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EFFECTS OF AGRONOMIC VARIABLES AND HINERITANCE ON AROMATIC COMPONENTS IN BASIL AND CHILI PEPPER

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FOREWORD

The plants, as sedentary organism, have to ad just to the surrounding environment during their life cycle. To compensate for the absence of mobility, plants have developed various mechanisms that allow them to interact with the environment, as the emission into atmosphere of volatile compounds (VOCs) from flowers, leaves and fruits, and underground from roots. To date, 1700 volatile compounds have been described from more than 90 families and they are mainly represented by terpenoids, phenylpropanoid/benzenoid, fatty acid and amminoacid derived.

The primary function of VOCs is ensuring to the plant a reproductive and evolutionary success, attracting pollinators and seed disseminator through the emission of a blend of VOCs species-specific. Moreover, the plants produce VOCs in vegetative tissue in response to damage and herbivore attack.

The chemical composition of VOCs blend emitted from plants and its intensity are influenced by physiologic status, for example flower age, pollination status, endogenous diurnal rhythms and developmental stage of the plant organs. Compositional changes of volatiles in fruit blend during ripening have a primordial role as ecological cues for attracting organisms engaged in seed dispersal in the crops' wild ancestors.

In addition to an involvement of plant volatiles in defense and reproductive process, volatiles isoprenoids are able to protect plants from abiotic stress. In last years many efforts were directed to understanding the biology of the plants adaptation to abiotic stress in order to identify the key functions of tolerance and transfer them in open field crops through breeding approach.

The plant response to abiotic stress is rarely stress-specific; more often the stress triggers a generic response as the production of reactive oxygen species (ROS).

The plants have a complex response system of antioxidants and enzymes that protect them in condition of excess of ROS; when the defense system is overloaded, the oxidative stress occurs.

The salinity is one of the most critical stress that affect yield and quality in various agricultural systems and it is characterized by oxidative stress.

The tolerance to salt stress is coped by the plants through three interconnected mechanism. First, the damage may be prevent or alleviate trough the activity of antioxidant compounds and enzymes that protect the cells structures and functions from excess of ROS. Then, the homeostatic conditions are re-established by accumulation of osmolytes (i.e proline, sucrose). Finally, the plant growth is restored, even though at reduced rate.

In the last years, the research on antioxidants have focused mainly on non–volatile isoprenoids, (i.e. carotenoids and tocopherols), although certain volatile isoprenoids are also involved in protective activity against the oxidative stress.

The recent knowledge on regulation plant volatiles emission and signal transduction pathways involved are still at their infancy. To date, less than 10% of the genes responsible for volatiles biosynthesis have been identified. Although the transduction signals of defense-induced volatiles emission has been under investigation, the exact signaling mechanism that control the variation of volatiles emission related to environmental and physiologic factors still needs more investigation. The identification of the major volatiles compounds in fruits and vegetables and the genetic basis of fruit quality trait, as aroma, will provide a useful support to development of flavor-targeted breeding programs.

The identification of volatile composition at different stages of maturity may facilitate producers and industry in selection of fruits and vegetables for the market, and enhance agriculture sustainability by reduction of waste.

Molecular markers for fruit aroma may be useful tools to efficiently create improved cultivars with novel combination of volatile compounds. Additionally, knowing the genetic control of the major fruit quality traits will provide breeders with a handle to optimize content of these compounds to encounter the consumer preference and acceptance.

ABSTRACT

This PhD Thesis focuses on how aroma profile in crops is affected by agronomic and genetic variables. In the first chapter we considered the effects of salt stress on main morphological and physiological traits of two cultivars of sweet basil (Genovese and Napoletano) Specifically, we focused on compositional changes in aroma profile and their possible significance in adaptation and tolerance to the oxidative stress.

In the second chapter, we considered the aroma profile of two chilli pepper species, Habanero (*Capsicum chinenese*) and Jalapeño (*Capsicum Annuum*), at different stages of maturity. Moreover, preliminary results of genetic mapping process were introduced.

Part of this research has been conducted at University of California-Davis (USA).

RIASSUNTO

Nel presente lavoro di tesi sono analizzate le variabili agronomiche e genetiche che influenzano il profilo aromatico di specie vegetali. Nel primo capitolo gli effetti dello stress salino sui principali tratti morfologici e fisiologici di due cultivar di basilico, Genovese e Napoletano, sono stati investigati. In particolare, sono state considerate le variazioni del profilo aromatico e la loro possible implicazione nell'adattamento e la tolleranza allo stress ossidativo.

Nel secondo capitolo, è stato caratterizzato il profilo aromatico di due specie di peperoncino, Habanero (*Capsicum chinenese*) and Jalapeño (*Capsicum Annuum*), a diversi staid di maturazione. In fine, alcuni dei risultati reliminari del processo di costruzione della mappa genetica sono stati presentati. Parte della ricerca è stata condotta presso l'Università della California- Davis (USA)

CHAPTER 1

SALT STRESS TOLERANCE IN BASIL (OCIMUM BASILICUM L.): MORPHOLOGICAL AND METABOLIC CHANGES AND THEIR ROLE IN OVERALL ADAPTATION MECHANISM.

INTRODUCTION

Salinity is one of the most critical abiotic stresses affecting crop yield and quality worldwide. Today, about 20% of world's cultivated land and nearly half of all irrigated lands are affected by salinity (Zhu, 2001). Salinization of soils is a natural phenomenon occurring in areas of the world where evaporation exceeds precipitation and has been aggravated by agricultural practices. In the last decades many efforts have been dedicated to understanding the fundamental biology of plant stress adaptation with the ultimate objective of identifying key stress tolerance functions that could be transferred via traditional breeding and/or trans-gene technology to crop plants.

Biological systems, however, have shown wide adaptation to environmental stresses including salt, and plants can be found growing in saline environments and indeed in seawater.

High salt stress disrupts homeostasis in water potential and ion distribution both at a cellular and at whole plant level. Furthermore, prolonged and extreme salt stress is responsible for damages of cellular structures, as well as the inhibition of enzymatic activities, nutrient

uptake, photosynthetic functions, growth arrest and even death. Establishing a link between basic mechanisms of salt tolerance and functional traits that may actually improve crop production in saline environments is a major task. Salt tolerance is achieved in many plants through three interconnected mechanisms (Zhu, 2001). To resume growth, homeostatic conditions should be re-established in the new stressful environment though the accumulation of osmolytes and ion compartmentalization and the damage may be prevented or alleviated by antioxidant compounds and enzymes that defend the cell structure against condition of excess reactive oxygen species (ROS) (Zhu 2001).

ROS are important signaling molecules and also serve to initiate defense responses. The cellular balance of ROS is normally kept under tight control (Dietz, 2003); however, when this control is lost, damage occurs. Plants have a complex response network of lipid-phase and aqueous-phase antioxidant compounds and enzymes that defend against condition of oxidative stress.

The implication of isoprenoids in protection against oxidative and other abiotic stress has also been investigated. In the past more emphasis has been given to non-volatiles isoprenoids, which have many roles in plant cells and act trough different mechanisms. Some of them can carry messages throughout the plant and elicit systemic responses as hormonal signal (i.e. ABA), whereas others act directly as antioxidant (for example, carotenoids and tocopherols). Recent research has revealed that certain volatile isoprenoids also play an important role in abiotic stress responses (i.e. isoprene). Volatile isoprenoids are generally lipophilic, low-molecular-weight compounds with masses under 300 Da (Dudareva *et al.*, 2006).

Production of volatile isoprenoids represents a substantial investment for the plant in terms of carbon and energy. Constitutive amount of isoprene or monoterpenes produced by emitting plants is equivalent to 1-2% of photosynthetic carbon fixation (Sharkey *et al.,* 2001).

Under stress conditions, even when the carbon budget becomes negative and photosynthesis is severely inhibited, isoprene emission is often sustained (Brilli *et al.,* 2007). This large cost suggests the possibility that isoprenoid emission also confers benefit to the plant.

Change in volatile emission patterns under biotic and abiotic stress conditions suggested that volatiles might be linked with stress responses. Subsequent experiments have demonstrated that volatile isoprenoids play a role in photoprotection, thermotolerance, and they are involved in protection under oxidative and drought stresses (Vickers *et al.,* 2009).

The VIPs (volatiles isoprenoids) may be involved at different steps of oxidative stress response process. They can physically stabilize hydrophobic interaction in membranes, reducing lipids peroxidation. In addition, they may have an antioxidant behavior, scavenging ROS and prevent further oxidative damage (Vickers *et al.,* 2009).

Volatile isoprenoids confer protection against abiotic stress and common mechanism driving abiotic stress protection may be an antioxidant effect of these compounds (Vickers *et al*., 2009). Numerous studies, including inhibition of the monoterpene-producing methyl erythritol phosphate (MEP) pathway by application of fosmidomycin, fumigation of non-emitting species with exogenous gaseous isoprenoids and use of transgenic plant in which terpene synthese genes were inserted or silenced, have provided evidences that volatiles isoprenoids confer protective effect to photosynthesis under thermal and oxidative stress (Delfine et al., 2000).

The emission of isoprene appears to be a primitive trait that has been replaced with enzymatically controlled light-dependent monoterpene emission in non-isoprene emitting plants (Harley et al., 1997). Monoterpenes were suggested to be more effective in scavenging antioxidants in the gas phase than isoprene and, because of their lower volatility, form larger pools in membranes and intercellular spaces. (Fares et al., 2008).

In groups of non-isoprene-emitting taxa, monoterpene and sesquiterpenes may play the same role as isoprene in protection against abiotic stress. The taxonomic distribution of isoprene emission is broad: mosses (Hanson et al., 1999), ferns (Tingey et al., 1987), gymnosperms and angiosperms all have members that produce isoprene but also have members that do not (Sharkey et al., 2008).

According to the literature, some members of *Lamiaceae* (or *Labiatae*) family emit isoprene, e.g. sage (*Salvia sp.*), rosemary (*Rosmarinus officinalis*) and lavander (*Lavandula sp.)* (see http://www.es.lancs. ac.uk/cnhgroup/iso-emissions.pdf for a comprehensive list).

In this study, we focused on the response to salt stress of basil in terms of variation in volatiles profile and plant morphological and physiological characteristics. Specifically we attempted to establish a link between volatiles profile and morpho- physiological adaptation features in two basil ecotypes differing in salt stress tolerance.

MATERIAL AND METHODS

Two ecotypes of basil (*Ocimum basilicum* L.) were exposed to NaCl stress in three experiments carried out over a three-year-period (2007-2009). Two experiments were carried out at the University of Naples Federico II (40°49' N, 14° 15' E, 30 m.a.s.l.) in a cold glasshouse, in the summer 2007. The third experiment was carried out at University of California-Davis in a glasshouse in the summer 2009. In the following sections, only the results of the third experiment will be discussed.

Two cultivar of sweet basil (*Ocimum basilicum*), Napoletano and Genovese, were sown and grown in a greenhouse. When 4 leaves were fully developed, the plants were transplanted in coconut fibre pots filled with sterilised soil and grown in hydroponic system. Each growing unit, containing 6 plants, was filled with 15 L of Hoagland half-strength nutrient solution, refilled every day and aerated with a pump with air-stones; planting density was 50 plant m^{-2}. The cycle was conducted in 3 months from seed-to-full blooming. Starting from 25 DAS (days after sowing) to each growing unit different levels of salinity were assigned: 0, 100 or 200 mM NaCl. Each treatment was replicated twice (total 12 plants per each salinity level). The growing units were randomly distributed on the greenhouse bench.

