UNIVERSITE DE CORSE – PASCAL PAOLI
UFR DES SCIENCES ET TECHNIQUES

THESE

Pour obtenir le grade de
DOCTEUR DE L'UNIVERSITE DE CORSE
Discipline : Biochimie et Biologie Moléculaire

Présentée par
Sajjad HUSSAIN

CARACTERISATION PHYSIOLOGIQUE DE GENOTYPES D'AGRUME : ETUDES DE TOLERANCE AU STRESS SALIN ET IMPACTS DE LA PRESENCE DE PORTE-GREFFES ZYGOTIQUES ET AUTOTETRAPLOÏDES

Soutenue le 16 Février 2011

Dr. Raphaël MORILLON
(directeur de thèse)
Dr. Francois LURO
(co-directeur de thèse)

JURY :

M	**Manuel TALON**	**IVIA, Valencia, Rapporteur**
M	**Jean-Luc REGNARD**	**SupAgro, Montpellier, Rapporteur**
Mme	**Liliane BERTI**	**Université de Corse, Corte, Examinateur**
M	**Felix TOMI**	**Université de Corse, Corte, Examinateur**
M	**Muhammad Akbar Anjum**	**BZ University, Multan, Examinateur**
M	**Francois LURO**	**INRA, San giuliano, Examinateur**
M	**Raphaël MORILLON**	**CIRAD, Valencia, Examinateur**

ACKNOWLEDGEMENT

This dissertation would not have been possible without the assistance and the advice of several individuals who in one way or another contributed and extended their valuable guidance in the preparation and completion of this study.

First I thank to jury, **Mr. Manuel TALON**, IVIA, Valencia, Spain, **Mr. Jean-Luc REGNARD**, SupAgro, Montpellier, France, **Ms. Liliane BERTI,** Université de Corse, Corte, France, **Mr. Felix TOMI** Université de Corse, Corte, France, **Mr. Muhammad Akbar Anjum** BZ University, Multan, Pakistan **Mr. Francois LURO,** INRA, San giuliano**,** France **and Mr. Raphaël MORILLON,** CIRAD, Valencia, Spain for accepting to devote their time in evaluating this work.

I would like to thank **Mr. Robert Domaingue**, Director of Research unit Genetic Improvement of Vegetatively Propagated Crops CIRAD, **Mr. Patrick Ollitrault** Group leader of genetic and study of genome CIRAD / IVIA, **Ms. Dominique Agostini** President of INRA in Corse and **Mr. Olivier Pailly** Director of Research Unit Genetics, Ecophysiology and quality of citrus, INRA San Giuliano for their warm welcome in their research units. I would like to thanks to **Higher education commission (HEC)** of Pakistan for agreeing to support my thesis and "Société Française d'Exportation des Resources éducatives" **SFERE**, for its help along my staying in France.

I would like to express my deep and sincere gratitude to my supervisor, **Dr. Raphael Morillon**, researcher HDR, CIRAD / IVIA, His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a good basis for my research activity.

I wish to thank my co-supervisor **Dr. Francois Luro**, researcher INRA de San Giuliano, for his important support throughout this work. I express my appreciation and gratitude for his scientific advice. I will not forget his kind guidance and moral support through all my ups and down in research activities.

I thank to **Dr, Laurent Urban** for his guidance in my research work. I would like to appreciate the effort of CIRAD team (**Dr. Yann Froelicher**, researcher and **Mr. Jean Bouffin)** at San Giuliano for their helping hand and encouragement.

I thank to **Mr.Camille Jacquemond** (INRA) for his availability and useful help for clementine trail. Now, At least I have my clementine. I am very grateful to **Mr.Frank Curk** (INRA), for attracting me to Corse and for interesting discussions on several topics and for his helping hand in my research work. You make my five year stay in Corse. You were very very formidable for me.

Sincere thank to **Ms Claudie Dhuique-Mayer**, researcher CIRAD for her assistance and guidance in Carotenoid quantification. My sincere thanks also go to **Mr.Gilles Constantino** (INRA) for his availability in molecular biology laboratory and help for DNA sequencer analysis and Join map. It was really king of him.

My special thanks to **Mr.Jean-Baptiste Baïly Bassene** for his helping hand when i newly came at INRA. You were the true gentleman for me and for others too. It was a really nice to meat you. I will not forget all the pleasant moment (Ha la la) which we spent at Batiment Social International

I thank the whole team at INRA in San Giuliano (Researcher, Technicians, SDAR, PhD students) for their good mood added to the weather in Corsica reminds me of my little home.

During this work I have collaborated with many colleagues for whom I have great regard, and I wish to extend my warmest thanks to all those who have helped me with my work in the IVIA, Spain, INRA, Corse, and CIRAD at Montpellier.

I would like to thank Elodie Carcouët for her generous help, Mourad Kamiri for his gentleness and availability whenever i needed, Wafa Mouhaya and Thierry Allario for help in Tetraploid and salinity. Sandrine Antoine and Florine Gonord for their help in sugar and acid quantification. Salutu!! to all those who came to the Batiment social internationale, for the good and worst moments.

My sincere gratitude to my friends Sardar Faisal Mahmood, Malik Muhammad Imran, Chaman Subhani, Shaghef Ijaz and Benoit Digard, for their encouragement and support. It was really kind of them.

Last but not the least, i would like to thank: my parents my brothers and sisters and all my family and the one above all of us, the omnipresent Allah, for answering my prayers for

giving me the strength to plod on despite my constitution wanting to give up and throw in the towel, thank you so much Dear Allah.

Sajjad HUSSAIN

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABA:	Abscisic acid
ACO_2:	CO_2 assimilation
AFLP:	Apmlified fragment length polymorphism
CAPS:	Cleaved Amplified Polymorphic Sequence
CDPKs:	Calcium-dependent protein kinases
CLC:	Cl^- channels
COD	Choline Oxidase
CTV:	Citrus Tristeza Virus
cpSSR	chloroplast SSR markers
DNA:	Deoxyribonucleic acid
EDTA:	Ethylene Dinitro Tetraacetic Acid
EMS:	Ethyl Methane Sulphonate
FAO:	Food and Agriculture Organization
Gs:	Stomatal conductance
GSA:	Glutamate-5-SemiAldehyde
HKT:	High-affinity K^+ Transporter
HSP	Heat Shock Proteins
ISSR:	Inter Simple Sequence Repeat
KORCs:	Outward Rectifying K^+ Channels
LCT1:	Low-affinity Cation Transporter
LEA:	Late Embryogenesis Abundant
MAPK:	Mitogen-Activated Protein Kinase
MAS:	Marker-Assisted Selection
MIP:	Membrane Intrinsic Proteins
NCED	9-Cis-Epoxycarotenoid Dioxygenase
NHX	Na+/H+ Antiporter
NIP:	Nodulin 26 Intrinsic Proteins
NSCC:	Non-Selective Cation Channels
P5CS:	delta1-Pyroline-5-Carboxylate Synthase
P5CR	delta1-Pyroline-5-Carboxylate Reductase
PCA:	Principle Component Analysis
PCR:	Polymerase Chain Reaction

PIP:	Plasma membrane Intrinsic Proteins
PSI:	Photosynthesis System I
PSII:	Photosynthesis System II
QTL:	Quantitative Trait Loci
RAPD:	Random Amplified Polymorphic DNA
RFLP:	Restriction Fragment Length Polymorphism
ROS:	Reactive Oxygen Species
SCAR:	Sequence Characterized Amplified Region
SIP:	Small Intrinsic Proteins
SIPK	Salicylic acid Induced Protein Kinase
SNP:	Simple Nucleotide Polymorphism
SOD:	SuperOxide Dismutase
SOS:	Salt Overly Sensitive
SSR:	Simple Sequence Repeat
STMS	Sequence Tagged Microsatellite sites
STS:	Sequence Tagged Site
TA:	Titratable Acidity
TBE	Tris/Borate/EDTA
THF	TetraHydroFuran
TIP:	Tonoplast Intrinsic Protein
TSS:	Total Soluble Solids
USDA:	United States Department of Agriculture

1 Introduction

Citrus fruits have been cultivated for over 4000 years. Originally from south-east Asia, citrus were scattered all around the world to their limit of expansion, i.e. between 40 degrees latitude north and south. The area of Citrus origin is located in tropical and subtropical regions of Southeast Asia, Northeast India, Southern China, the Indochinese peninsula and the Malay Archipelago (Nicolosi 2007). The production and global consumption of citrus steadily increased over recent decades with a world production of about 124 million tons in 2008-2009 (FAO): Oranges represent 55.2 % of total production, followed by group of small citrus fruits (mandarins, clementines: 23%), lemons and limes (11.2%), pummelos and grapefruits (4.4%) and other Citrus (6.2%). The main citrus producers are Brazil (20.6 MT) and China (19.6 MT), USA (10 MT), Mexico, (6.8 MT), India (6.2 MT) and Spain (5.7 MT).

1.1 Taxonomy

Citrus species and related genera are primarily evergreen species of subtropical and tropical origins belonging to the order *Geraniales* and family *Rutaceae*. Species of the *Rutaceae* family generally have four important characteristics: (1) the presence of oil glands, (2) the ovary raised to floral discs, (3) pellucid dots are presents in the leaves, and (4) fruits have axil placentation (Swingle and Reece 1967). The family *Rutaceae* is subdivided into six subfamilies including the *Aurantioideae*. The true citrus and related genera belong to *Aurantioideae* subfamily. The term "true citrus fruit" includes species of the genus *Citrus* and two related genera, *Fortunella* and *Poncirus* which are sexually compatible (Swingle and Reece 1967). The term "citrus" is used to designate both fruit and tree. The most consumed species belong to the genus *Citrus*. Many different classifications have been proposed, but those proposed by Swingle and Reece (Swingle and Reece 1967) and Tanaka (Tanaka 1961) are the most widely used. These two authors have different views regarding the classification of species. The classification of Swingle identifies 16 species, whereas the Tanaka identifies 156 species. According to the Tanaka classification, there are eight cultivated species: limes, *C. aurantifolia;* Sour orange, *C. aurantium* (L.); lemon, *C. lemon* (L.) Burm.; Pummelo, *C. maxima* (Burm.) Merr.; Citron, *C. medica* (L).; Grapefruit, *C. paradisi* Macf.; Mandarin, *C. reticulata* Blanco and orange, *C. sinensis* (L.) Osb. The genus *Fortunella* comprises of 2-4 species and the genus *Poncirus* include *Poncirus trifoliata* (L.) Raf. characterized by deciduous and trifoliate leaves.

Citrus taxonomy and phylogeny is often a subject of controversy because of high diversity of phenotypic characters, a long history of cultivation and a complex reproduction system. Indeed, the presence of self-incompatibility genes, facultative apomixis, gene of sterility and a large sexual compatibility between *Citrus* spp. and related genera can be observed (Froelicher *et al.* 2010; Nicolosi *et al.* 2000). Understanding taxonomy, phylogenetic relationships, and genetic variability in citrus is critical for determining genetic relationships, characterizing germplasm, controlling genetic erosion, designing sampling strategies or core collections, establishing breeding programs, and registration of new cultivars (Herrero *et al*. 1996). In recent years all the citrus scientific community has adopted hypothesis about citrus history and phylogeny proposed by Scora (1973) and Barrett and Rhodes (1976) where a large diversity of *Citrus* is represented by three basic species such as mandarin (*C. reticulata*), citron (*C. medica*) and pummelo (*C. maxima* (Burm.) Merr.). They are at the origin of many secondary cultivated species such as orange, lemon, sour orange, grapefruit, clementine by several sexual crosses which occourred spontaneously more or less recently during the citrus history. The current assumption now considers four main species citron (*C medica*), mandarin (*C. reticulata*), pummelo (*C. maxima* (Burm.) Merr.) and micrantha (*C. micrantha* Wester). From these four main species all other species were derived by hybridization. This hypothesis is supported by the results of studies using biochemical and molecular markers such as isozymes (Herrero *et al*. 1996), Restriction Fragment Length Polymorphism, (RFLP) (Federici *et al.* 1998), Random Amplified Polymorphic DNA (RAPD) (Corazza-Nunes *et al*. 2002; Nicolosi *et al*. 2000), Inter Simple Sequence Repeat ISSR (Fang *et al*. 1998), Simple Sequence Repeat (SSR) (Corazza-Nunes *et al*. 2002; Luro *et al*. 2001). This concept gained further support from various studies using cytoplasmic DNA markers (Froelicher *et al*. 2010; Nicolosi *et al*. 2000). Cytoplasm in *Citrus* is maternally inherited (Green *et al*. 1986; Masashi Yamamoto 1993). Most of the studies were realized with chloroplastic molecular marker. Using the chloroplast SSR markers (cpSSR) analysis, (Cheng *et al.* 2005) constructed molecular phylogeny of citrus, but relationships of many species were not resolved. Thus, orange and sour orange probably result from a backcross between the pool of citron and mandarin while that of the lemon is a hybrid between sour orange and citron, and grapefruit a hybrid between orange and citron (Nicolosi *et al*. 2000). The lime is the only specie for which a taxon grown outside of citrus was involved, it has indeed a chloroplast genome different from that of other cultivated species of *Citrus* (Nicolosi *et al*. 2000) and it suggests that the mexican lime (*C. aurantifolia*) probably comes from a hybridization between citron and *C. micrantha*. Mexican lime contains the cytoplasmic

organelles from *C. micrantha.* Also it should be noted that orange, sour orange, lemon and grapefruit have cytoplasmic organelles from the pummelo (Froelicher *et al*. 2010).

1.1.1 Inter-specific and intra-specific genetic diversity

Organization of genetic diversity and inter-specific relationships has provided vital information for understanding relationships between different crop species of *Citrus*. Phenotypic diversity of citrus is highly structured around three main taxa. The strong structuring still observed today, both at molecular and morphological level indicates that genetic exchange between the original three taxa remained limited. Polyembryony due to apomixis was certainly a limiting factor in gene flow. Other factors, such as structural differentiation of genomes have also facilitated the maintenance of gametic disequilibrium which limits the recombination over large portions of genome. On the other hand, intraspecific molecular diversity studies within citrus showed very contrasting structures (Federici *et al.* 1998). Citrons present very low allelic diversity due to high homozygosity and low polymorphism between cultivars. Grapefruit, orange and sour orange have similar intraspecific structures. They showed low allelic diversity and heterozygosity. Lemon trees showed high heterozygosity and low inter-varietal polymorphism. Limes are also heterozygous but show a higher inter-varietal polymorphism than lemon. Pummelo and mandarin offer high allelic richness mainly due to strong inter-varietal polymorphism. These two species also show no significant differences in panmixia, which likely reflects significant genetic exchange within each these two taxa. All other species, except citron presented high hetrozygosity.

1.2 Citrus genetic resources

There are several collections of citrus throughout the world. These collections are preserved and maintained with two main objectives: first, to preserve the diversity of citrus and their related genera over the long period of time, and second, to create orchards to provide grafts of valuable varieties. The collection of the Okitsu Branch (Fruit trees research station) in Japan is the most important for cultivated material from the zone of origin, while the conservatory of the University of Malaysia is remarkable for its collection of *Aurantioideae* of Southeast Asia. The collection of USDA (United States Department of Agriculture), IVIA (Instituto Valenciano de Investigaciones Agrarias) Spain, and the University of Adana in Turkey contain certain Rutaceae related to citrus but are regularly supplied for the most part

by new varieties created throughout the world. The INRA and CIRAD station of San Giuliano, in France, has a unique status because of the favorable phytosanitary conditions of Corsica (Ollitrault *et al.* 2003).

