Special Ocassions
1M	Once per Month
1W	Once per Week
NS	Non Smoker
OS	Occasional Smoker
RS	Regukar Smoker

SUBJECT	SURVEY 3 - Exposed Flowers				
	Rep unique				
	SL	AJ	FR	RO	PE
	712	437	156	673	394
1	6	no	5	no	7
2	8	5	8	5	6
3	8	8	7	no	8
4	8	5	7	5	8
5	6	5	5	5	9
6	8	5	7	8	4
7	8	7	9	5	8
8	7	5	9	8	9
9	6	5	7	6	8
10	5	5	3	7	7
11	6	4	7	6	8
12	8	5	5	8	9
13	4	no	7	6	8
14	7	7	8	6	8
15	9	8	9	8	9
16	8	5	4	8	7
17	8	5	9	6	9
18	9	5	5	9	8
19	8	8	6	5	9
20	8	5	8	7	9
21	8	no	8	9	3
22	4	5	5	7	3
23	9	8	9	9	8
24	no	no	7	8	7
25	6	7	9	5	9
26	4	7	8	8	9
27	7	5	8	6	7
28	8	5	7	7	6
29	9	7	7	8	9
30	7	7	8	5	8
31	8	7	9	7	8
32	7	7	8	6	8
33	7	5	8	7	4
34	7	6	8	7	8
35	7	no	6	6	3
36	6	no	7	5	4
37	6	6	5	4	8
38	7	7	6	5	2
39	4	4	8	6	8
40	7	6	7	7	7

Averages	**7,00**	**5,91**	**7,08**	**6,57**	**7,18**
STEDV	1,41	1,22	1,53	1,35	2,00
SE	0,23	0,21	0,24	0,22	0,32

Code	Meaning
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely
no	No Smell

SUBJECT	SURVEY 4				
	The character you appreciate more is				
	Flower size	Stem lenght	Flower colour	Floral scent	Vase life
1	3	3	5	5	5
2	2	4	5	4	3
3	5	2	5	3	4
4	4	2	5	4	3
5	2	2	4	5	4
6	3	4	5	3	4
7	3	2	5	5	2
8	2	2	5	5	1
9	4	1	5	4	3
10	4	3	4	5	5
11	3	3	3	3	3
12	4	3	5	5	3
13	3	3	4	5	4
14	3	3	5	4	4
15	3	3	4	5	4
16	4	3	5	5	3
17	2	2	5	4	2
18	3	3	5	5	5
19	4	4	4	5	5
20	3	4	5	3	5
21	4	3	5	5	4
22	2	2	4	3	3
23	5	5	5	5	5
24	4	1	5	5	3
25	4	3	5	4	3
26	3	2	5	5	4
27	5	3	4	4	4
28	2	2	5	4	5
29	2	2	4	4	3
30	4	4	5	4	5
31	3	3	5	5	4
32	3	2	5	4	5
33	3	4	5	5	4
34	3	3	5	4	3
35	4	1	5	4	3
36	1	1	4	4	3
37	5	5	5	5	5
38	4	3	5	4	5
39	3	2	5	5	5
40	3	3	5	4	1

Averages	3,28	2,75	4,73	4,35	3,73
STEDV	0,96	1,01	0,51	0,70	1,11
SE	0,15	0,16	0,08	0,11	0,18

Code	Meaning
5	Strongly agree
4	Agree
3	Neutral
2	Disagree
1	Strongly disagree

Comments included in the survey by the evaluators during the sensorial analysis.

SUBJECT	COMMENTS
	Please comment on any aroma you have just perceived
1	What I smelled/did not smell was contrary to what I expected. Where I expected (from prior experience) a strong nice aroma there was hardly anything and viceversa. You could have asked people for their favourite fragrance they usually buy/wear
2	Very weak smell in samples 437 and 673, Ajax and Rose exposed flowers
3	Sample 254 (Freesia-Enclosed) I felt that the smell was too strong. I prefer lighter floral scent
4	No comments
5	No comments
6	No comments
7	No comments
8	The flowers numer 156 (Freesia exposed) reminds me the beggining of spring!
9	I have had better smelling Freesias than the one on show. The Rose was disappointing (Very weak scent)
10	Samples 532 and 371 (Ajax and Rose covered) / 712 and 437 (Sweet Laura and Ajax exposed) couldn't detect much odour
11	No comments
12	No comments
13	Samples 437 and 532 (Ajax exposed and covered)
14	No comments
15	No comments
16	No comments
17	No comments
18	Shame they (156/Freesia exposed) don't smell like Freesias otherwise it could be like extremeley :). Sample 437 (Ajax exposed) not very fragrant or is this meant to be as is sample 156 (Freesia exposed)
19	I particularly liked the Peony aroma. It's very fresh and clean and slightly citron-like
20	No comments
21	No comments
22	No comments
23	The aromas I've just tested always remind me of summer as a child
24	Sample 673 (Rose exposed) is a little weak
25	I love the white carnation's scent the most
26	Some of the flowers are realy nice in term of appearance but the scent are not nice/odourless
27	Some smell like desinfectant not very pleasant. Roses had a nice smell but barely detectable. One of the flowers had no detectable odour, but no option on form to indicate this
28	The red rose smell was familiar and pleasant for it
29	Some looked better than they smell
30	Liked the scented ones, not the others which were quite leafy-smelling
31	I really liked 394! Had a fresh and floral scent (Peony exposed)
32	No comments
33	Sample 712 had a sweet and citrus scent, very weak though
34	From the first study some scents remind me orange flavourand rose scent
35	None of these flowers or aromas I particularly like. I generally choose flowers with low aroma. Experiment design I think a third one would be good: only the visual and no scents
36	No comments
37	Never buy flowers But grow them
38	The rose scent was very weak
39	The roses had a weaker smell than I was expected
40	No comments