Measurements of physiological status were done at plant establishment, 48 hours after stress and every week until harvest; the volatile compounds content and plant growth were assessed during the balsamic period at full-blooming (maximum yield in essential oils; http://www.hort.purdue.edu/newcrop/CropFactSheets/basil, 1995) Leaf area was measured with a scanner and the images were analysed using the ImageJ software (Abramoff *et al.*, 2004). Fresh and dry yield were measured at harvest and after drying at 60°C, respectively. Stomatal conductance, expressed in mmol $m^{-2}s^{-1}$, was measured 4 times (1, 3, 4, 7 weeks after salt application) on the abaxial surface of the youngest fully expanded leaves with a stady-state

porometer (Li-Cor LI-1600). Three measurements per plant and 3 measurements per each treatment were done. Leaf water potentials (Ψ_t) were determined using a pressure chamber and the osmotic potential (Ψ_π) was measured on frozen/thawed leaf samples (1x1 cm) with an osmometer (5500 Vapor Pressure Osmometer Wescor). Pressure potential (Ψ_p) was estimated as the difference between Ψ_t and Ψ_π at harvesting, assuming a matrix potential equal to 0.

The volatile compounds were extracted according to the procedure in Boatright et al. (2004) in six replicates per treatment. Leaves were ground in liquid nitrogen and dichloromethane was added to 4ml/g of tissue. Tissue was extracted on an Orbit shaker at 170 rpm for 1 h and the extract was centrifuged at 100,000 rpm for 10 min, followed by filtration through a 25 ml syringe with a 0.2μm sterile nylon filter. Anhydrous sodium sulfate was added to the filtered extract to remoce traces of water and the extract was evaporated down to 200 μl. Then 20 μ1 of butanoic acid, 2-methyl 3-methylbutyl ester (1.1 mM) were added as internal standard and sample were then analyzed by GC-MS.

The GC-MS analysis were performed on an Agilent 6890 GC/5975B MSD using a HP-5-MS non-polar capillary column (30 m X 0.25 mm; film thickness 0.25 μ m), injector temperature 220°C, splitless injection volume of 1μl and helium carrier gas flow rate of 1.2 ml/min. Initial column temperature was 40°C, then heated to 180°C at 6°C min^{-1}. MS analysis was performed with a transfer-line temperature of 230°C, a source temperature of 230°C, a quadrupole temperature of 150°C, an ionization potential of 70 electron volts, and a continuous scan range (m/z) from 40 to 300.

Spectral deconvolution was performed with the AMDIS software (Styczynsky et al. 2007) and the analysis of the spectra by MPP (Mass Profile Professional, Agilent).

The identification of the components was performed by matching their spectra with those present in the NIST library. Their occurrence in *Ocimum* species was confirmed by the

literature. Quantitative data were obtained from normalized area values with internal standard and fresh weight. The relative abundance of each component is expressed as the ratio on its peak area to that of the internal standard's.

All measurements were replicated on 5 different randomly selected plants. Data were analyzed with ANOVA and means were compared by the LSD test.

RESULTS

Growth response and water relations

Plant growth was affected by the salt concentration of the nutrient solution. The total leaf yield, leaf area and number of leaves decreased with increasing salinization in both cultivars (Table 1). Non-stressed GEN plants had a leaf number two times higher than NAP (240±10.7 vs 99±7.2, respectively). At 100 mM NaCl the leaves number in GEN was reduced by 20% compared to the control, but it was still double of that of NAP. The two ecotypes had different leaf area in absence of salt (Figure 1). The leaf area decreased similarly upon salinization in both cultivars. The specific leaf area (SLA) of NAP was three times that measured in GEN in absence of stress (Figure 2), and it decreased by 25% and 60% in GEN and NAP respectively upon salinization. In general the dry matter percentage increased in both cultivars upon salinization.

In absence of stress plants had the same values for the stomatal conductance (Figure 3). However, upon salinization the stomatal conductance was higher in GEN compared to NAP plants. At 100 mM, the stomatal conductance was reduced by 30% and 75% in GEN and NAP respectively compared to the non-stressed control. The differences between the two cultivars were reduced at higher salt level, however the stomatal conductance of GEN was always higher than NAP.

Leaf water and osmotic potentials were reduced upon salinization. Furthermore, the pressure potential reached its highest value in GEN at 100 mM NaCl, where it was affected more by osmotic component (Table 2). At this concentration, the water potential slightly increased with respect to the control.

Volatile compounds: effect of cultivars

Volatile compounds were analyzed for the two cultivars of *Ocimum Basilicum*, grown under salt stress conditions at full blooming stage. The list of all compounds identified, as well as their relative abundance, is presented in Table 3.

The total abundance of volatile compounds per gram of leaf fresh weight was higher in GEN than in NAP. Sixty-five components were detected in NAP and GEN leaf samples, 35-41% of which were monoterpenes; 23-17% were sesquiterpenes; 40% were phenylpropanes; 0.2-0.7% were aldeydes.

The main difference between the two cultivars in terms of volatile composition is represented by the abundance of two main phenylpropanoids, methychavicol and eugenol.

On total profile, methylchavicol and eugenol were relatively the most abundant, representing 40% and 34% of total compounds in NAP and in GEN, respectively.

Furthermore, the other components most represented were linalool (17-23%), eucalyptol (12-11%), ß-cubebene (6.6-2.3%), himachala-2,4-diene (2-4%), τ-cadinol (4.2-3.5%), τ-cadinene (1.5-1.3%), α-caryophyllene (1.1-1.5%) ß-ocimene (0.4-1.3%).

Differences between the cultivars in total relative abundance of monoterpenes were small and non significant. However, the main monoterpenes, such as eucalyptol, α-terpineol and ß-myrcene were significantly more abundant in NAP.

Himachale, ß-cubebene, α-caryophyllene, τ-cadinol were the sesquiterpenes relatively more abundant. ß-cubebene and τ-cadinene were significantly more abundant in NAP, while ß-cedrene was present only in GEN.

The main aldeydes, known as green leaf volatiles leaf, were 3-hexanal and 2-hexenal. The first was 5 times more abundant in GEN than in NAP, while 2-hexenal was not detected in NAP.

Volatile compounds: effect of salt stress

Upon salinization, the two cultivars revealed an opposite trend in relative abundance of total volatiles compounds per gram of leaf fresh weight: it increased in NAP and decreased in GEN. However, the total yield of volatiles per plant on dry weight basis showed similar behavior in both cultivars, increasing upon salinization; at highest salt concentration, 200mM NaCl, the total volatiles content was two times higher in NAP than in GEN.

The total yield of the main volatiles compounds, expressed on dry weight basis, is shown in Table 4.

The main phenylpropanoids were significantly affected by the salt treatments. In stressed plants, methylchavicol content increased significantly in NAP, whereas in GEN, where it was present in traces in the control, methylchavicol was not detected upon salinization. Eugenol content did not vary significantly in GEN on fresh weight basis in stress conditions, whereas it doubled compared to control when expressed on dry weight basis. In NAP at 100 mM it was 150 times more abundant relatively to the non-salinized control.

The relative content of all monoterpenes compared to total volatiles increased in both cultivars upon salinization. ß-ocimene, α-pinene and limonene content significantly increased in both cultivars, while the other monoterpenes were significantly more abundant in NAP.

The sesquiterpene pool showed the same trend in the two cultivars, with an increase upon salinization. The more representative compounds that have shown a variation in both cultivars upon salinization were α-caryophillene, ß-farnesene and α-bergamotene. τ-cadinene, τ-cadinol, α-cadinol and α-cubebene were enhanced by the salt stress in NAP.

The aldeydes were negatively affected by salt stress and they decreased upon salinization. 3-hexenal was always more abundant in GEN than in NAP, while 2-hexenal was not detected in NAP when salt was applied to nutrient solution.

Methyl Jasmonate concentration was found more abundant in GEN than NAP and was not significantly affected by salt stress.

DISCUSSION

Salt tolerance in cultivars of basil

Plant biomass was affected negatively by increasing NaCl concentration of the nutrient solution in both cultivars. At 100 mM NaCl, we observed in Napoletano a significant decrease in leaf area, specific leaf area and total leaves fresh weight, increase in percentage of dry matter, stomatal closure and reduction of leaf transpiration (data not shown). In Genovese the reduction in specific leaf area, leaf area and stomatal conductance were less severe and the dry matter percentage was not affected by salt stress.

These finding are supported by results from two previous experiments carried out on the same cultivars during summer 2007. The lower ABA accumulation observed at 100 mM NaCl in GEN indicates different ability of GEN and NAP plants to respond and adapt to salt stress, since ABA is one of the key mediator during plant stress adaptation (Zhu *et al.*, 1997). Therefore, the ABA function in the two cultivars may be different since they showed different ABA level and stomatal response to the salt stress. Moreover, the reduction in leaf transpiration through stomatal closure could also be enhanced by the control of stomatal size and density (Woodward *et al.*, 2002), which were significantly reduced in GEN. It has been shown that lower stomatal index enhances drought and salt tolerance (Aharoni *et al.*, 2004, Bray and Reid, 2002). The lower stomatal density of GEN compared to NAP at 100 mM, could have allowed the plant to cope with the salt stress more efficiently by optimizing water use efficiency. It has been recently demonstrated that the control of stomatal density is one of the genetic determinants that may affect water use efficiency (Masle *et al*, 2005).

Moreover, a greater increase in pressure potential, at this salt concentration, was observed in GEN, but not in NAP, indicating that the former was able to better adjust to the hyperosmotic environment. During osmotic adjustment cells tend to compartmentalize most of

the absorbed ions in the vacuoles and, at the same time, they synthesize and accumulate compatible organic solutes in the cytoplasm in order to maintain the osmotic equilibrium between these two compartments and with the external environment (Serrano and Gaxiola, 1994; Hare *et al.*, 1998; Hasegawa *et al.*, 2000). Our results in the previous experiments have assessed higher concentration of ions (Na^+, Cl^-) in GEN at 100 mM NaCl compared to NAP, and increased level of proline, an osmolyte typically involved in osmoregulation (Maggio *et al.,* 2002). The osmotic regulation contributes to maintain water uptake and cellular turgor, which are essential to sustain physiological processes such as cell expansion, stomatal opening, photosynthesis, and many others plant functions (Zhang *et al.*, 1999).

Overall these results indicate that at 100 mM NaCl, long-term mechanisms of adaptation to stressful environments are activated more efficiently in GEN than in NAP.

Antioxidant activity of phenylpropanoid volatiles.

The effect of salt stress on volatiles abundance per gram of leaf and on total leaves fresh weight was significant in both cultivars. Our results indicate that total volatiles yield per plant increased upon salinization in both cultivars.

At 100 mM, total phenylpropanoids content doubled compared to the control in both cultivars, but the composition of the pool changed significantly in NAP where, compared to GEN, a 40% reduction in methylcahavicol content was replaced by an increase in eugenol content.

Moreover eugenol derivates, such as isoeugenol and eugenol methyl ester, not present in the control, were detected at 100 mM NaCl, indicating that salt stress may specifically affect the biosynthesis of eugenol and its derivates. A large number of naturally occurring molecules having antioxidant properties are known to be phenolic compounds, like eugenol. Several

studies have demonstrated the antioxidant capacity of the eugenol and related compounds, like isoeugenol, to inhibit the lipidic peroxidation induced by reactive oxygen species (Toda et al., 1994; Hidalgo et al., 2009). According to Ogata and colleagues (2000), eugenol may inhibit lipid peroxidation by trapping active oxygen species, such as O_2 or hydroxyl radicals. These compounds may protect the membrane lipids from oxidation by scavenging the active oxygen species generated from oxidative stress. However, the mechanism of their scavenging reaction in vivo is still obscure.

The higher content of eugenol at the time of stress application may have been a constitutive advantage for GEN plants to better cope with the early stage of oxidative stress.