1.3 Biotic and abiotic constraint for citrus expansion

In recent years, citrus production has decreased in some major citrus growing countries such as Brazil and USA. In the Mediterranean area, the climate and geography are suitable for citrus growing. However, citrus is under intense biotic (Citrus tristeza virus (CTV), Phytophthora sp…etc) and abiotic (salinity, drought, clay soils ...etc) stress in this area. In the nineteenth and early twentieth century, citrus orchards were initially decimated by the emergence of new diseases (Gummosis (a fungus), due to Phytophthora sp (a fungus). and CTV), whose growth was fostered by increased trade between Europe and the Americas. As a consequence, since 1840, citrus rootstocks resistant to Phytophthora sp. were used. Nowadays, the decrease in water resources and salinity stress are major abiotic constraints in many countries and also major constraint for citrus expansion. Soil salinity affects large terrestrial areas of the world, nearly half of irrigated land and 20% of all cultivated land is salt-affected and reduce the different crops yield well below to their genetic potential (Flowers 2004; Munns 2002).

1.3.1 Citrus variability in resistance to biotic and abiotic stresses

The variability of resistance to biotic and abiotic stress is considerable in citrus and opens up broad prospects for breeding. Among responses to the abiotic factors we can cite: cold tolerance in Satsuma mandarin trees; salinity tolerance in *C. limonia* (Rangpur lime) trees and *C. reshni* (Cleopatra mandarin); calcareous soil tolerance in *C. jambhiri*, *C. macrophylla*, *C. volkameriana*, and sour orange; drought tolerance in *C. limonia*. Considerable variability for resistance to major pests and diseases is also present: tolerance to *Phytophthora* sp. in some *C. maxima*, *C. aurantium*, *C. volkameriana*, and *C. amblycarpa*; African cercosporioses tolerance in grapefruit, lemon, and Satsuma and beauty mandarin; tristeza tolerance in Cleopatra mandarin, *C. amblycarpa,* Rangpur lime, *C. jambhiri* and *C. volkameriana*; Blight tolerance in orange; tolerance to citric canker due to *Xanthomonas campestris* in *C. junos* and some mandarins (Dancy and Satsuma for example); resistance to phytophagous acarids in March pomelo and mandarins (Satsuma and Dancy) and resistance to nematodes in *Poncirus trifoliata* (Ollitrault *et al.* 2003).

1.4 Rootstock improvement: a response to salt stress

For rootstock improvement the main focus is to obtain genotypes resistant to biotic and abiotic stresses. In addition, the rootstock should be compatible with the different varieties and capable of producing large quantities of good quality fruit (Khan and Kender 2007). The main objectives of rootstocks selection vary from one region to another and the constraints encountered. In general, adaptation to different soil types and pathogens resistance is the first objective for citrus breeders. The sour orange (*Citrus aurantium* L.) rootstock was conventionally used worldwide until 50s because of its tolerance to some extent, salinity, high pH, and calcareous soils (Grosser *et al*. 2004). However, trees grafted on sour orange are more vulnerable to diseases caused by certain strains of CTV (Rao *et al*. 2008). Its replacement by another rootstock becomes necessary to integrate, more tolerance against CTV and salinity frequently present in most of the citrus growing areas. Unfortunately the existing rootstock such as trifoliate orange and its hybrids Troyer and Carrizo citrange, which are resistant to many biotic stress (CTV, nematodes, *Phytophthora* spp.), are sensitive to high calcium and also to salt stress. Rootstocks belonging to lemon group are tolerant to salinity but susceptible to CTV, *Phytophthora* spp and nematodes. Moreover, Cleopatra mandarin, which is very tolerant to salt and tristeza is susceptible to *Phytophthora* spp. The creation of new rootstocks cumulating tolerances to biotic (tristeza, *Phytophthora* sp, nematodes) and abiotic (limestone, salinity) stress, while conferring a high quality fruit, is therefore an important goal for citrus breeders.

1.5 Impact of salt stress in citrus

1.5.1 Effect on vegetative growth and yield

Like many other crops citrus fruits are classified as salt sensitive (Maas 1993; Storey and Walker 1998) and more particularly to chloride (Cl^-) (Brumos *et al*. 2009). Intensity of leaf symptoms (chlorosis) increases with time of salt stress and Cl^- foliar and sodium (Na^+) contents are correlated with toxicity symptoms (Atmane *et al*. 2003). Salinity adversely influences several aspects of plant vegetative and reproductive growth. Salinity induces reduction in plant dry weight, leaf area, leaf water potential, root hydraulic variables, and these effects vary with the salt type and rootstock. High salinity level reduces plant height, stem diameter, number of leaves per seedling, fresh top and root biomass, and dry top and root biomass (Melgar *et al*. 2008; Singh *et al*. 2004). The growth rate of two citrus rootstocks

Cleopatra mandarin and Carrizo citrage is decreased when grown in solution containing 50mM NaCl (Gimeno *et al*. 2010). The effect of salt stress is considered to be proportional to NaCl concentration and duration of salt treatment (Zekri 1991). It has been reported that above a threshold conductivity value of 1.4 dS m^{-1}, every 1 dS m^{-1} increase results in an average of 13% decrease in yield and 50% reduction in yield was observed at electric conductivity of 5 dS m^{-1} (Iglesias *et al*. 2007; Maas 1993). In citrus, salt sensitivity depends on various parameters such as age of plants (Sykes 1985), rootstock/scion combination (Banuls *et al*. 1990; Cerdá *et al*. 1990; Garcia-Legaz *et al*. 1993; Maas 1993; Zekri and Parsons 1992), irrigation water quality (Anjum 2008), soil type and climate (Cerdá *et al*. 1990; Gimeno *et al*. 2010). The deleterious effects of salt stress lead to reduction in fruit yield and quality (Jenks *et al*. 2007; Storey and Walker 1998). Reductions in fruit yield caused by salt stress are primarily due to decrease in number of fruits per tree but not decrease in fruit weight (Maas 1993).

1.5.2 Effect on fruit quality

High salinity in irrigation water has been reported to reduce flowering intensity, fruit set, number of fruits, and fruit growth (Cole and McCloud 1985; Howie and Lloyd 1989). Citrus fruits on trees irrigated with saline water often ripe earlier and are smaller (Iglesias *et al*. 2007). Despite of significant reductions in fruit yield of citrus, salinity has a minimal effect on fruit quality. Salinity reduces rind thickness but had no significant effect on other fruit characteristics such as size, length to weight ratio, fruit color, weight and percentage of total soluble solids (TSS), or titratable citric acid (Maas 1993). However, salinity affectes several juice quality parameters of 'Marsh Seedless' grapefruit but did not affect fruit weight or diameter, peel thickness, or juice content (Levy *et al*. 1979). Similarly, in lemons, a significant increase in total soluble sugars and the sugar/acid ratio is observed (Nieves *et al*. 1991).

1.5.3 Accumulation and distribution of sodium and chloride ions in citrus under salt stress

NaCl is the most abundant salt under salt stress and plants develop their mechanism against Na^+ and Cl^-. Citrus rootstocks have a marked affect on the quantity of Cl^-, Na^+ or both ions accumulated in the foliage of grafted and ungrafted trees. Moreover, increasing salinity

level, increased accumulation of Na^+ and Cl^- in aerial parts and decreased K^+ and Mg^{++} (Romero-Aranda *et al*. 1998). Some of the adverse effects of salinity have been attributed to K^+ deficiency (Camara-Zapata *et al*. 2004) but K^+ reduction in citrus leaves under salinity has not always been observed (Walker and Douglas 1983). Leaf defoliation was strongly correlated with chloride accumulation (Banuls *et al.* 1997).

1.5.3.1 Cl^- transport

It has been reported in the literature that both the scion and rootstocks may influence Cl^- accumulation in leaves (Banuls *et al*. 1990; Garcia-Legaz *et al*. 1993; Levy and Shalhevet 1990; Lloyd *et al*. 1989; Moya *et al*. 2002; Nieves *et al*. 1991). Scion effects may be apparent when rootstocks are poor excluders (e.g. Lloyd *et al*., 1989). Nevertheless, even with rootstocks divergent in Cl^- exclusion ability, a scion effect may be observed (Lloyd *et al*. 1990). However, rootstocks with significant Cl^- exclusion ability, have a greater impact on the level of Cl^- accumulated than the scion (Behboudian *et al*. 1986; Cooper *et al*. 1952). Rootstocks have a large diversity for Cl^- exclusion from roots. Maas (1993) ranked the best Cl^- excluders as Sunki mandarin, grapefruit, Cleopatra mandarin, Chinese Box orange and Rangpur lime. Generally different rootstocks can be classified as indicated below for their ability to induce Cl^- accumulation in scion: Macrophylla (*C. macrophylla*) < Cleopatra mandarin (*C. reshni* Hort. ex Tan.) < Appleby smooth Seville (*C. paradisi* x *C. sinensis*) < Ellendale (*C. reticulata* Blanco x *C. sinensis* (L.) Osbeck) x trifoliata (*Poncirus trifoliata* (L.) Raf.) < Rangpur lime (*C. limonia*) < Ellendale x Carrizo citrange (*C. sinensis* [L.] Osb. x *Poncirus trifoliata* [L.] Raf.) < Benton citrange (*C. sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.) < Sweet orange (*C. sinensis* (L.) Osbeck) < Troyer citrange (*Poncirus trifoliata* (L.) Raf. x *C. sinensis* (L.) Osbeck < Carrizo citrange (*C. sinensis* [L.] Osb. x *Poncirus trifoliata* [L.] Raf.) < Trifoliata (*Poncirus trifoliata* (L.) Raf.) < Rough lemon (C. *limon* L.) (Grieve and Walker 1983). This accumulation is linear and continuous for most of the rootstocks. In citrus damages caused by salinity are usually associated with chloride accumulation but not with sodium (Banuls *et al.* 1997; Moya *et al.* 2003; Tadeo *et al.* 2008). It could be due to fact that citrus plant withheld Na^+ very efficiently in roots and stem and are very good excluder from leaf blades (Brumós *et al*. 2010; Munns and Tester 2008). It has been shown in citrus under salt stress conditions imposed with NaCl and KCl that the accumulation of chloride doubled as compared to accompanying cations when a Cl^- sensitive rootstock was used (Brumos *et al*. 2009). Citrus tolerance to salt stress is mostly correlated with ability to restrict Cl^- transport from root to shoot and the mechanism to restrict the Cl^- at root level is strongly depend upon

rootstocks performance under salt stress (Brumos *et al*. 2009; Brumós *et al*. 2010; Iglesias *et al*. 2007; Moya *et al*. 2002; Romero-Aranda *et al*. 1998). Tolerant genotypes apparently possess more efficient chloride exclusion mechanism at root level compared to sensitive genotypes (Fernández-Ballester *et al*. 2003; Moya *et al*. 2003).

In citrus, the ability to exclude Cl^- appears to be the combined result of several traits since chloride tolerance has often been associated with molecular, biochemical, hormonal, physiological (Tadeo *et al*. 2008), and morphological factors (Moya *et al*. 1999). Very little is known about the mechanisms involved in uptake, sub cellular distribution and long distance transport of Cl^- by plants (Brumos *et al*. 2009). These unknown mechanisms probably rely on plasma membrane transporters that may regulate root Cl^- uptake in the epidermis and/or long-distance Cl- transport in the vascular cylinder (Brumos *et al*. 2009; Colmenero Flores *et al*. 2007). Recently Brumos *et al*. (2010) studied uptake, long-distance transport and distribution of Cl^- symplastic pathway in the citrus Carrizo citrange (Cl^- includer) and Cleopatra mandarin (Cl^- excluder) rootstocks. Net Cl^- uptake was higher in Cl^- includer, which correlates with a lower root-to-shoot transport capacity in Cl^- excluder. The patterns of tissue Cl^- accumulation indicated that chloride exclusion in the salt-tolerant rootstock Cleopatra mandarin was caused by a reduced net Cl^- loading into the root xylem. Also, *ICln* gene exhibited a strong repression to Cl^- application in the excluder rootstock, suggesting a role in regulating Cl^- homeostasis in plants. A *CCC* gene encoding for a secondary Cl^- transporter involved in developmental processes and long-distance Cl^- transport has been characterized (Colmenero Flores *et al*. 2007).

1.6 Salt tolerance improvement

1.6.1 Strategies for salt tolerance improvement

The importance to produce salt-tolerant crops is evident and needs no emphasis. Different biological strategies have been adopted for development or exploitation of plants capable of tolerating high levels of salts. These approaches include:

- Exploitation of natural genetic variations, either through direct selection in stressful environments,

- Taking advantage of known genetic diversity: appropriate combinations were achieved through recombinations brought by the sexual hybridization,

- Exploiting ploidy to alter expression levels of the existing genes for salt tolerance,

- Selection of spontaneous mutants,

- Through the mapping of quantitative trait loci (QTLs regions of a genome that are associated with the variation of a quantitative trait of interest) (Foolad 2004; Lindsay *et al*. 2004) and subsequent marker-assisted selection (MAS),

- Genetically modifying crops by breeding and selection for improved salt tolerance (Foolad 2004; Yamaguchi and Blumwald 2005).

1.6.2 Constraints in amelioration through breeding programme

Extensive research all over the world to improve crop performance under abiotic stresses has not been that successful because the fundamental mechanism of stress tolerance in plant is not yet completely understood. There are many biological and technical constraints to solve the problem of salt stress tolerance (Epstein *et al*. 1980). Different technical constraints have been overcome to some extent but the biological constraints are more difficult to handle because a prerequisite for the development of salt tolerant crop is the identification of genetic and molecular determinant of salt tolerance. In addition, salinity tolerance is a developmentally regulated, stage-specific phenomenon; tolerance at one stage of plant development is often not correlated with tolerance at other stages as shown in tomato (Foolad 2004). The environmental factors have a major influence on plant salt stress responses and hindered the direct selection of salt-tolerant genotypes under field conditions (Richards 1996). The existence of salt tolerant plants (halophytes) and difference in salt tolerance between genotypes within salt sensitive species (glycophytes) indicates the importance of genetic basis to salt stress. Hence, it is very important to understand the mechanisms of salt tolerance in order to be able to use them through breeding programs.

1.6.3 Citrus salt tolerance improvement

The sustainability of citrus under salt stress is indeed dependent on the use of rootstocks. Rootstocks have a pronounced effect on scion growth and fruit quality. Several aspects of scion growth and fruit quality are affected by rootstocks i.e. sugar, acidity, carotenoid content, pH, flavour, colour, texture of fruit and tolerance to stress caused by biotic and abiotic factors (Barry *et al*. 2004; Bassal 2009; Davis *et al*. 2006; Navarro *et al*. 2010). Therefore, many studies have been performed showing the influence of association rootstock/scion on many characters, well beyond the resistance to biotic and abiotic stress. The choice of rootstock in combination with varieties of interest is commonly made according to effect on yield, fruit quality, and resistance to biotic and abiotic stress. Thus, new and

improved scion and rootstock cultivars aimed at controlling these production and environment constraints have been the primary aim of citrus breeding efforts. From ancient China to today selection of spontaneous mutations has been the method most commonly used for citrus improvement. Good results were obtained for orange varieties Washington Navel, Valencia, Shamouti, Pera, Hamlin, also for Marsh grapefruit and Eureka lemon (Spiegel-Roy and Goldschmidt 1996).

1.6.3.1 Constraints for citrus improvement

The particular problems associated with citrus breeding are reflected by the genetic and reproductive behavior of the genus. The most unusual feature is, of course, nucellar embryony. This mechanism limits, and often essentially precludes, crossing and selfing in many varieties. When first generation progeny are obtained, their degree of nucellar embryony may be greater or less than in their parents, depending upon the cross. In addition, various varieties and hybrids such as Satsuma mandarin, Washington navel oranges, and Morton citrange, are highly pollen-sterile. Self and cross incompatibility are more common than previously suspected. On the other hand, large gene diversity, frequent bud variation, and the overall interfertility among *Citrus* species provide a wide range of material for breeding purposes (Erickson *et al*. 1968). Seeds of many cultivars are polyembryonic with only two species showing monoembryonic cultivars (citron and pummelo). Many cultivars have been selected for their gametic sterility (Washington Navel orange) or gametophytic self-incompatibility (grapefruit, tangelo, and clementine). This prohibits the use of many cultivars as genitor and limits the successive phases of selfing in breeding schemes (Ollitrault and Luro 1995). Moreover, length of the juvenile phase, the unfamiliarity of the genetic determinism of most characters, and inbreeding depression among polyembryonic cultivars, are all factors unfavorable for the implementation of improvement strategies by sexual means.