The existence of a functional link between the Ascorbate-Gluthatione cycle and the physiology of stomatal closure and dehydration protection has been documented (Maggio et al., 2002). An increase of the plant ascorbic acid pool by pretreatment of tomato root systems with ascorbate or its precursor L-galactono-1,4-lactone caused a dramatic protection against a gradually imposed water stress or severe osmotic shock (Maggio et al., 2002). It has been proposed that one component of the ascorbic acid-mediated protective effect was a rapid induction of stomatal closure, which in this case appeared to be ABA-independent.

In our investigation, we observed a reduction in transpiration rate in GEN due a partial stomatal closure at 100 mM NaCl. The salt treatment caused a partial stomatal closure before any detectable increase in ABA, which may have been caused either by a sub-cellular re-translocation, rather than ex-novo ABA synthesis (Zhang et al., 2001) or alternative signal transduction pathways involving H_2O_2 as signal molecule. Indeed, Pei et al. (2000) reported that H_2O_2 appears to act directly on guard cell behavior by activating Ca^{2+} channel through the activity of the *gac2* gene product, independently, or at least partially independently of ABA. Research on essential oil and their antioxidant activity *in silico* and *in vivo* have shown that

phenolic components can be, after penetrating in the cell, oxidized by ROS and generate additional radical species like phenoxil, hydroxyl, superoxide radicals and H_2O_2 (Bijur et al., 1997; Young and Lowe, 2001; Lowe et al., 2003). Antioxidants by interacting with ROS are converted into prooxidant which are able to oxidize lipids, proteins and DNA (Sakihama et al., 2002; Barbehenn et al., 2005; Atsumi et al., 2005). Volatile terpenic and phenolic components of essential oils can function as prooxidants by affecting the cellular redox status (Bakkali et al., 2006). In the cell, the redox balance is very sensitive. Probably compounds showing antioxidant activity can reduce the main load of oxidative stress but when there is an imbalance between oxidizing and reducing equivalents where the former predominates, for example when the antioxidant is oxidized and thus converted into a prooxidant, the antioxidant cellular defense cannot fully keep up with the oxidative stress and free radical are generated (Bakkali et al., 2008). In our experiment, the increase of eugenol upon salinization may have generated severe imbalance in cellular redox status and consequently enhanced the H_2O_2 levels in the cells. This may have triggered the ABA-independent mechanism of stomatal closure, that in GEN was translated in a partial stomatal closure, while in NAP the closure was more drastic, probably also supported by an ABA-dependent response.

Functions of Isoprenoids during salt stress: signaling response and ROS scavenger

An increase in total isoprenoids content upon salinization was observed in basil and some significant cultivar-specific trends were found.

The role of isoprenoids in plant response to salt stress is subject of discussion.

The enhanced biosynthesis of these compounds in the two cultivars of basil may be associated with their presumed involvement in stress response mechanisms in basil.

Monoterpenes are secondary metabolites formed in chloroplasts from freshly fixed carbon (Bohlmann et al., 1998) and their levels may, therefore, depend on CO_2 acquisition and formation of photosynthesis intermediates (Loreto et al., 1996). Our results show that the salt stress increased monoterpene concentrations while the plant dry weight accumulation was significantly affected in both cultivars. These contrasting trends were particularly evident when expressing monoterpenes concentration on a dry weight basis, suggesting that a larger fraction of carbon is allocated to monoterpene formation under stress condition in basil. The high costs of isoprenoids biosynthesis in stress condition, in terms of carbon allocated and energy indicates that they may have a significant ecological function in plants under oxidative stress (Peñuelas et al., 1998; in Delfine et al., 2005). In extreme environmental conditions between 5 and 40% of fixed carbon may be allocated into the biosynthesis of essential oils (Ross and Sombrero, 1991).

Evidences on isoprene's ability to protect the plant in abiotic stresses (thermal or oxidative stress) have been already shown (Loreto and Velikova, 2001). As in all taxa, also the *Lamiaceae* family comprises isoprene emitting and non-isoprene emitting members, although there is no information relative to basil (*Ocimum sp.*), to our knowledge. Moreover, there are strong phylogenic evidences that monoterpenes and sesquiterpenes may play the same role as that of isoprene in protection against abiotic stress in non-isoprene emitting plants (Harley et al., 1997).

The increased isoprenoids content increased under salt stress may be functional to protect and repair oxidative stress damages. α-pinene, β-ocimene, β-cariophillene and β-farnesene are some of the isoprenoids found in basil, for which antioxidant properties have been observed (Llusia and Pañuelas, 1998); they may act directly by scavenging ROS (Calogirou et al., 1999) and protect the cell from oxidative damages. Consistent with the hypothesis that an

increase in sesquiterpenes formation is a response to environmental stress (Charles et al., 1990), we observed an increase in the relative proportion of total sesquiterpenes upon salinization. As for the monoterpens, some compounds increased in both cultivars and no significant difference was found between GEN and NAP, with respect to α-caryophilene, β-farnesene and α-begamotene. Instead, significantly higher increases in τ-cadinene, α-cadinol and τ-cadinol were observed in NAP compared to GEN.

Even though the two cultivars have shown similar trends of increased total volatile content under stress, changes in concentration of some of isoprenoids, as was observed in NAP, may have a specific function in stress signaling.

In the transcriptome of Arabidopsis exposed to monoterpene volatiles several gene categories were identified as over- or under-represented. Exogenous application of myrcene or ocimene significantly affected the categories of transcripts associated with general, abiotic and biotic stress and transcription factors, that were over-represented in the transcriptome of treated plant (Godard et al., 2008).

Our results showed that salt stress conditions influenced myrcene and ocimene content. In stressed basil plants, β-myrcene content was positively affected by salt treatments and was significantly higher in NAP. The higher content of myrcene in NAP compared to GEN at 100 mM may be an indicator of more severe perception of salt stress.

The isoprenoids biosynthesis under stress condition may follow feed-back feed-forward mechanism: the stress response could be translated into an increase in VIP content and, in turn, an increased VIP content may trigger the stress response. Genes of the octadecanoid pathway and genes known to respond to octadecanoids (biosynthesis or response to JA) were among the two most prevalent within the stress-gene category both up-regulated by ocimene or myrcene (Godard et al., 2008). However, our results showed that increased contents in both of

monoterpens did not affect methyl jasmonate concentration upon salinization, while the total abundance in GEN was higher than in NAP.

CONCLUSIONS

The reduction of growth, osmoregulation and detoxification are the three responses associated to salt stress, which we observed in basil plants exposed to salinity. The Genovese plants have shown the ability to grow, even though at lower rate, adapting themselves to stressed conditions with reduction of leaf expansion and differentiation, lower stomatal conductance and traspiration rate. Increased pressure potential and ion accumulation observed in Genovese were representative of their ability to osmotically adjust, which in turn assured water uptake in hyperosmotic environment. A constitutively higher concentration of some volatile compounds with antioxidant capacity, such as eugenol and some isoprenoids, might have helped the plant to activate detoxification mechanisms and protect the photosynthetic process from oxidative stress. We also suggested a hypothetical link between increase of eugenol and ABA-independent mechanism of stomatal closure: the severe imbalance in cellular redox status consequent to increased concentration in eugenol upon salinization may have enhanced the H_2O_2 levels in the cells and triggered a partial stomatal closure in Genovese, while in Napoletano the more drastic stomatal closure may has also been supported by an ABA-dependent response.

The two cultivars revealed differences in salt stress tolerance in terms of response threshold. Constitutive morphological characteristics (stomatal index), physiological traits (growth adaptation) and specific metabolic profile (composition of the volatiles pool) may have been critical components for improving stress adaptation in Genovese plants.

The mode of action of isoprenoids during salt stress is still unclear. Isoprenoids can have direct and indirect action: protect the cell from oxidative damage scavenging ROS, and indirectly inducing transcription of stress-related genes. Volatile isoprenoids biosynthesis under stress conditions may follow a self-maintaining mechanism: the stress response is translated

into an increase in VIPs content and, in turn, the increased VIPs content may trigger the stress response. However, it is still unclear how their stress-induced accumulation in the plant may concomitantly act in stress response signaling.

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Table 1. Effects of NaCl treatments on main morphological indicators: Leaf area, Leaf yield, Number of leaves, Dry matter, Stomatal Conductance.

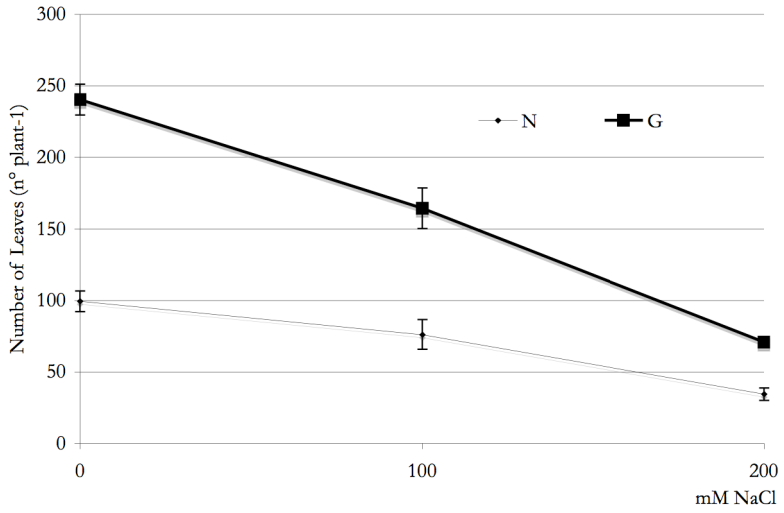
	Leaf Area	Leaf Yield	Number of leaves	Dry Matter	Stomatal conductance
	dm^2 $plant^{-1}$	g $plant^{-1}$	n° $plant^{-1}$	%	mmol m^{-2} s^{-1}
Cv					
GEN	18,2	51,7	158,4	17,3	199,5
NAP	21,2	87,6	69,9	12,2	149,5
Salt					
0	41,1	151,3	169,8	13,5	318,3
100	13,9	42,4	120,2	14,8	145,8
200	4,1	15,3	52,5	15,9	59,3
Significance					
Cv	ns	* (5,4)	** (3,1)	** (2,7)	** (9,8)
Salt	** (2.3 [1])	** (17,4)	** (7,1)	ns	** (16,1)
Cv x Salt	ns	* (18,8)	** (6,8)	ns	** (21,2)

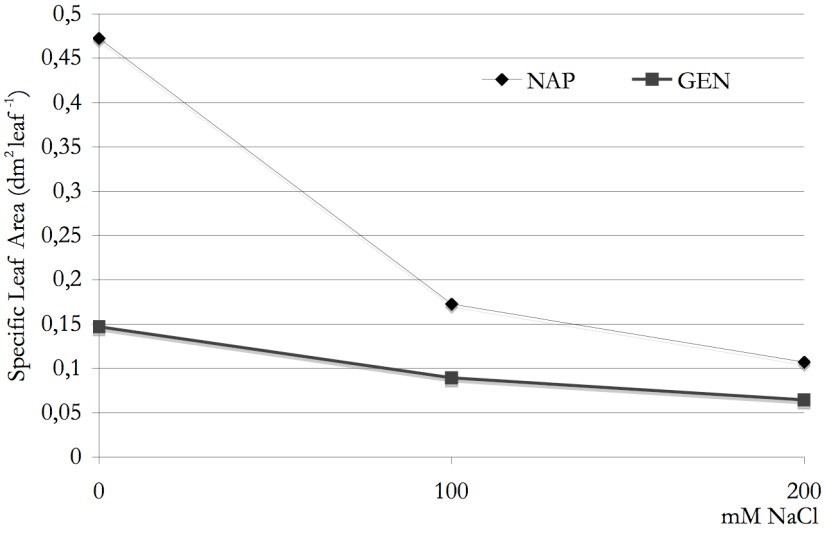
(Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01; lsd = [1])

Table 2. Influence of salt stress (0, 100 and 200 mM NaCl) on plant water status in two cultivars of sweet basil, Genovese and Napoletano.

	water potential	osmotic potential	pressure potential
		(MPa)	
Cv			
GEN	-0,43	-2,33	1,90
NAP	-0,36	-1,83	1,46
Salt			
0	-0,22	-1,10	0,88
100	-0,38	-1,99	1,61
200	-0,59	-3,14	2,55
Cv x Salt			
GEN 0	-0,25	-1,19	0,95
GEN 100	-0,30	-2,48	2,17
GEN 200	-0,75	-3,31	2,57
NAP 0	-0,20	-1,00	0,81
NAP 100	-0,46	-1,50	1,05
NAP 200	-0,44	-2,97	2,54
Significance			
Cv	ns	* (0,52)	ns
Salt	** (1,2 [1])	** (0,69)	** (0,2)
Cv x Salt	* (1,3)	ns	ns

(Mean values; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01; lsd = [1])





Figures 1-2. Influence if sal stress (0, 100, 200 mM NaCl) on Number of leaves and specific leaf area in the two cultivars of sweet basil, Npoletano (NAP) and Genovese (GEN).

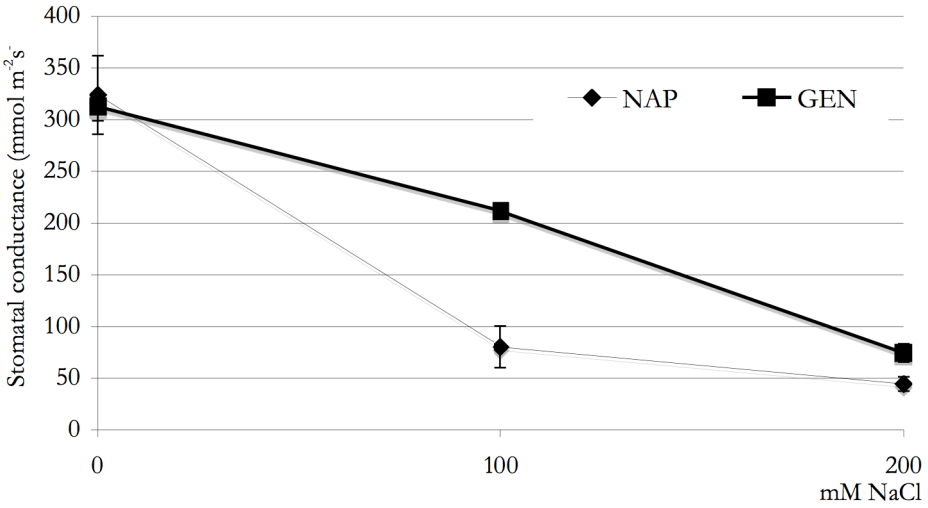


Figure 3. Influence of salt stress on stomatal conductance in two cultivars, Napoletano (NAP) and Genovese (GEN).

Table 3. Volatile compounds relative abundances of basil cultivars Napoletano (NAP) and Genovese (GEN) at different salt concentrations (0, 100, 200 mM NaCl).

Retantion time	Compound	References	N0	N100	N200	G0	G100	G200
5,33	3-Hexenal (Z)		11,72	1,32	4,17	49,18	24,89	26,61
6,74	2-Hexenal, (E)-		-	-	-	4,05	0,39	0,87
8,04	Heptanal		-	-	-	0,72	-	-
8,86	α-Pinene	a, b, c	11,97	21,31	25,03	12,24	16,06	16,95
10,00	Sabinene	a, b, c	18,55	32,21	35,45	17,51	17,43	23,82
10,21	1-Octen-3-ol		1,36	-	-	4,17	-	-
10,43	ß-Myrcene	a, b, c	14,69	27,21	31,76	36,32	33,85	33,14
10,86	Octanal		-	-	-	0,59	-	-
11,50	limonene	a, b, c	4,23	7,99	11,44	15,75	19,91	17,23
11,62	Eucalyptol		550,26	904,21	868,99	600,96	670,30	676,99
11,76	ß-cis-Ocimene	a, b, c	10,45	7,66	51,82	71,57	76,76	81,47
11,95	3-Carene	a, b	5,04	-	10,62	14,49	16,33	22,69
12,34	Mentha-1,4-diene		-	-	5,36	-	-	-
12,58	ß-cis-Terpineol		32,24	33,73	43,03	17,77	23,70	20,66
12,88	1-Octanol		-	-	-	1,02	-	-
13,12	Mentha-1,4(8)-diene		0,82	1,90	4,96	3,66	5,36	3,20
13,57	ß-Linalool	a, b, c	1005,71	1145,73	1031,95	1635,46	1136,33	1304,77
13,59	Nonanal		4,99	3,73	6,47	10,76	6,70	5,41

Table 3. (continued)

Retantion time	Compound	References	N0	N100	N200	G0	G100	G200
13,77	Octen-1-ol, acetate		-	-	0,39	3,22	3,29	3,25
14,61	Camphor	b, c	8,10	14,84	60,08	14,77	7,03	11,69
15,15	Borneol	b	-	-	-	5,57	-	-
15,43	Terpinen-4-ol	a, b, c	77,41	34,09	67,65	-	13,19	-
15,77	α-Terpineol	b, c	21,78	55,52	83,20	68,03	74,85	75,93
16,05	Estragole	a, b, c	2282,94	1540,20	1698,55	1,58	-	-
16,21	Acetic acid, octyl ester		2,56	13,41	22,35	17,16	26,20	26,50
16,95	ß-Citral		-	-	-	-	1,65	-
17,23	t-Terpineol		-	-	7,96	-	-	-
17,27	Chavicol		186,05	3,07	134,17	-	2,27	12,01
17,65	α-Citral		-	-	-	-	2,69	-
18,02	Bornyl acetate	a, b, c	1,69	6,07	25,50	51,99	77,61	86,05
18,34	Myrtenyl acetate		-	-	-	0,22	-	-
19,19	Elixene		5,97	10,58	8,34	8,53	6,08	6,68
19,43	α-copaene	a, c	1,69	2,17	7,78	-	0,57	-
19,55	δ-Elemene	b	-	4,74	14,58	0,15	0,69	0,80
19,77	Eugenol	a, b, c	22,71	1014,60	293,61	2290,50	1755,64	1971,69
19,90	cis-Isoeugenol		-	156,50	-	452,92	168,80	146,36

Table 3. (continued)

Retantion time	Compound	References	N0	N100	N200	G0	G100	G200
20,03	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester		4,24	2,45	1,67	4,53	1,42	1,97
20,09	Copaene		7,81	8,63	9,13	6,54	4,92	4,72
20,17	Geraniol acetate		-	-	-	-	3,31	-
20,42	ß-Elemene	b	34,38	49,60	41,88	47,65	33,47	35,02
20,63	Eugenol methyl ester		-	11,75	7,67	11,92	12,06	23,87
20,85	ß-Bergamotene		2,82	4,00	3,11	3,03	2,79	2,10
21,14	Himachala-2,4-diene		198,05	192,12	8,95	272,06	146,48	278,64
21,39	α-Bergamotene	b, c	8,26	8,85	254,73	109,16	10,21	12,30
21,45	α-Guaiene	b, c	14,41	16,86	13,87	17,43	13,63	13,23
21,47	ß-Sesquiphellandrene	c	4,19	6,09	8,11	1,46	1,90	0,46
21,50	ß-Cubebene		306,74	447,02	484,33	154,24	214,31	36,21
21,59	Isoledene		-	-	1,34	-	-	-
21,69	ß-Farnesene	c	17,41	6,93	17,53	17,32	13,60	13,39
22,01	α-Caryophyllene		90,57	84,98	29,62	78,19	77,37	110,70
22,67	τ-Elemene		108,09	74,28	129,67	40,13	31,37	68,40
22,80	δ-Guaiene	c	28,43	34,78	30,66	45,40	28,60	22,83
22,89	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-,1aR-(1a.,4b.,7a.,7b.,7a.)]-		25,57	78,36	62,54	10,05	4,94	-

Table 3. (continued)

Retantion time	Compound	References	N0	N100	N200	G0	G100	G200
22,92	τ-Cadinene		60,67	88,48	140,46	128,36	79,71	33,92
23,11	ß-Cedrene		-	-	-	19,60	13,98	7,72
23,15	Eugenol acetate		-	-	-	6,76	17,29	22,86
23,32	Germacrene D-4-ol		1,07	2,16	6,28	1,21	-	-
23,46	α-Muurolene		-	-	-	0,65	-	-
23,82	trans-Nerolidol	c	-	3,15	2,05	1,61	-	-
24,97	Cubenol		27,03	29,51	35,06	33,58	20,97	20,99
25,06	Dodecanoic acid, 1-methylethyl ester		0,84	-	0,67	2,96	1,09	3,07
25,19	Benzophenone		38,34	17,79	11,38	61,58	27,32	21,11
25,47	τ-Cadinol	b, c	227,77	252,94	292,90	272,76	172,70	182,34
25,53	Methyl jasmonate		2,28	3,48	0,68	7,30	4,57	2,76
25,67	ß-Eudesmol		-	1,44	1,27	-	-	-
25,71	α-Cadinol		3,82	4,00	8,52	4,69	3,05	2,07
	Total		**5495,7**	**6472,5**	**6159,3**	**6771,0**	**5129,7**	**5495,5**

The ralative abundance is expressed as the ratio of each compound peak area to that of the internal standard's. Identification confirmed by literature: a, Chalchat et al. (2008); b, Marotti et al. (1996); c, Viña et al. (2003)

Tab. 4 Major volatiles compounds and their relative abundance on dry weitght basis in basil cultivars Napoletano (NAP) and Genovese (GEN) at different salt concentrations (0, 100, 200 mM NaCl).

Compound	N0	N100	N200	G0	G100	G200	a	b	c
Phenylpropanoid									
Eugenol	1,4 ± 0,51	219,1 ± 12,9	211,7 ± 6,6	133,8 ± 31	290,2 ± 61,5	685,4 ± 77	**	**	**
Methylchavicol	148 ± 33,8	225,4 ± 15,9	1470 ± 190	0,1 ± 0,0	nd	nd	**	**	**
Monoterpene									
Eucalyptol	27,5 ± 3,9	188,3 ± 38,6	588,4 ± 89	38,8 ± 4	110,8 ± 10,8	241,0 ± 27	**	**	**
camphor	0,6 ± 0,6	3,8 ± 3,85	52,0 ± 13	1,0 ± 0,9	1,2 ± 0,59	4,3 ± 1,8	**	**	**
b-myrcene	0,8 ± 0,1	5,3 ± 1,38	24,7 ± 3,4	2,3 ± 0,3	5,6 ± 0,62	11,6 ± 1,5	**	**	**
sabinene	1,0 ± 0,2	6,9 ± 1,88	26,2 ± 6,7	1,1 ± 0,4	2,9 ± 1,05	7,9 ± 1,9	**	**	*
a-terpineol	1,6 ± 0,7	14,4 ± 5,72	54,9 ± 8,7	4,4 ± 0,5	12,4 ± 1,24	27,8 ± 4,3	*	**	**
b-linalool	57,3 ± 8,3	221,2 ± 33,1	654,1 ± 54	101,2 ± 15	187,8 ± 5,1	507,9 ± 46	ns	**	*
b-ocimene	0,8 ± 0,1	2,0 ± 0,95	44,8 ± 21	4,9 ± 1,2	12,7 ± 4,32	28,9 ± 8,5	ns	**	ns
a-pinene	0,7 ± 0,1	4,5 ± 1,04	33,5 ± 16	0,8 ± 0,1	2,7 ± 0,34	5,9 ± 0,6	ns	*	ns
limonene	0,2 ± 0,2	2,1 ± 1,06	7,7 ± 4,7	1,1 ± 4,7	3,3 ± 1,09	6,3 ± 1,6	ns	*	ns
Sesquiterpene									
Himachalene	14,5 ± 6,2	31,2 ± 17,7	6,7 ± 0,9	16,8 ± 7,4	24,2 ± 10,4	101,7 ± 14	**	**	**
t-cadinene	4,1 ± 1,7	22,0 ± 10,8	100,6 ± 23	8,1 ± 1,8	13,2 ± 3,34	14,1 ± 9,4	**	**	**
b-cubebene	11,7 ± 7,1	115,7 ± 46,7	330,6 ± 144	10,7 ± 7,8	35,4 ± 19,1	12,8 ± 4,5	**	*	*
a-caryophillene	4,1 ± 1,5	18,0 ± 4,84	21,9 ± 9,8	4,8 ± 1,4	12,8 ± 3,28	38,8 ± 6	ns	**	ns
b-fanesene	1,1 ± 0,3	1,4 ± 0,46	10,9 ± 5	1,1 ± 0,3	2,2 ± 0,65	5,2 ± 1,7	ns	**	ns
a-bergamotene	0,5 ± 0,1	2,0 ± 0,48	219,4 ± 137	7,5 ± 5,1	1,7 ± 0,39	4,4 ± 0,8	ns	**	ns