1.6.3.2 Strategies for salt tolerance improvement in citrus

1.6.3.2.1 Sexual hybridization

Sexual hybridization is an effective tool to improve salt tolerance in citrus. In citrus damages caused by salinity are usually associated with chloride accumulation but not with sodium (Banuls *et al.* 1997; Moya *et al.* 2003). Citrus rootstocks differ greatly in their ability to exclude Cl^- and Na^+, or both from the scions (Brumós *et al*. 2010; Maas 1993). Classification of citrus salt sensitivity on the basis of agronomic parameters, leaf symptoms

and leaf chloride analysis exists in literature (Chapman 1968). This data for major species in citrus gene pool can be used as a basis for plant breeding programs. It is also known that all traits for a rootstock are present within the citrus germplasm (Ollitrault *et al.* 2000). Now identification of complementary parents for tolerances to biotic and abiotic stress is possible. Until recent years, the citrus rootstocks selection for salt tolerance was based on the ability to exclude Cl^- and Na^+ which is a heritable character. The populations from sexual hybridization segregate widely for the ability to restrict the accumulation of these elements in the tissues. However, a good Cl^- excluder rootstock is generally not a good Na^+ excluder and vice versa. In addition, the hybrids obtained behave just like their corresponding parents (Ream and Furr 1976; Sykes 1992). Progress in genetic improvement of citrus rootstock by conventional sexual breeding is difficult, mainly because of the reproductive biology and heterozygosity of genitors. These induce important segregations of characters in progeny and a low probability of obtaining recombinant hybrids that combine all the desirable genes and traits of two parents (Ollitrault *et al.* 2000). Furthermore, the long juvenile period of citrus makes rootstocks-breeding programs very protracted and expensive. To overcome different constrained in sexual hybridization breeders in different areas of the world adopted new methods. The integration of biotechnology in breeding programs has allowed overcoming the limits of traditional breeding.

1.6.3.2.2 Somatic hybridization

Somatic hybridization (protoplast fusion) is an alternative to create new rootstocks. Indeed, fusion of protoplasts allows the addition of two genomes with combining of resistance genes from both parents (Mourão Filho *et al*. 2008; Ollitrault *et al*. 2007). It has been developed successfully by the Citrus Research and Education Center, University of Florida who has obtained and regenerated interspecific hybrids and intergeneric allotetraploid from a hundred parental combinations (Grosser *et al*. 1992; Grosser *et al*. 2000). Also, this strategy has enabled to create an inter-generic hybrid between *Poncirus trifoliata* and Common mandarin (*Citrus reticulata* Blanco) which proved to have resistance to tristeza and also supportive behavior towards calcareous soils (Ollitrault *et al*. 1998). The application of protoplast fusion technology in citrus improvement has resulted in the regeneration of somatic hybrid plants from more than 500 parental combinations (Grosser and Gmitter 2009).

Through somatic hybridization citrus allotetraploid rootstocks were developed by combining different resistance genes. Somatic hybridization allows the combination of characters of abiotic stress tolerance and resistance to pathogens such as tristeza and

Phytophthora spp. within the same genotype (Ollitrault *et al.* 2000). Polyploidization by somatic hybridization combines all nuclear genomes of different genotypes without any limitation. Beyond the enlargement of tetraploid species pool complex, the resulting hybrids are a novel tool for the analysis of rules of inheritance of phenotypic traits in citrus. Recently CIRAD created a somatic allotetraploid rootstock FLHORAG1 (Poncirus Pomeroy + *C. deliciosa*) and this somatic allotetraploid presented high resistance to salt stress (Mouhaya 2008; Mouhaya *et al.* 2010). For more efficient use of this technology in citrus breeding it is important to explore the salinity tolerance in large citrus diversity and especially in monoembryonic genotypes which are responsible for large genetic diversity in citrus.

1.6.3.2.3 Search for salt tolerance mutants in cell cultures

Many studies have been realized in order to regenerate plants from callus cell lines resistant to salinity. A NaCl tolerant cell line was selected from ovular callus of Shamouti orange (*C. sinensis* L. Osbeck), especially the strain R-10 selected in a medium containing 10 g/l of NaCl (Benhayyim and Kochba 1983; Kochba *et al*. 1982). But the regenerated plantlets from callus cell line have not showed a level of tolerance as high as expected (Spiegel-Roy and Ben-Hayyim 1985). This method to improve salt tolerance is not very popular because frequently weak relationship is observed between tolerance in vitro and at whole plant level after regeneration. Cell lines which were more tolerant to salt stress were not able to respond the same way after regenerated as plantlets. Salt tolerance depends on the association of anatomical and physiological mechanisms of whole plant but not to the mechanisms implemented only at the cellular level (Noble and Rogers 1992).

1.6.3.2.4 Induced mutation (chemicals or irradiation)

Induced mutation (either by chemicals or irradiation) has been established as a tool to generate variations in a number of seed and/or vegetatively propagated crops. Mutagenesis techniques have shown some promise for induction of salt resistance in citrus. For example, (Matsumoto and Yamaguchi 1984) obtained lines with a higher salt tolerance from *Poncirus trifoliata* material which had undergone the longest exposure to ethyl methane sulphonate (EMS). García-Agustín and Primo-Millo (1995) selected three NaCl resistant plantlets of Troyer citrange, regenerated from ovules treated with ethyl methane sulphonate, and vegetatively propagated a population of plants for further evaluation. To increase mutation rate, (Wan *et al*. 1991) reported that, in citrus a mutation frequency 300 fold greater than natural frequency when exposed callus to EMS. They obtained two salt resistant mutants after

treating callus with EMS (0.3-0.5%) and screening with sodium chloride *in vitro*. Zhanao *et al*. (1993) obtained NaCl tolerant lines from orange (*C. sinensis*) cultivars Jincheng and Taoyecheng by subjecting nucellar calluses to gamma ray treatments, followed by 10 generations of in vitro selection for salt tolerance. Lee *et al*. (2003) concluded that the combination of irradiation techniques with *in vitro* culture methods may be a worth approach in selecting salt-tolerant lines.

1.6.3.2.5 Genetics and genomics in citrus

For sexual and somatic hybridization, an important limitation for effective improvement programs result from the lack of available data about genetic determinism and inheritance of resistance to salinity. It has been reported that salt tolerance is a complex trait involving the function of many genes (Foolad 2004; Lindsay *et al*. 2004). Results obtained from different hybrids by different authors (Ream and Furr 1976; Sykes 1992; Tozlu *et al*. 2002; Tozlu *et al*. 1999) showed that salt tolerance is a heritable trait. Ream and Furr (1976) found 20 hybrids related to Cleopatra mandarin or Rangpur lime that had an ability equivalent to their parents to limit the transport of Cl^-. More recently, Tozlu (Tozlu *et al*. 2002; Tozlu *et al*. 1999; Tozlu *et al*. 2000) studied the distribution of Na^+ and Cl^- in the *Poncirus trifoliata* (L.) Raf., *Citrus grandis* (L.) Osb. and their F1 hybrids. However, these studies involve very limited plant materials and provide only preliminary information.

Genes encoding enzymes related to the effects of salinity on physiological mechanisms (oxidative stress, membrane components, water stress) begin to be unveiled. In Duncan grapefruit a gene *c-inol* was isolated which encodes for myo-inositol-phosphate synthase and have 84% homology with *tur*l and 61% homology to *ino*l from (*Spirodela polyrrhiza*) and yeast respectively (Abu-Abied and Holland 1994). *C-Lea*5 gene was isolated from cell suspensions of R-10 (a NaCl tolerant cell line) and in leaves of Shamouti orange. It has a strong homology (79.5%) with the *lea*5 gene product of cotton induced by water stress and with the hydrophilic protein encoded by *rd29B* in Arabidopsis induced by dehydration and salinity (Naot *et al*. 1995). A gene for Na^+/H^+ antiporter has been isolated from the fruit skin of grapefruit (*C. paradisi*) subjected to heat shock. It has been observed that the expression of the related gene increases after 24h of heat treatment. Also, *ICln* gene exhibited a strong repression to Cl^- application in Cleopatra mandarin, suggesting a role in regulating Cl^- homeostasis in plants (Brumós *et al*. 2010). Recently, a *CCC* gene encoding for a secondary Cl^- transporter involved in developmental processes and long-distance Cl^- transport has been characterized (Colmenero-Flores *et al*., 2007).

1.6.3.3 Ploidy manipulation: another way to improve salt tolerance

1.6.3.3.1 Polyploidy in plants

An individual whose chromosome number is greater than 2n (diploid) copies is called polyploid. Polyploidization events occur frequently during plant evolution. The most popular estimate of the proportion of polyploids in angiosperms is about 70% (Masterson 1994). Polyploids originate from either sexual reproduction via 2n gametes or somatic chromosome doubling. By traditional definition, there are two forms of polyploidy: allopolyploidy and autopolyploidy. These terms are often used to imply the mode of polyploid formation, but more accurately describe the degree of similarity between the subgenomes in polyploids (Stupar *et al*. 2007). Allotetraploid, derived from hybridization of two diploid gametes without meiotic reduction. In allopolyploids inherited subgenomes come from two different parents after interspecific hybridization. Autotetraploid result from somatic chromosome duplication of genetic material due to a reduction in non-equational mitosis. Subgenomes of autopolyploids are then considered as being identical.

In citrus, the chromosome number is n = 9 (Krug 1943; Stace *et al.* 1993). They are generally diploid (2n = 2x = 18), but there are some exceptions e.g. Hong Kong kumquat tetraploid and Tahiti lime (*C. latifolia* Tan.) triploid (Longley 1926). In citrus doubling of the chromosome number may occur during seed formation which leads to tetraploid seedlings. These tetraploid arise from chromosome set doubling of nucellar cells (Cameron and Soost 1969). Thus, spontaneous doubled diploid clones obtained from specific diploid citrus species are considered to be genetically identical, with the same genome expression profile. The triploid hybrids may come from a cross between diploid and tetraploid or between diploids. Triploids (3x = 27) are often sterile. The occurrence of spontaneous triploid and tetraploid seedlings is under genetic and environmental control. The frequency of spontaneous triploid hybrid seedlings varies from minimum 4% in *Citrus limon* to maximum 14% in *Citrus reticulata*. Spontaneous tetraploid plants arise in seedlings with a frequency varying from 1 to 7% (Barrett and Hutchinson 1982; Barrett and Hutchison 1978).

1.6.3.3.2 Impact of polyploidy (Physiological and anatomical changes)

Polyploidy in plant species is often associated with increases in mesophyll cell volume, leaf thickness (Byrne *et al*. 1981; Molin *et al*. 1982), the amounts of photosynthetic enzymes and pigments per cell (Joseph *et al*. 1981; Leech *et al*. 1985) compared to diploids. Polyploidy increases net CO_2 assimilation (ACO_2) expressed on a leaf area basis in *Atriplex confertifolia*

(Warner and Edwards 1989). In wheat with increase in ploidy levels, leaves were largeer, thiner and showed less ACO_2 per leaf area as compared to diploid (Evans and Dunstone 1970). In potato the leaf cell size and thickness of the cell organs are positively correlated with the ploidy level of plant (Stupar *et al*. 2007). Similarly size of stomata guard cells tends to increase with ploidy level (Masterson 1994). In muskmelon, the results showed that the leaves and flowers of the tetraploid plants were markedly larger, the plants were obviously higher, and the stems were thicker than those of the diploid plants. Transmission electron microscope observation revealed that the numbers of chloroplasts, granules and grana, and the length of chloroplast and granule of the tetraploid plant leaves were significantly more or longer than those of the diploid plants (Zhang *et al*. 2010).

Similar to polyploids of other agricultural crops, citrus tetraploid are characterized by slow growth, small tree size, dark and thick leaves, high chlorophyll content, large stomata with less stomatal density, big fruit, thick fruit peel with more oil glands presence, less fruit juice and increased tolerance to abiotic stresses (Costa *et al*. 2004; Romero-Aranda *et al*. 1997; Saleh *et al*. 2008; Spiegel-Roy and Goldschmidt 1996; Syvertsen *et al*. 2000). Tetraploid of Valencia sweet orange (*C. sinensis* (L.) Osb.) and Femminello lemon (*C. limon* (L.) Burm. species had thicker leaves, larger mesophyll cell volume and lower light transmittance as compared to their respective diploids (Romero-Aranda *et al*. 1997). Polyploid leaves present larger stomata and a decrease in stomatal density (Byrne *et al*. 1981; Chen 2007). Autotetraploid citrus seedlings were shown to have lower growth rates than their respective diploid parents, associated with a lower rate of whole plant transpiration (Syvertsen *et al*. 2000). More recently anatomy and physiology of diploid and autotetraploid Rangpur lime (*C. limonia* Osbeck) seedlings were studied and growth of diploid was more vigorous than tetraploid although leaves, stems and roots of tetraploid plants were thicker and contained larger cells than diploid. Moreover, leaf water content was higher in tetraploid than in diploid (Allario *et al* 2011 in press). Allotetraploid citrus obtained by somatic hybridization displays certain morphological vegetative traits (leaf thickness, density, and size of stomata, etc.) similar to autotetraploids arising from chromosome doubling in nucellar cells (Ollitrault *et al*. 2008).

1.6.3.3.3 Changes in gene expression

Polyploids are environmentally selected because of their genome plasticity (Leitch and Leitch 2008) leading to selective advantages over diploid usually associated with enhanced vigor. However, although a high percentage of speciation events are accompanied by an

increase in ploidy, there is no direct evidence that polyploid lines enjoy greater net species diversification (Wood *et al*. 2009). The genetic and epigenetic changes associated with polyploidization in different allotetraploid plants have been studied (Flagel and Wendel 2009; Rapp *et al.* 2009; Wang *et al.* 2005). Allopolyploids gain advantages from the control of different physiological and metabolic pathway which results in vigorous growth and increased biomass (Ni *et al*. 2009). These changes in gene expression could be very effective in allopolyploids because partitioning of ancestral functions or expression patterns may occur between duplicated genes (subfunctionalization) so both are retained (Adams *et al*. 2003). After duplication, two gene copies can become specialized to perform complementary functions (Ward and Durrett 2004). This may also favor adaptation to environmental changes. Currently, genome expression changes in allotetraploids are considered to be more strongly affected by genome hybridization than by genome ploidy changes (Auger *et al*. 2005). There has been much research into genome expression and evolution of allopolyploids in cotton (*Gossypium* L.*)* plants in the last decade (Adams *et al*. 2003; Flagel *et al*. 2008; Rapp *et al*. 2009). Identification of changes in genome expression patterns of autotetraploids are less numerous than for allotetraploids (Riddle *et al.* 2010; Stupar *et al.* 2007). The gene expression changes studied by microarrays was very limited in autotetraploid potato (*Solanum tuberosum* L.) (Stupar *et al*. 2007) and maize (*Zea mays* L.) (Riddle *et al*. 2010). Leaf transcriptome expression using a citrus microarray containing 21081 genes revealed that the number of genes differentially expressed in both diploid and autotetraploid Rangpur lime (*C. limonia* Osbeck) genotypes was less than 1% and the maximum rate of gene expression change within a twofold range (Allario *et al.* 2011 in press). The large phenotypic differentiation in tetraploid Rangpur lime compared to diploid is not associated with large changes in genome expression. Autopolyploids derive all of their alleles from a single species which may result in small changes in their regulatory networks and gene expression (Stupar *et al*. 2007). These changes may be due to nuclear dosage and ploidy driven cellular modifications leading to physiological changes such as cell size, division rate, or organellar composition. On the contrary of allotetraploids, alteration of the expression of any allele in autopolyploids may lead to phenotype changes since it is considered that no potential for homologous complementation or advantageous subfunctionalization could occur.

1.6.3.3.4 Citrus tetraploid impact on salt tolerance

The behavior of citrus tetraploid rootstocks under salt stress condition is not investigated extensively and few examples found in literature. Based on seedlings growth and

chloride accumulation in leaves under salinity stress, autotetraploid plants of trifoliate orange, Carrizo citrange and Cleopatra mandarin were shown to be more tolerant than their respective diploid parents (Saleh *et al*. 2008). Similarly it was found that Cleopatra mandarin autotetraploid plants subjected to salt stress presented a greater growth rate than diploid suggesting that autetraploid genotypes experienced a better adaptation to stress. Tetraploid Rangpur lime rootstocks grafted with Valencia orange (4x RL/V) were shown to be more resistant to water deficit than the respective diploid Rangpur lime rootstocks grafted with Valencia orange (2x RL/V) (Allario *et al*. 2010 submitted). It was previously reported that under moderate saline stress, citrus tetraploid genotypes were more tolerant than the diploid, but under progressively increase salt stress conditions (50-400 mM) up to 8 weeks, tetraploid seedlings of *Poncirus trifoliata* (L.) Raf.) and willow leaf mandarin (*Citrus deliciosa* Ten. were certainly more sensitive to salt stress than diploid, as they accumulated more toxic ions and were more affected than diploid. Chloride accumulation in tetraploid leaves was greater and the maximum quantum yield of PSII was more reduced in tetraploid than in diploid (Mouhaya *et al*. 2010).

1.6.3.3.5 Tetraploid and citrus fruit quality

Rootstock tetraploid effect on citrus fruit quality is not documented because much emphasis was given to tolerance to biotic and abiotic stress using diversity in diploid genotypes. In citrus tetraploid plants generally gave lower fruit production with thick and irregular fruit bark (Soost *et al*. 1975). Poncirus trifoliata “tetraploid no. 1” showed more dwarfing effect and lower fruit production than diploid form of trifoliate orange (Tutberidze and Kalandarishvili 1975). More recently attempts have been made to investigate the inheritance of fruit quality traits in allotetraploid citrus hybrid. The allotetraploid hybrid (*C. reticulata* Blanco x *C. limon* (L.) Burm.) produced the same carotenoid compounds as mandarin but at very low levels. The total carotenoid content is about 10-fold lower in the allotetraploid somatic hybrid juice sacs than in mandarin, leading to global quantitative dominance of lemon at the phenotypic level (Bassene *et al*. 2009). In other crops such as grapevine autotetraploid rootstocks showed slow growth and thick leaves and lower fruit quality (Motosugi *et al*. 2007). In muskmelon, the soluble solid, soluble sugar and vitamin C contents in the tetraploid fruit were distinctly higher than those in the diploid fruit (Zhang *et al*. 2010). Carotenoid content specially (lutein) were different among diploid, tetraploid and hexaploid wheat species (Leenhardt *et al*. 2006).

2 Mechanism of salinity tolerance

Plants differ greatly in their tolerance to salinity and adopt different mechanisms at the molecular, cellular, and whole plant level to survive. Salinity disturbs plant in two ways: first the high salt content in root areas makes it difficult for roots to absorb water, hence inducing osmotic stress, and secondly the high concentration of salt within the plant can be toxic (Byrt and Munns 2008). In saline stress condition, plants suffer from water stress due to increased retention forces of water in the soil. The high accumulation of salts around roots prevents them from capturing the water, which causes a decrease in plant water potential. Lack of water causes osmotic stress on the cells and tissues resulting in increased concentration of solutes and ions in the cell. In response to these changes, several regulatory mechanisms are in place to adjust the cell metabolism and cope with damage caused by the loss of water and the accumulation of ions to toxic levels in the different plant organs. The regulatory mechanisms are accompanied by the engagement of signaling cascades and activation of transcription factors causes changes in the expression of genes involved in the synthesis effectors of stress adaptation. These will help maintain ionic homeostasis, osmolytes biosynthesis, trapping toxic radicals, transportation of water and coordination of the response (Apse *et al*. 1999; Hasegawa *et al*. 2000; Zhu 2002). It is important to know whether plant growth is being restricted by osmotic effect of salts in soil or toxic effect of salts in plants cells and tissues. High concentration of salts in the outside of roots has an immediate effect on cell growth, and related metabolism but toxic concentration within plants takes time to reach at lethal concentration. The mechanisms of salinity tolerance (Fig.1) can be categorized in three ways: 1) Osmotic stress tolerance, 2) sodium exclusion from leaves, 3) tissue tolerance.

2.1 Osmotic stress

2.1.1 Growth

Osmotic stress affects growth due to increased salt concentration in plant roots. Osmotic stress reduces cell expansion in root tips, young leaves and casue stomatal closure. In saline conditions, the alteration of water status causes a reduction in the turgidity of the cells thereby reducing their expansion and size, and consequently reducing the use of water by the plant. This fact will lead to decrease the development of new roots, leaves and lateral branches (Spollen *et al*. 1993). This loss in cell turgor is transient. Within a few hours, cells regain their original volume and turgor owing to osmotic adjustment but despite this cell elongation rate is

reduced (Byrt and Munns 2008). To date the mechanism of reduction in leaf and shoot growth in saline condition is not clear. The reduction in leaf growth rate is not dependent on carbohydrate supply and water status (Fricke *et al*. 2006). Reduced growth rate must be regulated by distance signaling in the form of hormones or precursors.

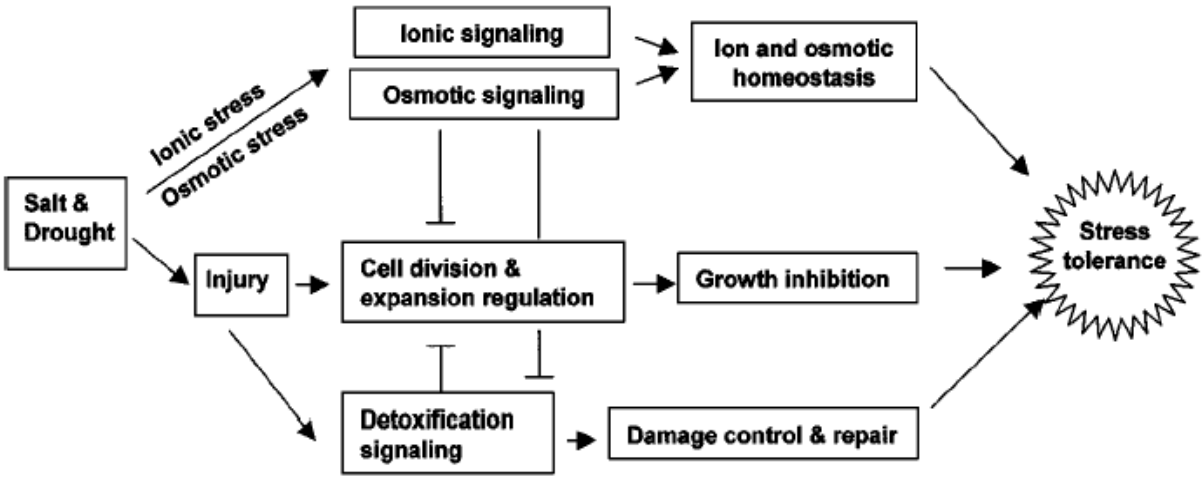


Fig. 1: Functional demarcation of salt and drought stress signaling pathways

(Ulm *et al*. 2002)

Accumulation of toxic ions and deficiency of essential nutrients reduce leaf growth rate, as evidenced by low concentration of Na^+ and Cl^- in expanding cells and tissues (Fricke *et al*. 2004; Hu *et al*. 2007; Neves-Piestun and Bernstein 2005). Abscisic acid (ABA) is the main hormone in cellular signaling in roots and shoots which plays important role in regulation of growth and stomatal conductance (Davies *et al*. 2005; Ulm *et al*. 2002). ABA concentration increases rapidly in growing zone of barley under salt stress and returns at original level after 24 h but leaf growth rate still remains reduced (Fricke *et al*. 2004). ABA deficient mutant in maize and tomato showed same leaf growth rate as wild type under drying soil and saline soil conditions (Mäkelä *et al*. 2003; Voisin *et al*. 2006). This evidences indicated that there are other limiting factors for reduced growth.

2.1.2 Stomatal conductance

The most prominent and readily measureable plant response to salinity is the decrease in stomatal conductance (Agastian *et al.* 2000; Brugnoli and Björkman 1992; Ouerghi *et al.* 2000; Parida *et al.* 2003). Stomatal closure is undoubtedly the osmotic effect of high salt concentration in plant root zone. When soil water potential decreases, leaves must lower their water potential to create the differential gradient that allows a maintaining of the colume of water from root to the aerial parts to limit embolism and limit cell water loss. During osmotic

stress when water deficit occurs, plants close their stomata to reduce water loss through transpiration. Signal perception induces mechanisms of adaptation or tolerance to salt tress. For example certain species living in an environment rich in salt survive by limiting the transpiration through closure of stomata (Sibole *et al*. 2003). The closure of stomata is mainly regulated by the hormone ABA, which is locally synthesized in the leaves and roots of stressed plants (Fricke *et al*. 2004). Pre-treatment with ABA increased salt tolerance in various species such as barley (Popova *et al*. 1995) and Citrus (Gómez Cadenas *et al*. 1998). In addition to the ABA effect, K^+ plays determinant role in stomatal closure. ABA induces the release of K^+ ions outside the stomatal guard cells causing the decrease of turgor and stomatal closure. The presence of Na^+ in the apoplastic space of guard cells could disturb the K^+ channels that participate in stomatal movement (Schroeder *et al*. 2001).

2.1.3 Photosynthesis

Several studies have showed the importance of photosynthesis system II (PSII) in response to environmental changes (Baker 1991). Salt stress reduces the rate of photosynthesis (Kawasaki *et al*. 2001), which ultimately causes a reduction of growth. Under saline conditions, photosynthetic carbon assimilation is seriously decreased by reduced leaf expansion. Some studies have shown inhibition of PSII activity (Bongi and Loreto 1989; Everard *et al*. 1994; Masojidek and Hall 1992; Mishra *et al*. 1991), while others have observed no negative effect of saline stress on PSII (Brugnoli and Björkman 1992; Morales *et al*. 1992; Robinson *et al*. 1983). Salinity reduces CO_2 assimilation via reduction in leaf area (Munns 2002; Papp *et al*. 1983). This reduction in assimilation rate can be beneficial instead of being disastrous for tolerant species which positively correlated the decrease of CO_2 assimilation with chlorophyll accumulation and soluble protein contents improving the photosynthesis under salt stress (Sibole *et al*. 2000). The salinity stress affects photosynthetic enzymes (Brugnoli and Björkman 1992; Reddy *et al*. 1992) and may inhibit repair of PSII (Allakhverdiev *et al*. 2002).

2.1.4 Oxidative stress

The salt stress inhibits photosynthesis rate which results in oxidative stress. Oxidative stress is caused by formation of reactive oxygen species (ROS). The ability of plants to scavenge ROS and to decrease their damaging effects is an important stress-tolerant trait. Mitochondria generate ROS that are thought to augment intracellular oxidative stress.

Mitochondria possess at least nine known sites that are capable of generating superoxide anion, a progenitor ROS (Andreyev *et al*. 2005). When plants face salinity stress, they try to adjust leaf morphology, chloroplast pigments composition, and activity of bio-chemical processes that help to prevent oxidative damage to PSII. ROS are usually generated by normal cellular activity such as photorespiration and ß-oxidation of fatty acids. Most of them include, for example hydrogen peroxide, hydroxyl radicals and superoxide anions. ROS level increase under salinity stress (Ulm *et al*. 2002). Plants immediately start producing the enzymes to detoxify these species such as superoxide dismutase (SOD), ascorbate peroxidase and catalase (Logan 2005). Elimination of ROS is achieved either by antioxidant compounds such as glutathione, thioredoxin, ascorbate and carotenoids or by ROS scavenging enzymes as superoxide dismutase, catalase, glutathione peroxidases and peroxiredoxins. Plant's tolerance capacity has evolved either to avoid the production of ROS, or to increase the detoxification or repair of ROS damage e.g. superoxide dismutase, catalyses the conversion of superoxide anions to H_2O_2 and H_2O. Over expression of this enzyme increased tolerance to abiotic stress such as salinity, low temperature (Bohnert and Sheveleva 1998). Transgenic Arabidopsis plants with reduced catalase activity revealed increased sensitivity to salt stress (Willekens *et al*. 1997).

2.1.5 Regulation of osmotic potential: synthesis of compatible solutes

Osmotic adjustment is one of the vital cellular responses to water deficit generated by drought, salinity or freezing temperature conserved by halophytes and glycophytes (Chinnusamy *et al*. 2005). This adjustment may contribute to maintain all turgor despite low water potentials and proceed to the uptake of K^+, compartmentalization of Na^+ and Cl^- into the vacuole or synthesis of compatible solutes such as proline, glycinebetaine, polyol, sugars etc. (Ashraf 1994). These solutes are called compatible because they are non-toxic at high concentration; they have low weight, are highly soluble and protect plants from stress by turgor maintenance, detoxification of ROS, and by stabilization of quaternary structure of proteins (Bohnert and Jensen 1996; Yancey *et al*. 1982). Different compatible solutes are synthesized according to the species. Glycinebetaine was found to be the major organic substrate accumulating under hyper saline growth conditions in the halotolerant cyanobacterium *Spirulina subsalsa*. In this species, it is shown that this osmolyte specifically protects enzymatic activity such as glucose-6-phosphate dehydrogenase which remained fully activated in the presence of NaCl (Gabbay-Azaria *et al*. 1988). Accumulation of

glycinebetaine has been also reported under conditions of salt stress for example barley and (*Atriplex*) (Jagendorf and Takabe 2001).

2.1.5.1 Aquaporins

Water is the major component of all living cells, and efficient regulation of water homeostasis is essential for many biological processes. Water passes through biological membranes by aquaporin water channels. Aquaporins are intrinsic membrane proteins characterized by six transmembrane helices that selectively allow water or other small uncharged molecules to pass along the osmotic gradient (Kruse *et al*. 2006; Postaire *et al*. 2010). Aquaporins are membrane proteins of low molecular weight (24-30 kDa) belonging to the family of membrane intrinsic proteins (MIP) (Agre *et al*. 1998). Membrane intrinsic proteins (MIP) have been classified into four groups based on the homology of their sequence. These are plasma membrane intrinsic proteins (PIP), tonoplast intrinsic protein (TIP), nodulin 26 intrinsic proteins (NIP) and small intrinsic proteins (SIP) (Johanson *et al*. 2001; Johansson *et al*. 2000; Weig *et al*. 1997). In plant cells, the cytoplasm is in fact enclosed between two membranes: the plasma membrane, which forms the outer boundary of the cell, and the tonoplast, which surrounds the vacuolar compartment. Aquaporins located in the plasma membrane (PIP, plasma membrane intrinsic protein) or tonoplasts intrinsic protein (TIP) contribute to intracellular water balance and transcellular water flow (Kruse *et al*. 2006). Because of the limiting role of plasma membranes in transcellular water transport, PIPs aquaporins represent the most likely candidates for protein-mediated hydraulic conductivity in roots and leaves (Heinen *et al.* 2009; Kaldenhoff *et al.* 2008; Maurel *et al.* 2008). The PIN NOD26 has been localized in the peribacteroid membranes in root nodules of soybean (Fortin *et al*. 1987). The SIP was localized at the endoplasmic reticulum membrane in Arabidopsis and is involved in water and ions transport (Ishikawa *et al*. 2005). Aquaporins regulate the hydraulic conductivity of membranes where they are integrated and can increase by ten to twenty times their water permeability coefficient. The diversity of aquaporins is attributed to the large degree of compartmentalization in the cell and their involvement in the control of water transport to adapt to environmental change (Johanson *et al*. 2001).