The ralative abundance is expressed as the ratio of each compound peak area to that of the internal standard's, normalized on dry weight. nd = not detected. Values are means **±** S.E.. c:= differences between cultivars , b= differences among salt treatments; c =differences among cultivars upon salinization; ns = not significant; * = significant at P≤0.05; ** = significant at P≤0.01

CHAPTER 2

AROMA POLYMORPHISMS IN CHILI PEPPER: CHARACTERIZATION OF VOLATILE PROFILES AND INFLUENCE OF RIPENING STAGE IN HABANERO (*CAPSICUM CHINENSE*) AND JALAPEÑO (*CAPSICUM ANNUUM*).

INTRODUCTION

Capscicum is a genus of plants belonging to the *Solanaceae* family, native to Mexico but now cultivated worldwide, whose fruits are mainly used as spices, food and medicines. Chili pepper is used in food preparation for its contribution in color, pungency and aroma (Mosquera et. Al, 1997). The main quality parameters considered in breeding programs in the past years for *Capsicum* varieties were color and pungency. However, current research is also focusing in the aroma as an important parameter for the quality of fresh fruit and vegetables (Luning et al., 1994; Cremer et al., 2000). Fruit aroma is important quality and marketability attribute, selected for during domestication and crop improvement (Zamir, 2001). This trait is apparently evolved from their primordial roles as ecological cues for attracting organisms engaged in seed dispersal in the crops' wild ancestors (Cipollini and Levey, 1997). There is great

variation for this trait in peppers due to a large number of crop types and varieties. The genus *Capsicum* comprises five main species: *C. annuum, C. frutescents, C. chinense, C. baccatum* and *C. pubescens* (Pruthi, 1980).

Jalapeño is a cultivar of the species *Capsicum annuum* originating in Mexico. It is named after the town of Xalapa, Veracruz, where it was traditionally produced. Domesticated in Central America, it is the most common species in Mexico and North America. The fresh market consists of green Jalapeños, and red Jalapeños are considered inferior. This variety is mildly pungent and its Scoville units range from 2,500 to 8,000. The Habanero chili (*Capsicum chinense*) is one of the most intensely spicy species of chili peppers of the *Capsicum* genus, its Scoville units range from 100,000 to 350,000. Like all *Capsicum*, the Habanero pepper originated in Meso- or South America, most likely the Yucatán and its coastal regions. Today, the crop is most widely cultivated in the Yucatán Peninsula of Mexico. Other modern producers include Belize, Panama, Costa Rica, and some U.S. states including Texas, Idaho, and California. While Mexico is the largest consumer of this spicy ingredient, Habanero flavor and aroma have become increasingly popular all over the world. Most peppers belonging to *C. chinense* species have a characteristic fruity smell, typical of the Habaneros.

In this study, two of the most popular hot pepper types, Jalapeño and Habanero were characterized for their major volatile compounds. Since they can be crossed with each other producing fertile hybrid (McLeod et al., 1983), and they have very different flavor, pungency, shape and size, these two species were selected to understand the genetic basis and inheritance of aroma compounds.

In general, fruit aroma is composed of a complex mixture of many, sometimes hundreds of volatile compounds arising from diverse biochemical pathways. The distinctive flavor of a fruit is a result of the relative abundances and interactions among volatiles, sugars and acids.

Flavor is therefore a complex fruit quality trait, which has been little investigated genetically. Most studies on pepper aroma have been done on bell pepper (*C. annuum*), where over 200 volatile compounds have been reported, (Van straten and Maarse, 1991; Lunning et al., 1994; Simian et al., 2004). Moreover, more than 125 volatile compounds have been identified in fresh and processed chili pepper (Nijssen et al., 1996), but their flavor significance is still unknown. A survey in 3 varieties of *C. chinense* revealed 34 volatiles involved in their aroma (Sousa et al., 2006).

The synthesis and emission of plant volatiles is under the control of a variety of factors, including developmental stage of the plant organ, diurnal endogenous rhythms and environmental conditions (Dudareva et al., 2006). Variations of aroma composition at different stages of ripening have also been investigated in chili pepper fruits. Recently, Pino and colleagues (2006) have studied the changes of volatile constituents in Habanero chili peppers during maturation and it was found that numerous volatile compounds decreased or even disappeared, while esters increased at the same time. Volatile constituents in *C. annuum var. glabriusculum* chili pepper have also been studied. Comparing the amount of total volatiles at two different stages of maturity, green and red, the authors concluded that green stage is better in terms of its flavor than the red stage for its higher content in volatiles (Forero et al., 2009).

As first step to identify the genes responsible for the synthesis of flavor-related chemicals, an attempt was made to identify loci that influence the chemical composition of ripe fruits. A few QTL (qualitative trait loci) mapping studies have been reported in *Capsicum*. 58 QTLs associated with nine yield-related traits, fruit parameters, flowering and maturity were detected in a *C. annuum* x *C. frutescens* segregating population (Rao et al., 2003). More recently Zygier et al. (2005) detected four QTLs associated with fruit shape, weight and size using

introgression lines of *C. chinense* and *C. frutescens*. QTLs associated with disease resistance have been reported by Ben Chiam et al. (2001).

As a precedent of QTL analysis for volatiles in Solanaceous crops, Tieman et al. (2006) carried out a genome-wide loci analysis of volatiles in tomato. Twenty-five loci were identified that significantly altered one or more of 23 different volatiles. However to date there is no information available for genetic basis and inheritance of flavor compounds in hot pepper.

The results presented in this thesis are part of a three-year project, the main goal of which is to characterize the major fruit quality determinants of the two most popular hot pepper types in USA market, Jalapeño and Habanero, and to understand the genetic basis and inheritance of fruit quality traits.

The long-term goal of this project is to improve fruit quality characteristics in pepper and to assist in the development of flavor-targeted breeding programs. The identification of molecular markers for pepper fruit quality can be used by breeders to efficiently create improved cultivars with novel combination of volatile compounds that could extend the market segment of the pepper industry. Additionally, knowing the genetic control of the major fruit quality traits (color, hot, flavor) will provide breeders with a handle to optimize content of these compounds. The specific objectives of the project are to identify the major fruit volatile compounds involved in Habanero and Jalapeño flavors; to determine chromosome segments controlling fruit volatile profiles in *Capsicum*; to analyze the genetic segregation of other fruit related traits and plant habit; to develop lines combining fruit traits from the two parental lines and to gain insight into the inheritance of these traits. In this thesis, the data on volatile aroma profiles of the two parental lines and the hybrid, and the influence of maturity stage on the profile composition will be discussed. Moreover, preliminary data of the scoring process to identify linkage group will be shown.

MATERIAL AND METHODS

Fruit volatile analysis

The experiment was carried out at University of California Davis during the years 2008 and 2009, on Jalapeño line 'JBS2MS' (*Capsicum annum*), Habanero line 'OR-HB-04IT' (*Capsicum chinense*), F1 hybrid generated by crossing these two lines; then a F2 population of 240 plants was generated in spring 2008. The plants were grown in the greenhouse of Harris Moran Experimental Station in Davis, California USA.

The volatile profiles of the parents, F1 hybrid, and F2 population generated in this study were determined as described below.

Fruit samples consisted of 4 fruits taken from 1 individual plants of the same accession/genotype. The fruits were sampled at two different maturity stages, green and colored (2 fruits per maturity stage).

For the sampling of pepper volatiles, the highly sensitive and quantitative closed-loop stripping method was used. Pepper fruits were cut in half and placed in an air tight chamber and volatiles were collected through a matrix trap (Porapak Q) during continuous circulation of headspace air inside the chamber for 1 hour. Trapped volatiles were eluted from the Poropak Q filters with 250 µl of dichloromethane (CH_2Cl_2). Then 20 µl of 3-methylbutyl 2-methylbutanoate 1.1 mM were added as internal standard and sample were then analyzed by GC-MS

The GC-MS analysis were performed with Agilent 6890 gas chromatograph (splitless, injector volume of 1µl) coupled to an Agilent 5975B quadrupole mass selective detector. Separation was performed on HP-5 non-polar capillary column (30 m X 0.25 mm; film thickness 0.25 µm) with column flow rate of 1.2 ml/min and helium as the carrier gas. Initial

column temperature was 40°C, then heated to 180°C at 6°C min^{-1}. Mass spectra were obtained in scan mode in the range (m/z) from 30 to 300.

The deconvolution of the spectra was performed by AMDIS and the analysis of the spectra by MPP (Mass Profile Profesional, Agilent).

A tentative identification was based on a search of the NIST library by comparison of the spectra of each compound with the spectra present in the library. Their occurrence in Capsicum species was confirmed by the literature

Quantitative data were obtained from normalized area values with internal standard and fresh weight. The relative abundance of each component is expressed as the ratio on its peak area to that of the internal standard's.

Due to human's variable sensitivity to different aroma compounds, not all constituents found in the GC-MS analysis contribute to pepper flavor and some compounds with low olfactory threshold weren't detected. To verify the presence of pyrazines in our samples, the aroma extract was also subjected to gas chromatography coupled with Olfactometry (GC-O) analysis. 2-isobutyl-3-methoxypyrazine was used as standard to monitor the presence of main ions.

A The GC-O analysis were performed with Agilent 6890N gas chromatograph (splitless, injector volume of 1μl) coupled to an Agilent 5973 quadrupole mass selective detector, equipped with sniffing port. Separation was performed on HP-5 non-polar capillary column (30 m X 0.25 mm; film thickness 0.25 μm) in constant pressure mode (25.2 psi), with column flow rate of 3.4 ml/min. Initial oven temperature was 40°C held for 5 min, then heated to 250°C at 8°C min^{-1}. Mass spectra were obtained in SIM mode, scanning four different ions: 94, 124, 127, 154.

Using this method, we analyzed the aroma of the parental lines and F1 samples at mature green stage. Three panellists sniffed from the sniffing port each extract in duplicate recording qualitative and semi quantitative (strong, medium, weak) information for each compound perceived in the sample. Panellists were trained to recognize 2-isobutyl-3-methoxypyrazine with bell pepper-like odour, using authentic standard.

After the identification of this odour in the samples, peak identification of 2-isobutyl-3-methoxypyrazine in the samples was performed by comparison of their spectra to that of authentic standard.

Genetic map

DNA was extracted from the leaves of the parental lines, F1 hybrid and 240 plants of F2 population by CTAB DNA isolation method.