Much of our information on the physiological relevance of aquaporins in plants comes from analysis of transgenic plants with modified expression of various aquaporins, or from analysis of aquaporin mutants (Kruse *et al*. 2006). The first evidence for a function in cellular water uptake and whole plant water transport came from plants expressing antisense RNA for PIP proteins, which developed a larger root system than the controls (Kaldenhoff *et al*. 1998).

The family of PIP consists of two groups of proteins PIP1 and PIP2 differentiated by the length of their N- and C- terminals. The PIP2 has a shorter extension at N- terminal then PIP1 and a longer C-terminal extension as compared to phosphorylation sites (Johansson *et al.* 2000; Schäffner 1998). The genes coding for different aquaporins, expressed or over expressed under stress have been described in Arabidopsis, rice, sunflower, cauliflower, wheat, barley, and corn (Jang *et al.* 2004; Kawasaki *et al.* 2001; Maathuis *et al.* 2003; Seki *et al.* 2002). In tobacco, the plasma-membrane aquaporin NtAQP1 was shown to be important for hydraulic conductivity and water stress resistance in roots (Siefritz *et al.* 2002).

In addition to their role in water transport in plants, some aquaporins facilitate the transport of gases such as CO_2 (Flexas *et al.* 2006; Prasad *et al.* 1998), ammonium NH_3 and NH_4^+ (Holm *et al.* 2005) and H_2O_2 (Bienert *et al.* 2007) through membranes. Plants with impaired expression of a PIP1 aquaporin showed several differences, not only in water transport (Maurel *et al.* 2008; Siefritz *et al.* 2002) but also in CO_2 limited processes such as photosynthesis and stomatal conductance (Uehlein *et al.* 2003).

2.2 Ion stress homeostasis

NaCl is the main salt in soil salinity stress; both Na^+ and Cl^- can be cytotoxic. However, research focus has been the intracellular Na^+ homeostasis mechanism (Hasegawa *et al.* 2000). The capacity of plants to tolerate salt stress is related to how efficiently they regulate ionic imbalance in cytosol. Intracellular ion homeostasis requires determinants that control toxic ion uptake and facilitate their compartmentalization into the vacuole (Hasegawa *et al.* 2000; Niu *et al.* 1995; Zhu 2003). Since the vacuole is a focal compartment for cell expansion, vacuolar compartmentalization contributes to maintaining water relations of cells hypertonic medium that drives growth with minimum deleterious impact on cytosol and other organelle machinery. When ion accumulation exceeds the capacity of vacuolar compartmentalization, ions are accumulated in cytosol at toxic level. Plasma membranes have an inside negative potential of -120 and -200 mV (Borsani 2001a; Niu *et al.* 1995). It facilitates passive influx of Na^+ which results in the increase concentration of ion in the cytosol to > 10^3 fold relative to apoplast (Niu *et al.* 1995). High Na^+ concentration (> 100 mM) in cytoplasm disturbs the normal functioning of the cell (Serrano *et al.* 1999). The accumulation of Na^+ limits the absorption of other ions essential for plant growth. Indeed, Na^+ enters into competition with K^+, Ca^{2+}, Mg^{2+} and Mn^{2+}, while Cl^- limits absorption of NO^{3-} $PO4^{2-}$ and $SO4^{2-}$ (Munns and Termaat 1986). Morphological and physiological changes produced by salinity are often associated with the accumulation of both Na^+ and Cl^-. Maize accumulates the same amounts

of Na^+ and Cl^- (Izzo *et al*. 1991) and in rice a linear accumulation of both ions were observed (Lessani and Marschner 1978). In Citrus accumulation of Cl^- is greater and more rapid than that of Na^+ (Romero-Aranda *et al*. 1998). The ability of citrus to tolerate salinity is related to ability of the rootstock to exclude Cl^- (Banuls *et al.* 1997; Cooper *et al.* 1952; Maas 1993; Romero-Aranda *et al.* 1998; Storey and Walker 1998; Walker and Douglas 1983). In saline medium leaf chlorosis and defoliation are directly related to the toxicity of chloride in citrus (Banuls *et al.* 1997; Cooper 1961; Romero-Aranda *et al.* 1998).

2.2.1 Na^+ influx and efflux across the plasma membrane

In case of high salinity, control of homeostasis consists of Na^+ efflux from the cytoplasm while maintaining K^+ concentration. Sodium uptake across the plasma membrane has been attributed to low Na^+ affinity of systems that transport K^+ (Amtmann and Sanders 1998). Transport systems that have high affinity for K^+ but also low affinity for Na^+ include AKT1, outward rectifying K^+ channels (KORCs) and the KUP/HAK family of K^+ / H^+ symporters (Maathuis and Amtmann 1999). But there is no direct evidence of this function. The high-affinity K^+ transporter (HKT1), low-affinity cation transporter (LCT1) and non selective channel are considered to be the most likely transport system for Na^+ influx (Amtmann and Sanders 1998; Zhang *et al.* 2010; Zhu 2003). The main site of Na^+ entry in roots is uncertain. Most of the Na^+ that passively enters roots cells is likely pumped back out again. Energy-dependent Na^+ efflux is obligatory to maintain a low Na^+ concentration in the cytoplasm. At the tonoplast, the vacuolar H^+-ATPase generates an H^+ gradient that energizes the NHXtype Na^+/H^+ antiporter which induces Na^+ efflux in the vacuole and contributes to Na^+ detoxification. The vacuolar Na^+/H^+ antiporter SOS1 (Salt Overly Sensitive 1) localized in the plasma membrane has been identified in Arabidopsis (Shi *et al*. 2000; Zhu 2003). Na^+ is also indirectly excluded from the cytoplasm by proteins regulating K^+ transport. Overexpression of *AtNHX1* in Arabidopsis has increased salt tolerance and increases vacuolar sequestration of Na^+ (Apse *et al*. 2003). Similarly, in Brassica (Zhang *et al*. 2001) and tomato (Zhang and Blumwald 2001) overexpression of the *AtNHX1* has demonstrated improved salt tolerance. Over expression of the *OsNHX1* gene increased salt tolerance in rice (Fukuda *et al*. 2004). *AtNHX1* has a critical involvement in the subcellular partitioning of K^+, which in turn affects plant K^+ nutrition and Na^+ tolerance (Leidi *et al*. 2010). Also, *cNHX1* gene expression has been reported in citrus under salt stress and high temperature. However, in spite of numerous reports of improved salt tolerance by the overexpression of NHX-type exchangers

in various plant species, the mechanism underlying the enhancement of salinity tolerance by these transporters is not yet clear (Pardo *et al.* 2006; Tester and Davenport 2003). Several reports failed to find the anticipated correlation between increased salt tolerance and enhanced accumulation of Na^+ by NHX proteins (Fukuda *et al.* 2004; Ohta *et al.* 2002; Wu *et al.* 2009), whereas others found greater K^+ contents, rather than greater Na^+ contents, in tissues of the transgenic plants (Rodríguez Rosales *et al*. 2008; Wu *et al*. 2005; Xue *et al*. 2004).

2.2.2 Cl^- transport

Cl^- channels reside both in the plasma membrane and intracellular organelles. Their functions range from ion homeostasis to cell volume regulation, transepithelial transport, and regulation of electrical excitability (Jentsch *et al*. 2002). The transport of Cl^- is interacting with various ion channels such as antiporters Na^+/H^+ and HCO_3^-/Cl^- and H^+/ATPase pumps. The regulation of cell volume requires the activation of K^+ and Cl^- to induce efflux of salts and thus regulate cytoplasmic pH. Very little is known about the role of Cl^- channels in regulating the transport of Cl^- under saline conditions. According to (Hechenberger *et al*. 1996), the Cl^- channels voltage-dependent (type CLC) are found in all prokaryotes and eukaryotes. They are responsible for passive transport of Cl^- under control of the electrochemical gradient. In plants, genes encoding the type CLC channels were first identified in tobacco (Lurin *et al*. 1996). In Arabidopsis, seven genes encoding for channels type CLC have been identified (*AtClCa-AtCLCg*) (Hechenberger *et al*. 1996) and some channels have been localized. The AtClCa playing the role of antiport NO_3^-/H^+ at the tonoplast and *AtCLCe* and *AtCLCf* located respectively at the chloroplast thylakoid membrane and Golgi vesicles (Marmagne *et al*. 2007). In situations of salt stress, the expression of vacuolar *OsCLC1* is induced in Cl^- tolerant rice and is repressed in Cl^- sensitive rice (IR29 strain) (Diedhiou and Golldack 2006).

2.2.3 Na^+ and Cl^- compartmentalization in vacuole and outside the cytoplasm

Compartmentalization of Na^+ and Cl^- into vacuole is a salt-adaptation mechanism. Na^+ can be leaked back to cytosol passively from vacuole probably through tonoplast non selective cation channels (Byrt and Munns 2008). This passive leakage requires constant resequencing of Na^+ form cytosol. The vacuolar Na^+/H^+ antiporter SOS1 (Salt Overly

Sensitive 1) localized in the plasma membrane has been identified in Arabidopsis (Shi *et al.* 2000; Zhu 2003). The vacuolar Na^+/H^+ antiporter NHX is a transport system specific not only for Na^+ cations but it has a broad substrate specificity for at least four alkali metal cations (Na^+, Li^+, K^+ and Rb^+) (Kinclova-Zimmermannova *et al.* 2004; Leidi *et al.* 2010; Teakle and Tyerman 2010). Compartmentation in vacuoles is achieved by tonoplast Na^+/H^+ antiporters. NHX-type antiporters in the tonoplast have been reported to increase the salt tolerance of various plants species, and are thought to mediate the compartmentation of Na^+ in vacuoles (Leidi *et al*. 2010). This H^+ chemical gradient is generated by the vacuolar H^+-translocating enzymes, H^+-ATPase and H^+-pyrophosphatases. The electrochemical difference for H^+ increases the pumping of Na^+ into vacuole, which ultimately results in increased Na^+ accumulation and Na^+ tolerance. This phenomenon of efficient Na^+ influx towards vacuole may increase tissue tolerance by reducing cytosolic Na^+ concentration (Apse *et al.* 1999; Munns and Tester 2008). HKT transporters including *AtHKT1;1* and *OsHKT1;5* also play a vital role in preventing shoot Na^+ over-accumulation by mediating Na^+ exclusion from xylem vessels in the presence of a large amount of Na^+ thereby protecting leaves from salinity stress (Hauser and Horie 2009).

2.2.4 K^+/Na^+ selective accumulation

K^+ channels are very interesting to physiologists because they are universal regulators of cellular functions. The function of many organs and many different cell types is modulated via activation or inhibition of K^+ channels. In plant cells, K^+ is a major macronutrient essential for many cell processes, including enzymatic activation, turgor pressure formation, regulation of stomatal movement and maintenance of osmotic homeostasis (Hauser and Horie 2009; Shabala 2003). Counterbalancing the large excess of negative charge, K^+ is also equilibrated with Na^+ to provide a correct environment for protein synthesis in conditions of hyperionic stress (Hattori *et al*. 2005). Several types of transporters and channels have been identified that mediate K^+ uptake into the plant at micromolar and millimolar external K^+ concentrations (Leidi *et al*. 2010). In high salt concentration environment, Na^+ competes with K^+ for sites in the cell and enters in the cytosol through High affinity K^+ Transporter (HKT1) and non-selective cation channels (NSCC-VI), which trigger stress response following an increase in the concentration of cytosolic Ca^{2+} (Hasegawa *et al.* 2000; Horie *et al.* 2009; Lan *et al.* 2010; Leidi *et al.* 2010; Yokoi *et al.* 2002; Zhang *et al.* 2010).

2.3 Salt stress signal transduction

A generic signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g. inositol phosphates and ROS). Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes (Fig. 2) (Kudla *et al.* 2010; Mahajan *et al.* 2008; Xiong *et al.* 2002)

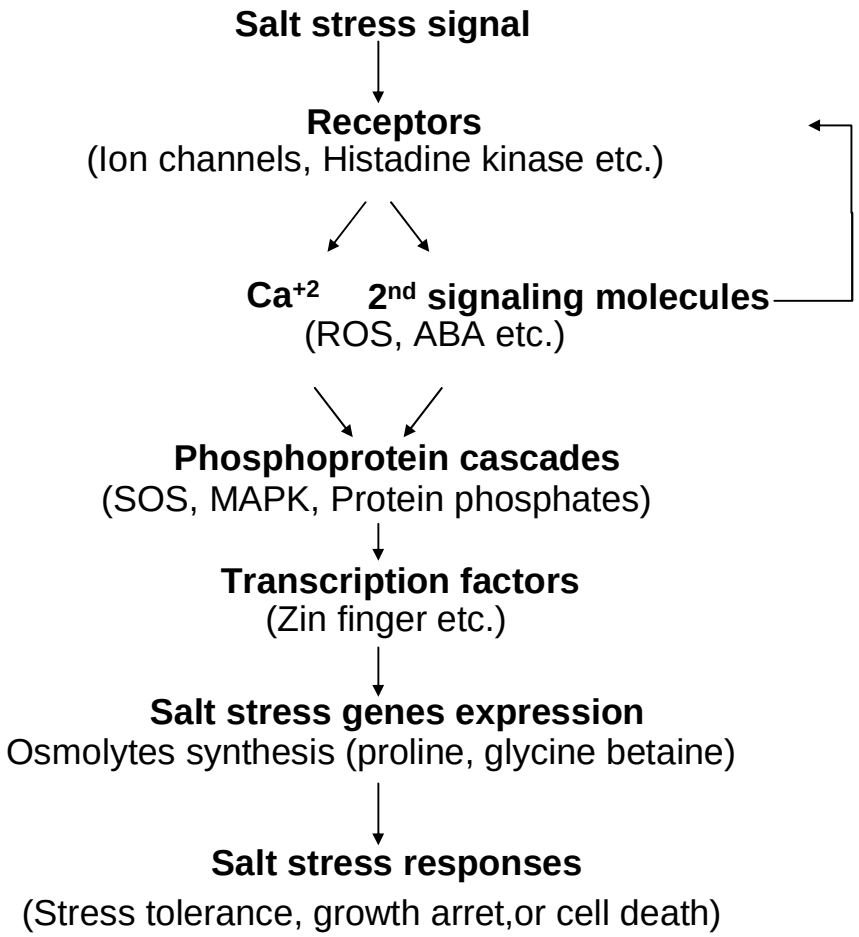


Fig. 2: A generic pathway for the transduction of cold, drought, and salt stress signals in plants.

(Mahajan *et al.* 2008; Xiong *et al.* 2002)

2.3.1 Ca^{2+} intracellular signaling

Various aspects of plant growth and development and stress physiology are mediated by chemical signaling through many chemicals such as calcium ions (Ca^{2+}), which function as a major secondary-messenger signaling molecule and plays a fundamental role in plant growth and development under normal as well as stress conditions (Kudla *et al.* 2010; Mahajan *et al.* 2008). Three major families of Ca^{2+} sensors have been identified in higher plants:

calmodulins (CaMs) and CaM-like proteins (McCormack *et al*. 2005); calcineurin B-like (CBL) proteins (Luan 2009; Weinl and Kudla 2009); and calcium-dependent protein kinases (CDPKs) (Harper and Harmon 2005). The extracellular stress signal is first perceived by the membrane receptors, which then activates large and complex signaling cascade intracellularly including the generation of second messengers such as Ca^{2+} (Qi *et al*. 2010). This increased cytosolic Ca^{2+} initiates the stress signaling pathways for stress tolerance. The concentration of intracellular Ca^{2+} is carefully regulated (Kudla *et al*. 2010). Changes in cytosolic free calcium are apparent during the transduction of a very wide variety of abiotic and biotic signals (McAinsh and Schroeder 2009). Ca^{2+} concentration in the cytosol is low, and upon stimulation (salt stress), calcium signals are generated through the opening of ion channels that allow the downhill flow of Ca^{2+} from a compartment in which the ion is present at relatively high electrochemical potential (either outside the cell, or from an intracellular store) to one in which Ca^{2+} is at lower potential. Whole-plant Ca^{2+} measurements have suggested a direct correlation between the strength of NaCl stress and the magnitude of Ca^{2+} elevation (Tracy *et al*. 2008).

Studies with animal cells have shown that there are several paths that mediate Ca^{2+} transient increase in the cytosol. Ca^{2+} can enter the cells from outside by voltage-gated, receptor operated or store operated Ca^{2+} channels. Also, Ca^{2+} in intracellular stores can be released through ligand messenger-sensitive channels (Anil and Sankara Rao 2001; Sanders *et al*. 2002). Physiological evidences demonstrated that Ca^{2+} favored K^+ over Na^+ uptake through a high affinity system (Zhu 2003). Ca^{2+} activates phosphorylation cascades Mitogen-Activated Protein Kinase pathway (MAPK) (Boudsocq *et al*. 2010), leading to subsequent activation of transcription factors that act specifically on promoters to initiate the response (including SOS gene transcription and genes for biosynthesis of osmolytes) (Mahajan *et al*. 2008; Yokoi *et al*. 2002).

2.3.2 Abscisic acid (ABA)

Abscisic acid is a ubiquitous plant hormone in vascular plants. Within the plant, ABA has been detected in every major organ or living tissue from the root tip to the apical bud. ABA is synthesized in almost all cells that contain chloroplast or amyloplasts. A large body of evidence indicates that ABA plays a major role in adaptation to abiotic environmental stresses, seed development, and germination (Adie *et al.* 2007; Bari and Jones 2009; Davies 2010; Wasilewska *et al.* 2008; Zeevaart and Creelman 1988). The ABA biosynthesis takes

place in chloroplast via the pathway of carotenoid. In higher plants, ABA is synthesized from a C40 precursor β-carotene via the oxidative cleavage of neoxanthin and a two-step conversion of xanthoxin to ABA via ABA-aldehyde (Wasilewska *et al*. 2008). Environmental stresses such as drought, salt and to a lesser extent, cold stimulates the biosynthesis and accumulation of ABA by activating genes coding for ABA biosynthetic enzymes. Stress activation of ABA biosynthetic genes is probably mediated by a Ca^{2+}-dependent phosphorelate cascade (Kudla *et al*. 2010; Xiong *et al*. 2002; Zou *et al*. 2010) (Fig. 3). In addition, ABA can feedback stimulate the expression of ABA biosynthetic genes, also likely through a Ca^{2+}-dependent phosphoprotein cascade (Xiong and Zhu 2001). Substantial evidence supports that the increased ABA levels limit water loss through transpiration by reducing stomatal aperture (Leung and Giraudat 2003; Zou *et al*. 2010). Gene *CPK10,* possibly by interacting with *HSP1,* plays important roles in ABA and Ca^{2+}-mediated regulation of stomatal movements *(Zou et al*. 2010). ABA is also involved in other aspects of stress adaptation. For instance, ABA-deficient mutants of Arabisopsis are impaired in cold acclimation (Mäntylä *et al*. 1995) and in a root morphogenetic response to drought (drought rhizogenesis) (Vartanian *et al*. 1994). The role of ABA in signaling stress conditions has also been extensively documented by molecular studies showing that ABA-deficient mutants are affected in the regulation of numerous genes by drought, salt, or cold (Giraudat *et al*. 1994; Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 1996; Yasuda *et al*. 2008). Loss of function mutants *adc1* and *adc2* display reduced freezing tolerance and alterations in ABA content and ABA-dependent signalling pathways under low temperature, compared to wild type plants (Cuevas *et al*. 2009). In rice *OsHSP71.1* gene was induced by ABA while *OsHSP24.1* was suppressed by ABA under heat shock (Zou *et al*. 2009).

The perception of ABA in stomatal guard cells induces the accumulation of cytosolic Ca^{2+} due to influx through NSCC channels in plasma membrane (Murata *et al*. 2001). Meanwhile, in tonoplast activation of H^+/ATPase induced alkalinization of the cytosol and promotes the efflux of Ca^{2+} from the vacuole. ABA closes stomatal pores by inducing net efflux of both K^+ and Cl^- from the vacuole to the cytoplasm, and from the cytoplasm to the outside of guard cells (Leung and Giraudat 2003). The ABA is involved in slow growth response to osmotic stress (Giraudat *et al*. 1994). The ABA is also involved in regulating Late Embryogenesis Abundant (LEA) protein synthesis and the key enzyme in the biosynthesis of proline (delta1 pyroline carboxylate synthase (P5CS). In Arabidopsis, exogenous ABA induces the expression of LEA genes (Xu *et al*. 1996) and P5CS in leaves (Yoshiba *et al*. 1997). Expression analysis revealed that ABA, salt, cold and osmotic stresses induce

expression of *LEA4-1* gene in leaf tissues of rape (*Brassica napus)* which plays a crucial role in abiotic stress tolerance during vegetative stage of plant development (Dalal *et al*. 2009). The accumulation of ABA in leaves is correlated with the accumulation of gene transcripts NCED (Xiong and Zhu 2003) under water stress. In transgenic plants overexpressing the gene NCED accumulates high amounts of ABA and increased tolerance to water deficit in tobacco (*Nicotiana tabacum*) (Thompson *et al*. 2000).

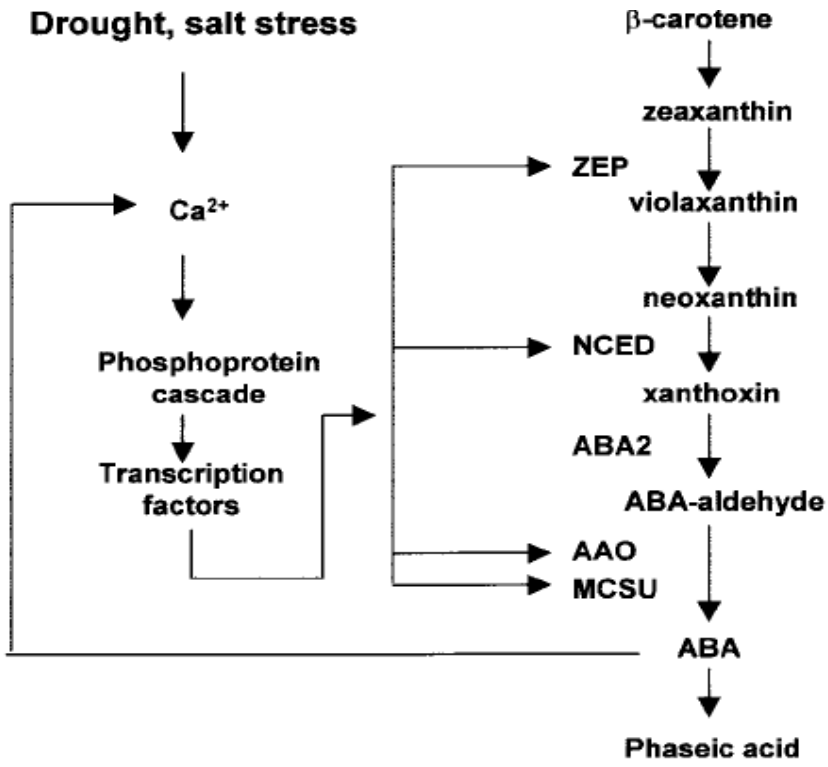


Fig. 3: Pathway and regulation of ABA Biosynthesis

(Xiong *et al*. 2002)

In citrus under salt stress, the accumulation of ABA has been reported in the roots, xylem and leaves (Gómez Cadenas *et al*. 1998). Abscisic acid reduces leaf abscission and increases salt tolerance in citrus plants (Arbona *et al*. 2006; Gomez-Cadenas *et al*. 2002). In citrus ABA concentration was increased under deficit irrigation (Melgar *et al*. 2010). In vitro shoots accumulated similar levels of chloride when cultured without roots and exhibited similar leaf damage and no difference was observed in ABA in citrus. Gene expression analysis revelead the induction of response to ABA stimulus under salt stress in salt tolerant cleopatra mandarin rootstock (Brumos *et al*. 2009). *PtrHOS1* expression was down regulated both by cold and ABA in *Poncirus trifoliata* (L.) Raf. (Liu *et al*. 2010). In the orange, the accumulation of ABA was correlated with the induction of gene CsNCED1 in leaves (Rodrigo *et al*. 2006). Transgressive concentration of abscisic acid (ABA) was observed in the

allotetraploid somatic hybrid and transgressive over expression was observed for *CitNCED1* and *CitNCED2* (Bassene *et al*. 2009).

2.4 Sensing and regulation pathway for ion homeostasis

Adaptation to environmental stresses is dependent upon the activation of cascades of molecular networks involved in stress perception, transduction, transcription and the expression of specific stress-related genes and metabolites. These activated stress response mechanisms re-establish ion homeostasis and protect and repair damaged proteins and membranes (Ashraf and Harris 2004; Hasegawa *et al.* 2000; Ulm *et al.* 2002).

2.4.1 Salt Overly Sensitive Pathway (SOS)

The work on discovery of SOS genes was initiated by Zhu and coworker and first SOS1 gene was discovered (Wu *et al*. 1996) and later on SOS2 (Zhu *et al.* 1998), SOS3(Liu and Zhu 1998), SOS4 and SOS5 (Zhu 2000) are discovered. Overexpression of *SOS* genes increased salt tolerance in transgenic Arabidopsis (Yang *et al*. 2009). SOS pathway induces the activation of the protein SOS1, Na^+ / H^+ plasma membrane and the activation of the Na^+ / H^+ exchanger (NHX1) of the vacuole (Shi *et al*. 2000; Ulm *et al*. 2002). The SOS pathway is composed of three proteins (SOS1, SOS3 and SOS2) involved in the response to salt stress. Ca^{2+} activates the pathway through binding to SOS3 protein, which in turn activates a serine / threonine kinase, SOS2. SOS2 phosphorylates SOS1, which activates its Na^+/H^+ antiporter function (Mahajan *et al*. 2008; Oh *et al*. 2010) (Fig. 4). By this mechanism plants have the capacity to control net Na^+ flux across the membrane (Ajay *et al*. 2008).

Furthermore, this complex SOS2-SOS3 interacts with and influences other salt-mediated pathways essential for ionic homeostasis. This complex inhibits HKT1 activity (a low-affinity Na^+ transporter) thus restricting Na^+ entry into the cytosol. SOS2 also interacts and activates NHX (vacuolar Na^+/H^+ exchanger) resulting in sequestration of excess Na^+ ions, further contributing to Na^+ ion homeostasis (Guo *et al.* 2004). Overexpression of *SOS1 or AtNHX1* has been reported to improve the plant salt tolerance (Apse *et al.* 1999; Apse *et al.* 2003; Shi *et al.* 2003; Zhang and Blumwald 2001). Because SOS1 requires the SOS2/SOS3 complex for its maximal activity, and AtNHX1 activity has been shown to be controlled by the SOS pathway (Qiu *et al*. 2004), the full activities of both SOS1 or AtNHX1 in transgenic plants may require SOS2 and SOS3. More recently six different transgenic *Arabidopsis* plants that overexpress *AtNHX1*, *SOS3*, *AtNHX1 + SOS3*, *SOS1*, *SOS2 + SOS3*, *SOS1 + SOS2 +*

SOS3 were tested under salt stress. Result showed that contrary to earlier reports, transgenic *Arabidopsis* plants overexpressing *AtNHX1* alone did not show any significant increase in salt tolerance. Meanwhile, transgenic plants overexpressing *SOS3* exhibit increased salt tolerance similar to plants overexpressing *SOS1*. Moreover, salt tolerance of transgenic plants overexpressing *AtNHX1* + *SOS3*, *SOS2* + *SOS3*, *SOS1* + *SOS2* + *SOS3*, respectively , appeared similar to the tolerance of transgenic plants overexpressing either *SOS1* or *SOS3* alone (Yang *et al*. 2009).

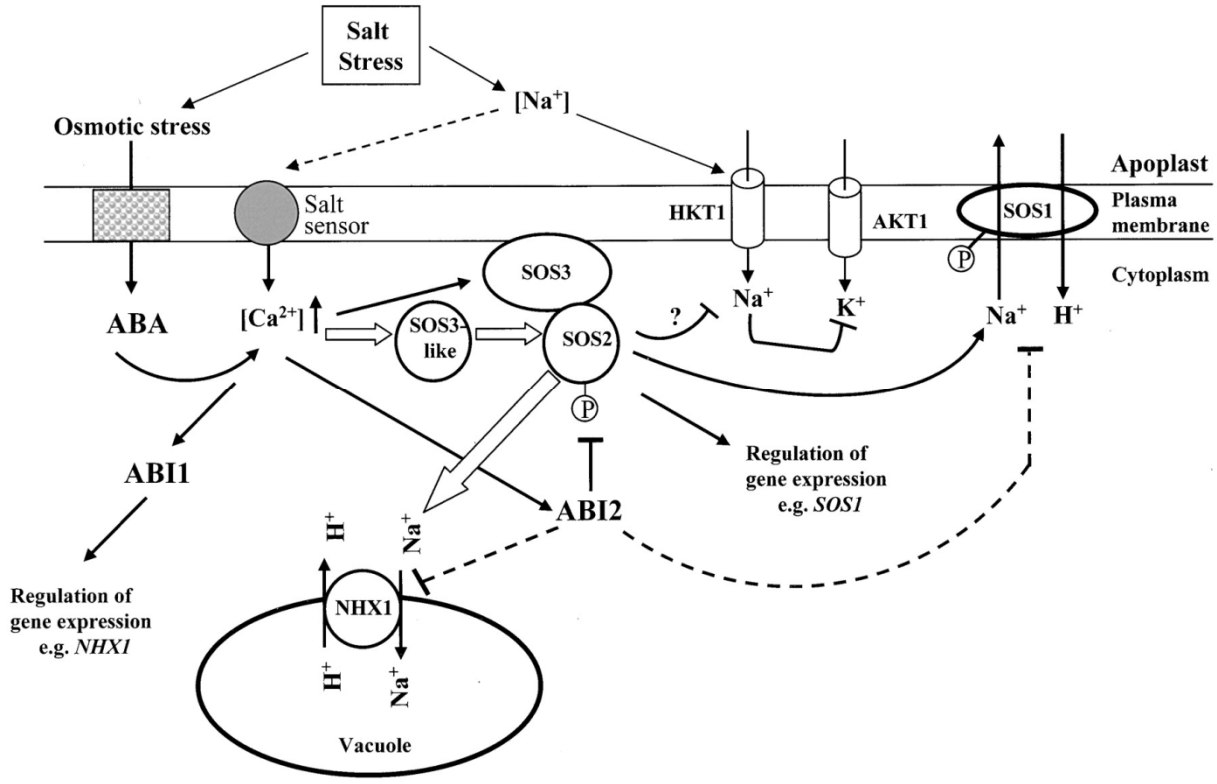


Fig. 4: SOS signaling pathway for ion homeostasis under salt stress in Arabidopsis.

(Chinnusamy *et al*. 2005)

The overexpression of SOS1 gene (Ajay *et al.* 2008) and SOS2 (Guo *et al.* 2004) have improved salt stress tolerance in Arabidopsis. A mutation of AtSOS1 in *Arabidopsis thaliana,* leads to a salt-sensitive phenotype accompanied by the death of root cells under salt stress (Oh *et al*. 2010).