0.5 g of tissue was ground in a cold 20 ml Eppendorff tube with a mini pestle in liquid nitrogen. 0.9 ml of 2% preheated CTAB buffer at 65°C were added to the tube. The tubes were placed in water bath at 60°C for an hour. After, the samples were cooled down to room temperature and equal volume of chloroform was added; they were mixed for 3 min and centrifuged for 5 min at 14,000 rpm. The supernatant was removed and washed three times in 76% ethanol. After overnight drying, 200 µl of distilled water was added to each tube. The concentration of DNA was measured with a spectrophotometer (NanoDrop 3300 Fluorospectrometer, Thermo Scientific) on sample size of 1µl, and adjusted to 10 ng/ml.

The CTAB buffer (100 ml) was prepared as follow: 2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide),10.0 ml 1 M Tris pH 8.0 , 4.0 ml 0.5 M EDTA pH 8.0 (EthylenediaminetetraAcetic acid di-sodium salt), 28.0 ml 5 M NaCl , 40.0 ml H2O 1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) 40,000 Mw).

The pH was adjusted 5.0 with HCl and the solution was made up to 100 ml with H2O.

SSR primers were synthesized based on the public sequences available at the Sol Genomic Network (http://sgn.cornell.edu/), including those reported in various maps (Nagy et al 2007, Wu et al 2009). This allowed aligning the linkage groups to those cited above and to the Capsicum AC99 and FA03/COII maps at Sol Genomic Network, constructed with over 900 SSR markers, many of which are publicly available.

For SRAP markers we follow the procedure reported by Li and Quiros (2001). The amplified DNA fragment are separated by denaturing acrylamide gel and detected by autoradiography. We used EM2 (5' GAC TGC GTA CGA ATT CTG C 3') as the forward primer labeled with fluorescent dye IRDye 800, in combination with either ODD50 (5' GAA TGC CAT CTA TCT CTT GA 3') or GA6 (5' GAG AGA GAG AGA TCA GC 3') as reverse, unlabelled primers. For band separation we used a LI-COR sequencer IR2 model 4200 (LICOR, Lincoln, Nebraska). Standard PCR procedures for DNA amplification were used depending on the type of marker.

RESULTS

Volatile profiles of parental lines and their variation at different stages of maturity.

The analysis was conducted on two different stages of maturity, green (MG) and colored (MR). In total sixty-six compounds were found in the two stages of maturity. At mature green stage, 74-36% of the volatiles are esters and 5-61% are sesquiterpenes, in the Habanero and Jalapeño respectively. Instead at mature coloured stage, the 86-42% of the compounds were esters while 5-57% were sesquiterpenes, in the Habanero and Jalapeño respectively (Figure 1.)

Moreover, about 20% of total volatile compounds in Habanero at mature coloured stage was represented by a cyclic compound, cyclohexanol, 3,3-dimethyl. At coloured stage, the content of this cyclic compound decreased, representing 7% of the total components found in Habanero. The list of all compounds, as well as their relative abundance, is presented in Table 1. The total abundance of volatile compounds per gram of fresh fruit detected in Habanero was 16 and 50 times higher than in Jalapeño, at green and coloured stage of maturity respectively. With regards to the variation of the total volatile content at different stages, opposite trends were observed in the two parental lines: the total volatile content increased in Habanero at the coloured stage, while it decreased in Jalapeño (Figure 2).

The composition of the volatile profile differed as the colour changed from green to orange/red. Esters increased in the parental lines when the fruit turned colour, from 74 to 86% in Habanero and from 36 to 42% in Jalapeño. Aliphatic esters represented 74% of all the esters in Jalapeño, while they accounted for only 9% of the total esters in Habanero.

At the mature coloured stage, sesquiterpene abundance decreased in Jalapeño from 61 to 57%, while no change was observed in Habanero. Compositional changes in the sesquiterpene

pool were observed in Habanero according to maturity stage; 8 new compounds, among which α-cubene, longifoliene were identified at orange stage. Moreover, while allo-aromadendrene was the main sesquiterpene at MG stage (40%), it was absent from the volatile profile when the fruits turned orange.

Monoterpenes and noroisoprenoids were not detected in Habanero at mature green stage. The norisoprenoid β-ionone was detected only in coloured fruits of Habanero.

In Jalapeño, the contribution of monoterpenes to the overall aroma profile decreased at the coloured stage, while norisoprenoids were not detected in either stage.

The main volatile component found in Jalapeño was isocaryophillene, which represented 60% of the total volatile profile. (Z)-3-hexadecene was detected only in Jalapeño. (Z)-3-hexen-1-ol and pentanoic acid, (2E)-2-hexen-1-yl ester were only detected at the green stage, while (E)-2-hexenal, 2-ethyl-hexan1-ol and tetradecane were present when the fruit turned red.

Three compounds, butanoic acid, 3-methyl-, hexyl ester, cyclohexanol, 3,3-dimethyl, isovaleric acid, hexenyl ester, represent 65% of the total volatile profile in Habanero.

Some compounds such as pentanoic acid, (2E)-2-hexen-1-yl ester and isocaryophillene were found only at mature green stage in Habanero, while new compounds such as α-longipinene and β-ionone were detected in coloured fruits. The compounds α-cubebene, β-cubebene and β-ionone were found in Habanero but not in Jalapeño.

F1 hybrid

Pepper fruits from the hybrid plant generated by crossing the Habanero and Jalapeño parents had higher total volatile content at both stages compared to the parents; the total abundance of volatiles decreased at the mature coloured stage, as was observed in Jalapeño.

Esters represented 85% of the total volatile profile, 11% of which were aliphatic esters, and 11% were sesquiterpene esters. Butanoic acid, 3-methyl-, hexyl ester, butanoic acid, 2-methyl-, hexyl ester and isocaryophillene were the most abundant components on the total volatiles profile at mature green stage.

Butanoic acid, 3-methyl, 3-hexen-1-yl ester and cyclohexanol, 3,3-dimethyl- were common only to the Habanero parent and the F1, suggesting that they may be inherited from Habanero, while heptadecane and ocimene, common only to Jalapeño parent and F1, may be inherited from Jalapeño. New compounds, such as (Z)-butanoic acid, 3-hexenyl ester and benzoic acid, hexyl ester were found in F1 at mature coloured stages, but no in the parents.

GC/MS-Olfactometry: 2-isobutyl-3-methoxypyrazine identification

Since pyrazines were not detected in the three lines by GC/MS, volatile samples at MG stage were analyzed by GC/MS-Olfactometry (GC-O). The authentic standard of 2-isobutyl-3-methoxypyrazine was injected and analyzed in SIM mode; it eluted at 15.30 minutes after injection. In the volatile samples of the parents and F1, the panel detected the bell pepper-like odour in the three samples at the same elution time of the authentic standard. Analysis of the MS spectrum of the three samples revealed the presence of 2-isobutyl-3-methoxypyrazine in the Jalapeño, Habanero and F1 profiles.

Analysis of polymorphisms

The hybrid nature of the F1 plant was confirmed using three primer sets of multi-locus DNA markers called SRAP. The primer combinations used have shown that the F1 genome combined DNA segments of both parents (Figure 3).

To identify linkage groups in Habanero, Jalapeño and F1 DNA, 50 SSR primer pairs were synthesized based on sequences reported in Nagy et al. (2007) and Wu et al. (2009), and were used in the screening of the three lines. Thirteen of them showed single polymorphisms and were selected for the screening of 240 plants of F2 population.

The results of the screening with SO13 marker on Habanero, Jalapeño, F1 and some of the F2 plants DNA, are shown in figure 4.

The position of the SSR marker on Pepper-FAO3v31 map, published on http://sgn.cornell.edu/, is 104.02 cM on the chromosome 3.

This marker showed a difference between the parents in the size of the segments amplified, whereas the hybrid combined bands of both parents. Further screening with SSR and other markers will be necessary to identify linkage groups.

DISCUSSION

Influence of ripening stage on volatile profile in chili peppers

Earlier studies have shown that during bell pepper ripening, a majority of volatiles decreased or even disappeared (Lunning et al., 1994). Until now, changes in volatile constituents in Habanero chilli pepper during maturation have been poorly investigated.

As expected, Habanero and Jalapeño peppers have qualitatively and quantitatively different volatile profiles. In Habanero, the amount of total esters, along with most other compound classes, increased at the coloured stage, while ester abundance decreased in Jalapeño at the coloured stage. Esters, with their fruity odour notes, contributed to 74% of the total volatiles profile in Habanero mature orange stage. Consistent with another study (Pino et al, 2006), Habanero at green stage had high concentration of butanoic acid, 3-methyl-, hexyl ester and cyclohexanol, 3,3-dimethyl. Pino and colleagues had also found that cyclohexanol, 3,3-dimethyl had a tendency to decrease as the colour changed from green to orange, while a opposite behaviour was found in C. annuum var. glabriusculum (Forero et al. 2009).

In contrast to previous study which showed predominance of aliphatic esters in the aroma profile and an increase in their content at orange stage (Pino et al, 2006), our results showed that ripening from green to orange had no effect on this class of volatiles which represented only 9% of the total esters.

The abundance of aliphatic esters has been not reported in other *Capsicum* species (Nijssen et al., 1996). Interestingly, the highest level of such esters was identified in Jalapeño in both stages and their amount increased as the fruits turned red, from 65 to 83% of the total esters.

Moreover, isoprenoids increased at maturity and formation of 8 new compounds was observed in Habanero. Among the compounds newly formed in Habanero at MR stage, the

norisoprenoids β-ionone and β-ciclocitral were identified. The norisoprenoids or apocarotenals are aroma compounds derived from breakdown of carotenoids (tetraterpenes). These two norisoprenoids are apparently oxidative breakdown products of β-carotene (Lewinsohn et al., 2005). The carotenoid degradation pathway is considered a key route for the formation of aroma compounds in many plant and plant products. In this pathway, carotenoids serve as substrates, but the nature of the biochemical mechanism (whether enzymatic or non-enzymatic) mediating this oxidative degradation is still to be elucidated in each particular case (Lewinsohn et al., 2005).

Moreover, allo-aromadendrene was the main sesquiterpene at MG stage (40%) in Habanero, but it was not detected when the fruits turned orange. This compound has not been identified in *Capsicum chinense* (Sousa et al., 2006; Pino et al., 2006), while it has already been detected in *Capsicum annuum* var. *glabriusculum* at green and red stages (Forero et al., 2009). Contrarily to this result, we did not observe it in our Jalapeño line in either stage.

At green stage, volatiles responsible of green notes, such as 3-hexen-1-ol and hexanal were not detected in Habanero, while they were identified in Jalapeño and F1 fruits.

In both parents and F1 fruits, (E)-2-hexenal was detected at the coloured stage of maturity, while 3-hexen-1-ol was found only at the mature green stage. These results are in agreement with the study of evolution of volatiles in varieties of *C. annum* (Lunning et al., 1994; Mazida et al., 2005). In fresh *Capsicum* fruits, these compounds, as well hexanal, are typically produced by enzymatic action upon tissue disruption (Wu et al., 1986). As assessed in tomatoes, the activity of several enzymes changed during ripening of chilli pepper, especially the ones involved in formation of these lipid-degraded products (Gaillard et al., 1977).

The amount of green descriptors such as hexanal and 2-isobutyl-3-methoxypyrazine has been shown to decrease upon maturation in other investigations (Chitwood et al., 1983; Luning

et al., 1994), and it was suggested that these compounds are responsible for green aroma in chilli pepper. These two compounds have extremely low aroma threshold of 0.0045 and 0.000002 ppm, respectively (Buttery et al., 1969), so they may be present in very low concentration and still contribute to the perceived aroma. Hexanal and (Z)-3-hexenal were detected only in F1 fruits at both stages, even though their content decreased at MR stage. 2-isobutyl-3-methoxypyrazine could not be detected in the samples by GC/MS. Further analysis using GC-O was conducted at MG stage to assess its presence in the samples. The result will be discussed in detail successively.