2.4.2 Mitogen-Activated Protein Kinase pathway (MAPK)

In situations of oxidative stress, a high production of ROS and particularly H_2O_2 induces the MAPK pathway (Desikan *et al*. 1999; Kovtun *et al*. 2000; Rodriguez *et al*. 2010)**.** Phosphates play the role of regulators for the pathway of MAPK. MAPK activation appears to

be mediated by Ca^{2+} signaling because Ca^{2+} channel blockers can inhibit ozone or H_2O_2-mediated SIPK activation (Salicylic acid Induced Protein Kinase) (Rodriguez *et al*. 2010). In all eukaryotic MAPK pathways are involved in the regulation of growth, cell death, differentiation, proliferation and stress responses (Chang and Karin 2001; Garrington and Johnson 1999; Rodriguez *et al*. 2010). The signal transduction of oxidative stress is controlled by phosphorylation of proteins involved in the MAPK pathway which is composed of three types of kinases: MAPKKK, MAPKK and MAPK. The MAPKKK are serine/threonine kinases that activate MAPKK by phosphorylation of two serine/threonine of the motif 'S/T-X3-5-S/T' (Chang and Karin 2001; Garrington and Johnson 1999). Similarly kinases MAPKK will in turn activate the MAPKs by phosphorylation of threonine and tyrosine residues reasons 'T-X-Y'. The MAPK last components of the cascade, are serine/threonine kinases that phosphorylate various substrates such as transcription factors and protein kinases (Chang and Karin 2001). The MPK1 (MAPKKK) interacts with the MPK3 and MPK6 to transmit the signal of salt stress condition (Ulm *et al*. 2002), and it induces the expression of the Na^+/H^+ vacuolar NHX1 and membrane SOS1 (Apse *et al*. 1999; Shi *et al*. 2000). In Arabidopsis *mkk2* mutants had reduced tolerance to salinity, transgenic lines that overexpress MKK2 exhibited enhanced tolerance to salt and cold (Teige *et al*. 2004), also MKK2 interacts strongly with MPK4 and MPK6 and weakly with MPK5. MAPK cascade involving MKK9-MPK6 is shown to play an important role in regulating leaf senescence in Arabidopsis (Zhou *et al*. 2009). MPAK implicates in hormone signaling (auxin, ethylene, ABA, salicylic acid and jasmonic acid), in mediating synergistic or antagonistic effects between different hormones, and in the regulation of phytohormone synthesis (Rodriguez *et al*. 2010; Santner and Estelle 2009).

2.4.3 Activation of the genes expression for adaptation to salt stress

At the end of signal cascades, the expression of different genes will be regulated contributing to adaptation of the plant to salt stress. These genes may encode enzymes involved in pathways of osmoprotectants biosynthesis, proteins and membrane transporters or for detoxification enzymes. Genes that could increase salt tolerance fall into three main functional groups: (1) those that control salt uptake and transport; (2) those that have an osmotic or protective function; and (3) those that could make a plant grow more quickly in saline soil (Munns 2005).

2.4.3.1 Genes that control salt uptake and transport (Na^+ homeostasis)

Although all transport system necessary for ion homeostasis have not been verified by molecular criteria but sufficient advancement has been gained for Na^+ homeostasis (function of plasma membrane and tonoplast H^+ pumps and Na^+/H^+) (Apse and Blumwald 2002; Zhu 2003). HKT1 gene mediates Na^+ influx across the plasma membrane in wheat, rice and barley (Huang *et al*. 2008; Rus *et al*. 2001). Genetic and physiological data establish that Arabidopsis AtHKT1 facilitates Na^+ homeostasis in planta and by this function modulates K^+ nutrient status (Rus *et al*. 2004). The *hkt1* mutation results in higher Na^+ shoot content and lower Na^+ root content (Mäser *et al*. 2002; Rus *et al*. 2004). *SOS1* was the first locus identified in salt hypersensitive mutants of Arabidopsis (Wu *et al*. 1996). *SOS1* encodes a Na^+/H^+ antiporter SOS1 that control energy-dependent Na^+ transport across the plasma membrane (Qiu *et al*. 2004; Shi *et al*. 2000). The plasma membrane SOS1 protein from Arabidopsis thaliana has also revealed surprising interactions with K^+ uptake mechanisms by roots. The function of individual members of the large CHX family remains largely unknown but two CHX isoforms, AtCHX17 and AtCHX23, have been shown to affect K^+ homeostasis and the control of chloroplast pH, respectively (Pardo *et al*. 2006). Vacuolar compartmentalization of Na^+ is controled by *NHX* antiporters (Apse *et al*. 2003). NHX antiporters in Arabidopsis are encoded by a gene family including six members (Aharon *et al*. 2003). Physiological role under salt stress of certain NHX antiporters are revealed but for others still to be determined. AtHKT1 antiporter localized in tonoplast and utilizes the H^+ electrochemical gradient generated by H^+ pumps, ATPase and pyrophosphatase. More recently in Arabidopsis, six vacuolar Na^+/H^+ antiporters (*AtNHX1-6*) have been identified. Among them, *AtNHX1*, 2 and 5 are functional Na^+/H^+ antiporters with the most abundant expression levels in seedling shoots and roots. AtNHX3 is also a functional antiporter responsible for salt tolerance by mediating K^+/H^+ exchange in higher plants (Liu *et al*. 2008).

2.4.3.2 Genes involved in osmotic homeostasis (synthesis of compatible solutes)

The osmotic adjustment in higher plants refers to the maintenance of turgor by lowering the osmotic potential through the accumulation of solutes in response to water deficit (Guei and Wassom 1993). When leaf water potential decreases: turgor potential, stomatal conductance and photosynthetic activity are maintained through the accumulation of intracellular solutes. Osmotic adjustment is also involved in delaying leaf senescence and in improving water extraction by roots (Turner 1997; Turner *et al*. 2001). The osmotic

adjustment is obtained by synthesis of different osmoprotectants by plant. In the following section we discuss two amino acids playing a vital role as cellular osmoprotectors, proline and glycine betaine.

2.4.3.2.1 Proline

Proline is one of the twenty amino acids involved in protein sequences and of the genetic code. In addition to being a osmoprotectant, it plays an important role in regulating the redox potential (Alia and Saradhi 1991). The accumulation of proline seems to be correlated with the tolerance of plants to oxidative stress induced by water or salt stress (Kishor *et al*. 1995). Proline provides a protection against NaCl-induced cell death via decreasing level of ROS accumulation (Banu *et al.* 2009). The proline synthesis is accompanied by renewal of stocks of $NADP^+$ that occurs in the maintenance of redox potential during stress. Proline is involved in the regulation of cellular pH (Bellinger and Larher 1985) to protect and stabilize macromolecules in conditions of water deficit or high ionic strength (Handa *et al*. 1986). Proline protects membranes against peroxidation by scavenging free radicals (Alia *et al*. 2001; Smirnoff and Cumbes 1989) and is a handful source of carbon and nitrogen to the plant during stress (Bellinger and Larher 1985; Hare *et al*. 1998). There are two alternative pathways for proline biosynthesis in higher plants. In osmotic stress conditions, the L-glutamate pathway is considered the major source of proline (Delauney *et al*. 1993). Glutamate is converted into glutamate-5-semialdehyde (GSA) by enzyme delta1-pyroline-5-carboxylate synthase (P5CS). The P5CS by its activity semi aldehyde glutamic dehydrogenase (GSA-HGD) converts the GSA into pyrolline-5-carboxylate (P5C) which is subsequently reduced to proline by P5C reductase (P5CR). In normal conditions, production of proline is achieved by means of ornithine.

Citrus accumulate proline constitutively and preferentially in the leaves (Kato 1986; Maas 1993; Nolte *et al*. 1997). The increased production of proline has been observed in response to water stress (Laborem *et al*. 1995), salt stress (Anjum 2008; Gómez Cadenas *et al*. 1998; Mademba-Sy 2004; Piqueras *et al*. 1996) and cold stress (Purvis and Yelenosky 1983). According to (Mademba-Sy *et al*. 2003) there is a positive correlation between proline accumulation and the degree of salt sensitivity in citrus. The salt sensitive genotypes such as Etrog citron and Marumi kumquat accumulate more proline as compared to salt tolerant genotypes such as common mandarin, clementine and Shamouti orange. In citrus when grafted plants were subjected to salt stress, no difference was found between the level of proline accumulated in leaves of rootstock and scion (Purvis and Yelenosky 1983).

The *P5CS* gene expression is upregulated by salt stress (Liu and Zhu 1997; Ueda *et al*. 2004), dehydration (HU *et al*. 1992), treatment with exogenous ABA (Xin and Li 1993; Yoshiba *et al*. 1995), and cold (Chen and Li 2002). The overexpression of *P5CS* gene helps to maintain leaf osmotic potential, the root biomass, and confers tolerance to osmotic stress in tobacco (Kishor *et al*. 1995), and tolerance to salt and drought in rice (Zhu *et al*. 1998). In citrus, overexpression of the *P5CS* gene induces better tolerance to drought stress in Citrange carrizo (Molinari and Marur 2004). Recently it was indicated that *P5CS1* is required for proline accumulation under osmotic stress (Székely *et al*. 2008) by contrast, *P5CS2* mutations cause embryo abortion during late stages of seed development. Salt stress increases the expression of *P5CS2* and induces proline accumulation in cactus pear (Silva-Ortega *et al*. 2008)

2.4.3.2.2 Glycine betaine

The glycine betaine is an amino acid synthesized and used as a nutrient by the plant. Its properties allow it to interact with the hydrophobic and hydrophilic macromolecules e.g. enzymes. Under stress conditions, glycine betaine is considered to be the most efficient osmoprotectant, capable of improving water availability and protect the enzymes of growth and photosynthesis (Sakamoto and Murata 2002). The glycine betaine also protects the membranes from cold (Krall *et al*. 1989), high temperatures (Jolivet *et al*. 1982) salt (Jolivet *et al*. 1983) and also prevents the dissociation of extrinsic polypeptides of photosystem II (PSII) in the presence of high salt concentrations (Murata *et al*. 1992). The level of accumulation of glycine betaine correlates with the capacity of plant tolerance to salt stress (Saneoka *et al*. 1995). Citrus accumulates proline and glycine betaine under salt stress (Piqueras *et al*. 1996). Different species showed more tolerance to stress by overexpressing *COD* (the gene coding for choline oxidase in bacteria) e.g. salt stress tolerance in Arabidopsis (Hayashi *et al*. 1998) and in tobacco (Nuccio *et al*. 1998), cold tolerance (Alia *et al*. 1998a) and tolerance to high temperatures in Arabidopsis (Alia *et al*. 1998b). Recent findings indicate that simultaneous transformation of BADH and SeNHX1 genes into tobacco plants can enable plants to accumulate betaine and Na^+, thus conferring them more tolerance to salinity than either of the single gene transformed plants or wild-type tobacco plants (Zhou *et al*. 2008).

2.4.3.3 Genes that regulate plant growth in saline soil

Genes that could increase the growth rate of plants in saline soil would influence the rate of production of new leaves and roots (by controlling the rate of cell division; the development of new primordia for shoot or root branching; the rate of cell wall expansion; or the dimensions of differentiated cells), or they would influence the rate of photosynthesis (by controlling stomatal aperture; or the dimensions of mesophyll cells, which influences leaf morphology and transpiration efficiency) (Munns 2005). Candidate genes controlling growth are probably involved in signaling pathways that start with a sensor and involve hormones, transcription factors, protein kinases, protein phosphatases and other signaling molecules such as calmodulin binding proteins. The *SOS2* gene encodes a serine/threonine protein kinase with an N-terminal catalytic domain similar to that of yeast SNF1 kinase (Liu *et al*. 2000). A Ca^{+2} transient activate *SOS3* which in turn interacts with *SOS2* suppressing auto inhibition of the kinase activity. NaCl and ABA but not KCl induces the expression of *AtGSK1* and over expression of *AtGSK1* showed the increased salt tolerance in transgenic Arabidopsis (Piao *et al*. 2001).

3 Objectives

As the continuity of the work already initiated on salt stress tolerance in citrus by CIRAD at San giuliano and IVIA, the work proposed in thesis has following objectives.

1. To highlight the physiological mechanisms that may be specific to citrus genotypes representing a large diversity of citrus and may explain the different behaviors of sensitivity/tolerance to salt stress.

In citrus large intra and inter-specific genetic diversity is present but salt stress studies were mostly oriented to polyembryonic species because of their utilization as rootstocks and not much attention was given to monoembryonic species (Citron and Pummelo) which are responsible for large citrus diversity. On the basis of genetic diversity i may expect the expression of different physiological behaviors regarding toxic ions accumulation, central metabolic processes such as carbon utilization and oxidative stress damage. Also, i will observe different intra and inter-specific physiological behaviors within three genera *Citrus*, *Poncirus* and *fortunella* and therefore i may wonder how this diversity in physiological behaviors is related to genetic diversity. At the end, identification of diversifying mechanisms at the origin of tolerance among genotypes would be usefull to increase the genetic gain in breeding scheme for rootstocks and varieties improvement.

To fulfill my first objective, i have selected 22 different genotypes of citrus representing a large diversity of *Citrus* genera completed with one intergeneric hybrid Carrizo citrange (*C. sinensis* x *P. trifoliata*) and one representant of the *Poncirus* and *Fortunella* genera. At first place i aimed to characterize the physiological and molecular responses of a limited number of genotypes that represent the citrus diversity exposed to moderate salt stress (75mM) (article n°1). In a second step, i sought to re-explore the diversity among citrus based on the physiological response and molecular response towards the salt stress in all genotypes studied (article n°2).

2. To investigate the genetic determinant invloved in salt stress tolerance.

To dissect the genetic mechanisms responsible for tolerance to salt stress in citrus, i obtained a F2 progeny by self-pollination of F1 which is obtained by crossing between two different parents (Cleopatra mandarin (*Citrus reshni* Hort. ex Tan) and *Poncirus trifoliata* (L.) Raf.). Poncirus genotypes are very sensitive to salt stress and limestone soils but resistant to CTV virus and nematodes, while Cleopatra mandarin is tolerant to salt but susceptible to *Phytophthora* spp. From this F2 population a genetic map would be to be established which

will be helpful in future to analyze QTLs allowing identification of genes involved in salt stress tolerance.

The work carried out by CIRAD showed that tetraploid rootstocks presented an increased tolerance to drought and salt stresses in citrus. However, the impact of the use of tetraploid rootstocks on productivity and fruit quality has never been investigated in the citrus literature. Third objective of this thesis is:

3. To investigate despite of showing tolerance to salt stress, what are the impacts of autotetraploid rootstocks on fruit yield and quality when compared to the use of the respective diploid parental rootstocks? Also what is impact of diploid and autotetraploid rootstock on clementine scion physiological parameters?

The effect of diploid rootstocks on scion growth, fruit yield and quality is relatively well described but effect of autotetraploid rootstocks is not clear. Doubling of chromosomes may increase certain plant function because of nuclear dosage. Autotetraploid rootstocks give better tolerance to salt and water stress and therefore we may expect changes in fruit yield and fruit quality. So when autotetraploid rootstocks are used for clementine what are the changes occurring in fruit quality? What changes occurr in carotenoid content, organic acids and sugar content? These changes in fruit yield and fruit quality could be positive or negative or autotetraploid rootstocks have no effect on scion horticultural performance of clementine. Similarly what is the impact of autotetraploid rootstocks on clementine pysiology and scion/rootstock sink relationship?

To answer this question, i reanalyze a rootstock trial planted nearly 36 years ago. Production and fruit quality of clementine grafted on 36 genotypes of Trifoliate orange (*Poncirus trifoliata*) were reanalyzed. I initially sought to characterize the impact of the presence of zygotic and spontaneous autotetraploid genotypes in a citrus orchard on rootstock selection for clementine (article n°3). Similarly, i had to compare the clementine yields and quality (composition of sugars, acids, carotenoids, phenolic compounds etc. ...) when using two autotetraploid rootstocks genotypes of Poncirus and their respective diploid genotypes. To end, i analyzed the physiology (photosynthesis, stomatal conductance and leaf sugar and nitrogen content) of these two pairs of genotypes to check if it would be possible to explain the fruit yield and fruit quality obtained with specific functioning of the rootstock/scion association.