The total volatile content in F1 hybrid was five fold higher than in Habanero. The profile was characterized by high level of esters and, similar to the Habanero parent, aliphatic esters accounted for only 13% of the total esters content. As in the Jalapeño parent, isocaryophilene was the main sesquiterpene identified in the hybrid and it represented 98% of the total sesquiterpenes.

2-isobutyl-3-methoxypyrazine

In general, although pyrazines are present in minute quantities in natural samples, their contribution to flavour is considerable (Luning, 1994) due to their extremely low odor threshold. For example, the pyrazine compound 2-isobutyl-3-methoxypyrazine exhibits a characteristic bell-pepper-like odor, associated with an olfactory threshold of 2 ppt (Buttery et al., 1969).

2-isobutyl-3-methoxypyrazine was not detected in our samples by analysis on GC/MS. Samples of the three lines at MG stage were then analyzed using GC-O and presence of the pyrazine was detected in the Jalapeño, Habanero and F1 samples. This compound has previously been found in bell pepper (Buttery et al., 1969), Jalapeño (Kollmannsberger et al.,

2007) and Habanero (Pino et al., 2006) is also likely an important constituent of chili pepper aroma.

QTL mapping

The process of genetic mapping can be defined as the determination of the linear order of molecular markers or genes along a stretch of DNA. The result is a genetic map, which may be described as a graph depicting the relative positions of markers along so-called linkage groups, based on their frequency of crossover or recombination during meiosis (Weising et al, 2005). The steps involved in genetic map construction are: selection of parent plants and population size; selection of molecular markers; scoring process; linkage analysis.

In this study, the parents were selected on the basis of their divergences in fruit quality characteristics, such as flavor, shape, size and pungency. In absence of any polymorphism neither segregation analysis nor linkage mapping is possible (Kang et al, 2002). In order to exhibit a sufficient polymorphism, parents have to be divergent but not so distant as to cause sterility of the progeny. If knowledge about the map position of a certain trait is wanted (e.g. volatiles compounds), the parents should be polymorphic for that trait.

Although the taxonomics boundaries of *Capsicum* are still uncertain, based on crossing relationship and biochemical markers, two species lineages can be resolved. Most of the *Capsicum* species are self-compatible and facultative in-breeders. According to Mcleord et al. (1983), the species we selected, *C. annuum* and *C. chinense,* are in the same gene pool and can be crossed with each other producing fertile hybrids.

Moreover, the resolution of the map and the ability to determine marker order largely depend on this size of the mapping population. A lower threshold that can localize QTL is a

size of 100 individuals (Weising et al, 2005), but generally, the larger the mapping population is, the better. 240 plants composed our mapping population, generated from F1 hybrid.

Then, SSR markers that had shown polymorphism in parents and F1 were selected and we started the scoring process: DNA from each progeny was isolated and tested for the state of those DNA sequence polymorphism that distinguished the parents.

To date, we are carrying out the scoring process on the mapping population; more detailed results will be available in the future. Additionally, we will add enough SRAP markers to construct a map of approximately 500 markers using the procedure of Li and Quiros (2001).

All the data accumulated from scoring the mapping population sequentially with a series of markers will be used to construct the linkage map. The linear arrangement of linked loci will represent the so-called linkage group; all of the linkage groups will represent the genetic map.

CONCLUSIONS

The fruit aroma is important quality and marketability attribute, selected for during domestication and crop improvement. Fruit aroma is composed of a complex mixture of tens, sometimes hundreds of volatile compounds arising from diverse biochemical pathways.

The understanding of the key chemical, enzymatic and molecular mechanisms that control the formation of the aroma volatiles in crop plants has still been little investigated. To date more than 125 volatile compounds have been identified in fresh and processed chili pepper, but their flavor significance is still unknown. The synthesis and emission of plant volatiles is under the control of a variety of factors, including developmental stage of the plant organ. Compositional changes of volatiles in fruit blend during ripening have a primordial role as ecological cues for attracting organisms engaged in seed dispersal in the crops' wild ancestors.

The individuation of those compounds involved in formation of fruit aroma profile at maturity may help, with a support a new technologies of quality assessing, to harvest the fruit at their peak maturity or ripeness to provide the best possible flavor to consumers.

In our study, we characterized the major volatiles determining Habanero and Jalapeño chili pepper aroma at two different stage of ripeness. In Habanero the total volatiles content enhanced during maturation, and esters, with their fruity aroma, were the compounds that more characterized the fruit profile at maturity. Fruit maturation in Jalapeño negatively affected the aroma profile; the total volatile content decreased, many compounds disappeared and weren't replaced by other detectable compounds. The identification of volatile composition at different stages of maturity may facilitate producers and industry in selection of fruits and vegetables for the market, and enhance agriculture sustainability by reduction of waste.

The identification of the major volatiles compounds in fruit and vegetable and the genetic basis of fruit quality trait, as aroma, will provide a useful support to development of flavor-targeted breeding programs. Molecular markers for pepper fruit quality may be useful tool for breeders to efficiently create improved cultivars with novel combination of volatile compounds that could extend the market segment of the pepper industry.

Additionally, knowing the genetic control of the major fruit quality traits (color, hot, flavor) will provide breeders with a handle to optimize content of these compounds to encounter the consumer preference and acceptance.

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Table 1. Volatile compounds relative abundances of Habanero, Jalepeño and F1 hybrid at two different stage of maturity, mature green (MG) and mature colored (MR) in order of their retentian time (RT).

RT	Compound	Ref.	MG Hab	MG Jal	MG F1	MR Hab	MR Jal	MR F1
4,92	3-Hexenal, (Z)-		-	-	2,67	-	-	0,72
4,95	Hexanal	a,c,d	-	-	1,24	-	-	0,22
5,91	4-methyl-1-Pentanol		-	-	1,90	0,27	-	2,07
6,33	2-Hexenal, (E)-	a,c,d	-	-	-	0,59	0,03	2,15
6,40	3-Hexen-1-ol, (Z)-	c,d	-	0,08	1,22	-	-	-
10,50	Butanoic acid, 3-methyl-, 2-methylpropyl ester	a,b,c	0,61	-	-	0,72	-	0,11
10,68	Propanoic acid, 2-methyl-, 3-methylbutyl ester		-	-	2,02	-	-	2,19
11,10	2-ethyl-1-Hexanol		-	-	2,66	-	0,02	-
11,61	Ocimene	a,d	-	0,37	30,74	-	0,01	25,11
11,72	Butanoic acid, pentyl ester	a	-	-	10,23	-	-	5,65
11,92	Butanoic acid, 4-pentenyl ester		-	-	-	-	-	0,67
12,22	Propanoic acid, hexyl ester		-	-	-	-	-	0,23
13,02	Butanoic acid, 2-methyl-, 3-methylbutyl ester	b,c	-	-	-	0,44	-	-
13,10	Butanoic acid, 3-methyl-, 2-methylbutyl ester		5,78	0,09	15,21	7,12	0,02	8,40
13,31	Propanoic acid, 2-methyl-, hexyl ester	a,b	3,04	0,63	237,88	6,83	0,12	188,17
14,09	Butanoic acid, 2-methyl-, pentyl ester	a,d	-	-	84,18	5,96	-	52,96
14,88	1,3,5,8-Undecatetraene		-	-	1,70	-	-	-
15,05	Butanoic acid, 3-hexenyl ester, (Z)-	d	-	-	-	-	-	0,85
15,51	Butanoic acid, 2-methyl-, hexyl ester	a,b,c	8,17	0,82	321,91	13,70	0,23	216,04
15,63	Butanoic acid, 3-methyl-, hexyl ester	a,b,c	120,71	0,69	467,18	231,83	0,24	501,45
15,82	Isopentyl hexanoate	a	-	-	0,75	0,14	-	1,64

Table 1. (Continued)

RT	Compound	Ref.	MG Hab	MG Jal	MG F1	MR Hab	MR Jal	MR F1
15,94	α-Citronellol		-	-	-	3,76	-	1,60
16,00	Propanoic acid, 2-methyl-, heptyl ester	b,c	-	-	0,50	-	-	1,73
16,08	β-Cyclocitral	d	-	-	3,02	0,23	-	1,04
16,30	n-Valeric acid cis-3-hexenyl ester		1,00	-	63,97	39,58	-	32,71
16,34	Butanoic acid, 3methyl 3 hexen-1-yl ester		33,01	-	81,41	135,75	-	6,61
16,46	Pentanoic acid, (2E)-2-hexen-1-yl ester		53,85	0,10	54,23	-	-	2,29
16,71	Pentanoic acid, hexyl ester		-	-	49,11	0,67	-	51,79
17,66	Cyclohexanol, 3,3-dimethyl-	a,b,c,d	72,86	-	-	42,37	-	0,30
18,08	Hexanoic acid, hexyl ester	a,b,c,d	1,65	0,41	46,76	10,41	0,17	69,40
18,50	Butanoic acid, 2-methyl-, heptyl ester	a,c	3,89	-	0,54	6,44	-	0,27
18,65	Hexanoic acid, 3-hexenyl ester, (Z)-	c,d	0,57	-	4,14	-	-	-
19,22	2-methyl-Tridecane		6,74	1,29	71,89	13,18	0,85	36,25
19,51	Ylangene		2,62	0,08	-	0,33	0,08	-
19,59	Copaene		0,30	0,04	2,34	0,29	-	1,35
19,82	Butanoic acid, 3-methyl-, phenylmethyl ester	a	1,79	-	13,34	0,74	-	3,06
19,82	β-Cubebene	c,d	1,28	-	-	1,85	-	-
19,99	Tetradecane	a,b	0,74	-	8,24	0,03	0,02	7,21
20,01	α-Cubebene		-	-	-	4,73	-	-
20,04	Butanoic acid, 3-methyl-, octyl ester	b	5,66	-	-	0,77	-	-
20,69	Thujopsene		0,34	-	-	-	-	-
20,76	Butanoic acid, 2-methyl-, octyl ester	c,d	0,63	-	-	0,31	-	-
20,99	E-2-Hexadecacen-1-ol		9,44	1,07	58,07	18,43	0,73	26,36
21,22	α-Himacalene	a,c,d	0,26	0,02	-	0,83	0,03	0,35

Table 1. (Continued)

RT	Compound	Ref.	MG Hab	MG Jal	MG F1	MR Hab	MR Jal	MR F1
21,22	β-Farnesene	a,c,d	0,55	0,02	-	1,69	0,01	0,16
21,34	Tetradecane, 2-methyl-	b,c,d	5,02	1,07	50,29	12,61	0,78	30,73
21,67	Longifoliene		4,76	0,02	5,60	1,18	0,01	2,58
21,83	Allo-Aromadendrene	a	7,82	-	-	0,55	-	-
21,88	β-Ionone	a,c,d	-	-	-	0,17	-	-
21,97	(Z)-β- caryophyllene	c,d	0,77	12,88	276,88	-	6,24	102,67
22,07	Pentadecane	a,b,d	1,21	0,11	15,69	2,65	0,07	8,87
22,21	α-Longipinene		-	0,04	-	19,51	0,03	0,20
22,44	γ-Cadinene	c,d	-	-	-	0,22	-	-
22,91	Benzoic acid, hexyl ester	a,c,d	-	-	-	-	-	0,31
22,97	Octanoic acid, hexyl ester		0,63	-	-	-	-	-
23,10	3-Hexadecene, (Z)-		-	0,02	-	-	0,01	-
23,37	2-methyl-Pentadecane		0,34	0,54	9,36	0,80	0,49	3,60
23,68	Squalene		-	-	-	0,09	-	-
23,91	2-(2-Methyl-propenyl)-cyclohexanone		0,94	-	6,43	3,24	0,01	4,78
23,99	Hexadecane	a,d	-	0,19	11,58	0,38	0,05	4,97
24,46	Longifolenaldehyde		-	-	-	-	0,01	-
24,96	2-Methyl-Z-7-hexadecene		-	0,16	-	-	0,17	-
25,24	2-Methyl-Hexadecane		-	0,44	2,84	0,05	0,44	1,25
25,45	Pentadecanal	a,c,d	-	0,04	-	0,11	0,11	0,19
25,89	Heptadecane	a	-	0,20	1,95	-	0,17	1,12
26,04	Decanoic acid, hexyl ester	a,d	-	-	-	-	0,01	-
	Total		**357,0**	**21,4**	**2019,7**	**591,5**	**11,2**	**1412,3**

The ralative abundance is expressed as the ratio of each compound peak area to that of the internal standard's. Identification confirmed by literature: a, Forero et al., 2009; b, Sousa et al., 2006; c, Pino et al., 2007; Pino et al., 2006.

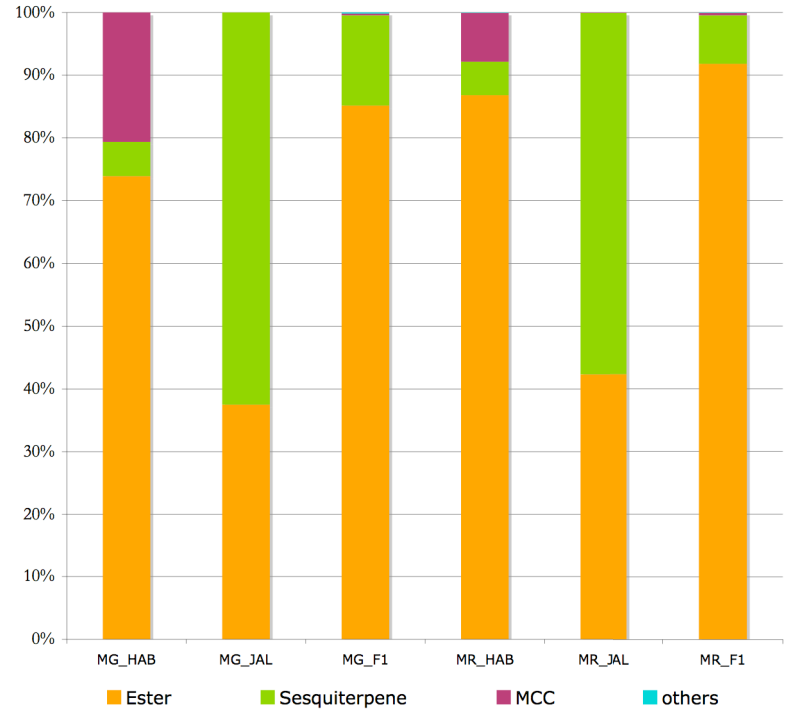

Figure 1. Changes of the major chemical classes in two ripening stages, mature green (MG) and mature colored (MR), of Habanero, Jalapeno and F1 hybrid chili peppers. Percentage on total volatile content. (MCC= miscellaneous of cyclic compounds; others= monoterpenes, norisoprenoids, aldeydes, alcohols).

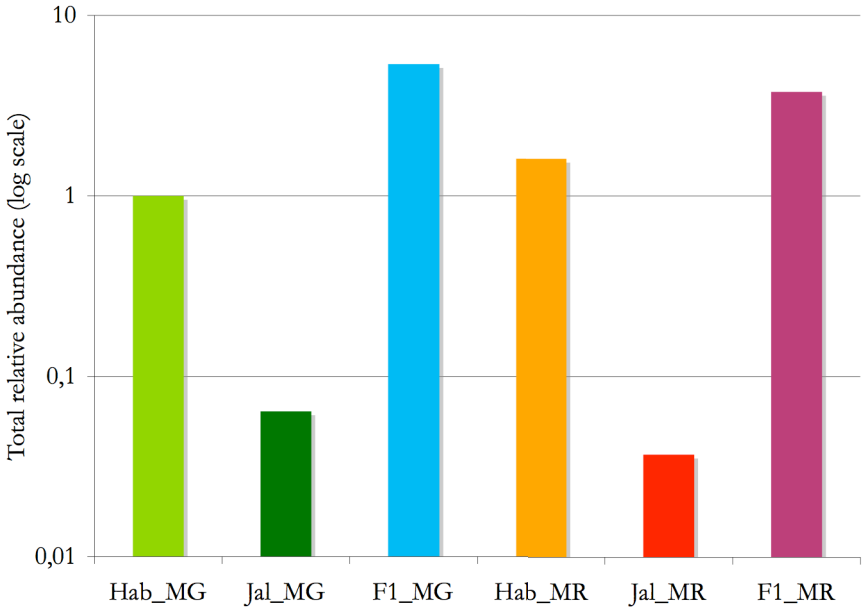

Figure 2. Volatile compounds relative abundances of Habanero, Jalepeno and F1 hybrid at two different stage of maturity, mature green (MG) and mature colored (MR). The ralative abundance is expressed as the ratio of each compound peak area to that of the internal standard's. Values normalized are expressed in log scale.

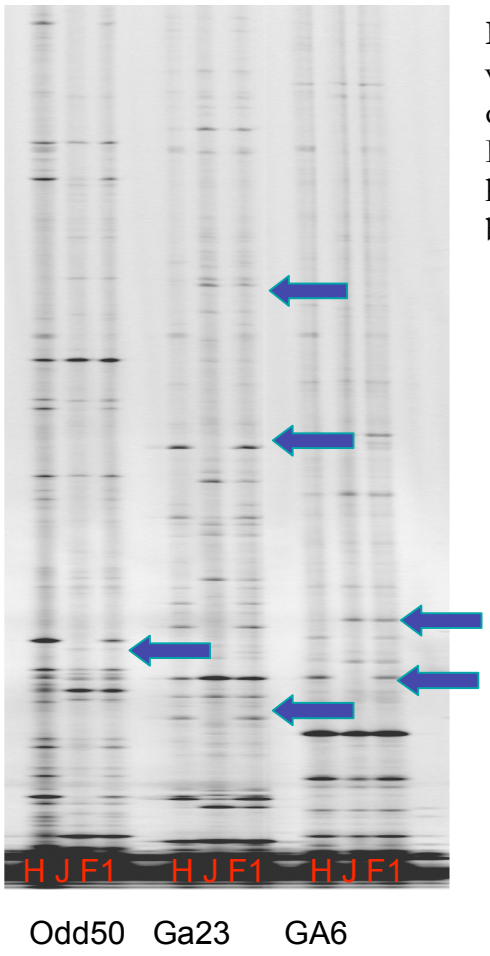

Figure 3. Confirmation of hybrid nature of F1 with multi-locus SRAP markers. Primer combination with Em2-ODD50, Em2-Ga23and Em2GA6. H= Habanero; J= Jalapeno; F1= hybrid. The arrows indicated bands in F1 from both parent.

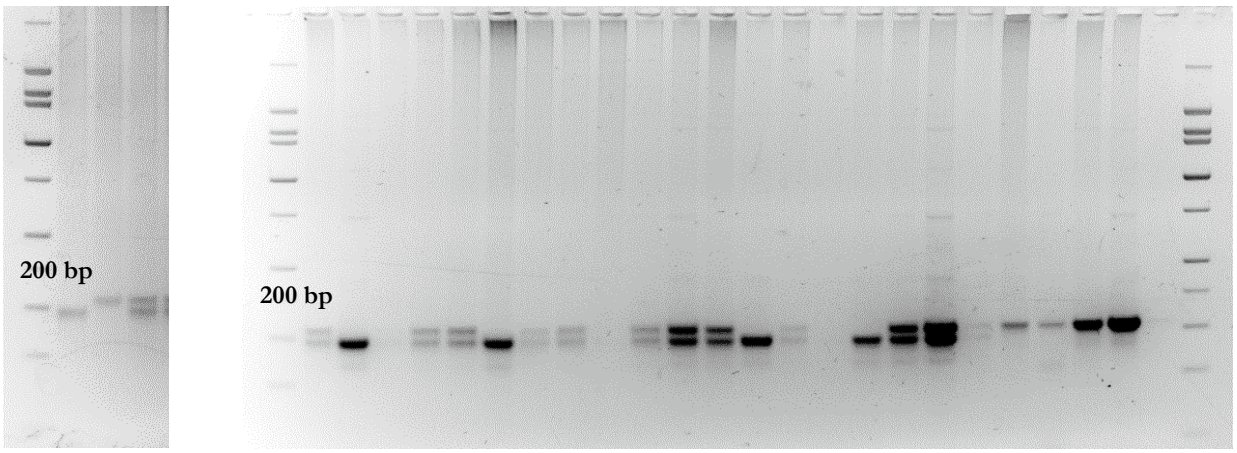

Figure 4. Results of the scoring process with SO13 SSR marker. On right: DNA marker (200 base pairs), Habanero, Jalapeno, F1 hybrid bands. On left: some of the results of F2 progeny.
Forward primer: 5' ATTGTGATAGCAACCCCTGG 3';
Reverse primer 5' CACAGATGAGGGCACAAATG 3'.

CHAPTER 3

CONCLUSIONS

Compositional changes in volatiles profile in fruit and vegetables play a fundamental role in sedentary life of plants. Volatiles involvement in plant reproduction and defense in biotic stress has been widely assessed, while the understanding of their protective effect against abiotic stress is still at its infancy. In first chapter of this thesis we focused on volatile involvement in abiotic stress and their role as antioxidant and/or stress signaling in salt stress adaptation mechanism. Isoprenoids can have direct and indirect modality of action: protect the cell from oxidative damage scavenging reactive oxygen species, and indirectly inducing transcription of stress-related genes. Volatile isoprenoids biosynthesis under stress conditions may follow a feedback-feed forward mechanism, where the stress response is translated into an increase in VIPs content and, in turn, the increased VIPs content may trigger the stress response.

However, it is still unclear how their stress-induced accumulation in the plant may concomitantly act in stress response signaling. Furthermore, we discussed the role of eugenol as prooxidant and its hypothetical involvement in ABA-independent mechanism of stomatal closure by generating severe imbalance in cellular redox status and consequently enhancing the H_2O_2 levels in the cells. The two cultivars of basil have shown differences in salt stress tolerance in terms of response threshold. We can assume that constitutive morphological characteristics (stomatal index and growth adaptation) and metabolic profile (composition of the volatiles pool) may have been critical component for improving stress adaptation in Genovese plants.

In the second chapter we characterized the aroma profile of two of the most common chili peppers and the influence of the ripening stage in determination of the total profile. Fruit maturation and turning in color have different effects on volatile flavor of the two species. In Habanero the total volatiles content enhanced during maturation, and the profile at orange stage is richer in esters with their fruity notes. On contrary, fruit maturation in Jalapeño negatively affected the aroma profile; the total volatile content decreased, many compounds disappeared and weren't replaced by other detectable components.

The identification of volatile composition at different stages of maturity may facilitate producers and industry in selection of fruits and vegetables for the market, and enhance agriculture sustainability by reduction of waste. Moreover, based on polymorphism in aroma profile we have chosen these two species to identify loci affecting flavor volatiles in chili pepper. Molecular markers for pepper fruit quality may be useful tool for breeders to efficiently create improved cultivars with novel combination of volatile compounds that could extend the market segment of the pepper industry.

Additionally, knowing the genetic control of the major fruit quality traits will provide breeders with a handle to optimize content of these compounds to encounter the consumer preference and acceptance.

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