4 Plant materials and methods

4.1 Plant materials for salt stress experiment

We investigated the physiological and molecular responses of 22 different citrus genotypes (including rootstock and scion) representing the major citrus diversity under moderate salt stress. The list of genotypes used for salt stress tolerance experiment is presented in Table 1.

Table 1: List of genotypes used to study the diversity in different citrus genotypes under salt stress.

Common name	Tanaka system	ICVN[a] and SRA[b] no.
Diamante citron	*C. medica* L.	SRA540
Digite citron	*C. medica* L.	SRA 640
Etrog citron	*C. limonimedica* Lush.	SRA 709
Poncire commun citron	*C. medica* L.	SRA 601
Eingedi pummelo	*C. maxima* (Burm.) Merr	SRA 610
Kao Pan pummelo	*C. maxima* (Burm.) Merr	SRA 321
Timor pummelo	*C. maxima* (Burm.) Merr	SRA 707
Eureka lemon	*C. limon* (L.) Burm.	SRA 4
Lisbon lemon	*C. limon* (L.) Burm.	SRA 16
Brazilian sweet lime	*C. limettioïdes* Tan.	SRA 697
Mexican lime	*C. aurantifolia* (Christm.) Swing.	SRA 140
Rangpur lime	*C. limonia* Osb.	SRA 777
Cleopatra mandarin	*C. reshni* Hort. Ex Tan.	SRA 948
Sunki mandarin	*C. sunki* Hort. Ex Tan	ICVN0110076
Willow leaf mandarin	*C. deliciosa* Ten.	SRA 133
Duncan grapefruit	*C. paradisi* Macf.	SRA 470
Star Ruby grapefruit	*C. paradisi* Macf.	SRA 293
Carrizo citrange	*C. sinensis* x *Poncirus trifoliata*	SRA 796
Australian Sour orange	*Citrus aurantium* L.	SRA 851
Combava	*C. hystrix* D.C.	SRA630
Marumi kumquat	*Fortunella japonica* (Thunb.) Swing.	SRA 488
Pomeroy poncirus	*Poncirus trifoliata* (L.) Raf	ICVN 0110278

[a] International Citrus variety numbering.

[b] station de research agronomic

Plant materials were propagated in two ways: for polyembryonic genotypes, propagation was performed by sowing seeds in a neutral substrate (perlite), while for citron and pummelo that are monoembryonic genotypes, stem cutting was required to produce true-

to-type plants. Synthetic auxin (Rhizopon, 4% auxin) was used for root induction. Seedlings were transplanted three months after germination in 2.5 liters pots in a mixture of river sand and soil both from Corsica (classified as Cambisol with 15–25% clay, 34% loam and 42% sand) and peat (1 : 1 : 1) at a pH of 6.5 for 6 months in a greenhouse. Plants were irrigated thrice a week with half diluted nutritive solution (fertilizer 28-14-14, ref 205, Fertil, France).

4.2 Plant material: impacts of the presence of zygotic and autotetraploid rootstocks

Seeds of 36 trifoliate oranges have been introduced to the research station of San Giuliano in Corsica (France) from all over the world. From these 36 trifoliate orange rootstocks 32 rootstocks were used to study the impact of facultative apomixis and chromosome doubling in field trial (Table 2) and two autotetraploid and their corresponding two diploid trifoliate orange rootstocks were selected to study the impact of autotetraploid rootstocks on scion (Table 3). All nursery germination was performed in a greenhouse, with a cooling system. The temperature was about 25°C and the relative humidity at about 75%. Seedlings were transplanted three months after germination in individual plastic bags located in a greenhouse with an electrical airing system. The substrate used was composed with recent alluvium. Seedlings were manually irrigated based on the daily water balance. Before grafting, visual screening was done in order to have a homogenous population. Following parameters were used for screening: plant height, leaf thickness and homogenous aspect of the plants. After screening, plants were T-budded with virus-free clementine “Commune” (*Citrus reticulata* Blanco x *Citrus sinensis* (L.) Osb.) buds.

Table 2: List of the 32 trifoliate orange genotypes used to study the impact of facultative apomixis and chromosome doubling in field trial

#.	Common Name	Latin name	ICVN	Origin
1	Rubidoux	*Poncirus trifoliata* (L.) Raf.	110126	South Africa
2	Boufarik	*Poncirus trifoliata* (L.) Raf.	110507	Algeria
3	Menager	*Poncirus trifoliata* (L.) Raf.	110116	France
4	Rusk	*Poncirus trifoliata* (L.) Raf.	110437	USA (Texas)
5	Ferme blanche	*Poncirus trifoliata* (L.) Raf.	110435	Algeria
6	Jacobsen	*Poncirus trifoliata* (L.) Raf.	110107	USA(California)
7	English	*Poncirus trifoliata* (L.) Raf.	110097	South Africa
8	SEAB	*Poncirus trifoliata* (L.) Raf.	110438	Algeria
9	Beneke	*Poncirus trifoliata* (L.) Raf.	110436	USA
10	Pomeroy	*Poncirus trifoliata* (L.) Raf.	110081	USA (California)
11	Rusk	*Poncirus trifoliata* (L.) Raf.	110443	USA (Texas)
12	Christian	*Poncirus trifoliata* (L.) Raf.	110338	South Africa
13	Ferme blanche	*Poncirus trifoliata* (L.) Raf.	110087	Algeria
14	Kryder	*Poncirus trifoliata* (L.) Raf.	110446	USA (Florida)
15	Luisi	*Poncirus trifoliata* (L.) Raf.	110088	France
16	Rusk	*Poncirus trifoliata* (L.) Raf.	110082	USA (Texas)
17	Brazil	*Poncirus trifoliata* (L.) Raf.	110510	Brazil
18	Argentine	*Poncirus trifoliata* (L.) Raf.	110505	Argentine
19	Morocco	*Poncirus trifoliata* (L.) Raf.	110511	Morocco
20	Boufarik	*Poncirus trifoliata* (L.) Raf.	110506	Algeria
21	Morocco	*Poncirus trifoliata* (L.) Raf.		Morocco
22	Beneke	*Poncirus trifoliata* (L.) Raf.	110083	USA
23	Kryder	*Poncirus trifoliata* (L.) Raf.	110110	South Africa
24	Christian	*Poncirus trifoliata* (L.) Raf.	110084	South Africa
25	Boufarik	*Poncirus trifoliata* (L.) Raf.	110508	Algeria
26	Luisi	*Poncirus trifoliata* (L.) Raf.	110448	France
27	Christian	*Poncirus trifoliata* (L.) Raf.	110085	USA
28	Menager	*Poncirus trifoliata* (L.) Raf.	110449	France
29	Kryder	*Poncirus trifoliata* (L.) Raf.	110108	USA (Florida)
30	Christian	*Poncirus trifoliata* (L.) Raf.	110447	USA
31	Pomeroy	*Poncirus trifoliata* (L.) Raf.	110442	USA (California)
32	Morocco	*Poncirus trifoliata* (L.) Raf.	110512	Morocco

After two years, nine blocks each containing 36 trifoliate orange rootstocks was planted at a planting distance of 6 m x 4 m at the INRA-CIRAD Research Station in Corsica, France. The experimental plot was surrounded with one guard row. The plots were tilled (70 cm deep) three months before planting. From 36 trifoliate oranges two auto tetraploid and their corresponding diploid were selected to analysis the fruit yield and quality and impact on clementine physiology and scion/rootstocks relationship. (article n°4)

Table 3: Plant material used for evaluation of scion fruit yield and quality on tetraploid rootstocks.

Sr no.	Name	ICVN no.	Ploidy level	Origin
1	Poncirus SEAB	110086	2x	Algeria
2	Poncirus Boufarik	110509	4x	Algeria
3	Poncirus Rubidoux	110125	2x	USA (California)
4	Poncirus Rubidoux	110440	4x	USA (California)

4.2.1 Cultural practices

During the two first years after plantation, formation pruning, basin irrigation and a topsoil removing (15 cm) were performed. From the third year, trees were hand pruned for fruiting, the weeds were controlled with chemicals, and the plants were irrigated by sprinkler irrigation, the irrigation frequency was adjusted according to the weekly water balance. Fertilizers (N and P) were applied, 40% of N was applied in early spring and 60% in early summer. The soil used in this trial is classified as fersiallitic with 15-25% clay, 34% loam and 42% sand. The soil pH was 6. San Giuliano is located at latitude 42°05N, 9°25E with a Mediterranean climate. The Clementine grove was only sprayed with oil (4,000 l/ha at 2%) just after pruning and at the end of the summer (against scales). No spraying against Phytophthora, and no auxins were used. No fruit degreening was performed. Standard agronomical measurements were taken on each tree.

4.3 Plant materials: Gene mapping and positioning of candidate gene for salt tolerance

For the study of co-location of candidate genes with QTLs for salt stress tolerance, an intergeneric F2 population was established from genotypes with the more distinct behavior under salt stress: Cleopatra mandarin and *Poncirus trifoliata*. F2 population was generated by self-pollination of an F1 hybrid of (*Citrus reshni* Hort. Ex Tan x *Poncirus trifoliata*). The seeds resulting from the F2 generation were sorted compared to their morphology in order to isolate monoembryonic seeds. Plants were propagated by sowing seeds in a neutral substrate (perlite).

4.4 General methods

4.4.1 DNA extraction and amplification

In all the different experiments, same DNA extraction method was used. Total DNA was extracted on 0.5 cm^2 of leaf of each plant seedlings according to Doyle and Doyle (1987) and as adapted to citrus by Cabasson *et al*. (2001). The leaf material was crushed in the presence of liquid nitrogen and mixed with 750 µl of extraction buffer [2% (w/v) MATAB, 1.4 M NaCl, 0.2 M Tris-HCl pH = 8, 50 mM EDTA, 1% (w/v) PVP, 0.5 % sodium bisulphate (w/v)]. Then it was transferred into a 2 ml eppendorf tube and incubated for 30 min at 65°C. After 30 min, 750 µl of chloroform / isoamyl alcohol (24 / 1, v v) were added to the tube and the mixture was subjected to mild agitation (orbital shaker) for 5-10 min. After centrifugation for 5 min at 8000 g, the aqueous phase was transferred to a new eppendorf tube and 500µl of isopropanol was added to precipitate DNA. After centrifugation for 10 min at maximum speed, the supernatant was removed and DNA pellet was washed with 70% ethyl alcohol and dried with vacuum drier. At the end 100 µl of sterile distilled water was added and kept at -80°C.

The DNA was amplified by Polymerase Chain Reaction (PCR) using primers. The reaction mixture for one sample contained DNA 2 ng/µl 5µl; Tampon 10X 1.5 µl; $MgCl_2$ 25 mM 0.9 µl; dNTP 25 mM 0.12 µl; forward primer 25 µM0.12 µl; reverse primer 25 µM 0.12 µl; Taq polymerase 3u/µl 0.3 µl and made the final volume at 15µl. PCR performed in a programmed thermocycler PTC-200, consisted of an initial DNA predenaturation cycle at 94°C for 5 min. and 50 cycles for following stages: 94°C for 30 sec for separation of DNA strands, 50° or 55°C depending upon primers for 1 min. for annealing, 72°C for 45 sec for polymerization, and a final extension cycle at 72°C for 4 min. when the program for the amplification of DNA samples was completed, these were stored at 4°C.

4.4.2 Polyacrylamide gel electrophoresis

The separation of PCR products were done by means of vertical denaturalized polyacrylamide gel electrophoresis (5%, urea 7 M). The gel was placed in a tank containing **Tris/Borate/EDTA** (TBE) buffer 0.5X (5X TBE: 54 g Tris base, 27.5 g of boric acid, 20 ml of 0.5 M EDTA pH 8, distilled water qs 1l) for a pre-migration of 30 min under a constant power of 60W. The denatured DNA samples (5 min at 94°C) were loaded on the gel. Migration was relaunched under the same conditions as the pre-migration for certain period of

time depends on the size of expected fragments. The revelation of DNA fragments was done with silver nitrate staining as described by Beidler *et al.* (1982). The DNA was fixed in alcohol (10%) for 30 min and then bath in nitric acid (1%) for 5 min, the gel was then washed in two successive baths of ultrapure water (18 MΩ) for 5 min. Staining was performed in a bath of silver nitrate 0.2% (w/v) for 30 min and then DNA fragments was revealed by two successive baths with a solution of sodium carbonate ($NaCO_3$ 30g/l + 600 µl formaldehyde). Revelation was stopped by a bath of 5% acetic acid for 1-2 min and then gel was washed with deionized water.

4.4.3 Ploidy status

Ploidy status of all plants used in our experiments was determined by flow cytometry using leaf samples according to Froelicher *et al*. (2007). Approximately 0.5 cm^2 of leaf was chopped in 250 µl of nuclei extraction buffer (Partec Cystain UV Precise P). The solution obtained was filtered (pore size of 30 µm) to eliminate cell debris. After filtration, 400 µl of staining buffer (Partec, Cystain UV Precise P Staining Buffer) was added for DNA staining. The samples were incubated for 5 min and analyzed by flow cytometer PA1 (PARTEC, Germany). The nuclear DNA contents were evaluated according to Seker *et al*. (2003). A tetraploid citrus plant was used as internal control. Only true to type plants were selected for further measurements.

4.5 Methods for Salt stress experiment

4.5.1 Verification of genetic status of plants

To verify genetic material propagated from seed, total DNA was extracted as described above and genetic constitution of the seedlings was analyzed using four Inter-Simple Sequence Repeat (ISSR) primers: $HVH(CA)_7T$, $DBDA(CA)_7$, $BDB(CA)_7C$, $HVH(TCC)_5$ (Fang and Roose 1997)(Table 4). The conditions and separation of PCR products were done by means of vertical denaturalized electrophoresis polyacrylamide as described earlier.

Table 4: ISSR primers used for the identification of the zygotic genotypes among the different *Citrus* genotypes

Name	Sequence	Hybridation T°C	Fragment length (bp)
ISSR 1	$HVH(CA)_7T$	55.5	300-1100
ISSR 4	$DBDA(CA)_7$	55	200-1500
ISSR 5	$BDB(CA)_7C$	58	200-1000
ISSR 8	$HVH(TCC)_5$	55.5	450-1400

4.5.2 Salt treatments

After the period of acclimation in greenhouse, plants were divided as a non-saline control and salt treated. Six plants per genotypes according to availability were used for salt treatment and three plants were used as non-saline control. The conditions of salt stress were applied during 12 weeks (from mid-May to 10th of August). Salt-treated plants were watered three times a week with the nutrient solution plus 75 mM NaCl. The frequency of watering controls plants was identical to that of stressed plants. Growing conditions were 18°C min 38°C max and a relative humidity of 55% on average.

4.5.3 Plant diameter and leaf symptoms

Plant diameter was measured at the start and at the end of experiment for each non-saline control and salt treated plant. Plant diameter was measured with the help of vernier calliper in mm. The diameter increase percentage was calculated after 80 days of salt stress for non-saline and salt treated plants. Leaf symptoms were observed throughout the experiments representing different physiological disorders: leaf necrosis, stem necrosis, leaf pointed depigmentation and green leaf fall down.

4.5.4 Physiological measurements

4.5.4.1 Maximum quantum yield of PSII

4.5.4.1.1 Principle

The light energy absorbed from the chlorophyll molecules at the reaction centers: (1) can be used to drive photosynthesis through the photosynthetic electron chain, (2) the energy can be dissipated as heat or, (3) the energy can be re-emitted as red light which is chlorophyll fluorescence. Since these three processes occur in competition, the measurement of

chlorophyll fluorescence yield gives information on the potential energy used in photochemistry or other dissipative processes (Epitalawage *et al.* 2003; Maxwell and Johnson 2000). Therefore, any increase in the efficiency of one component will diminish the other two.

4.5.4.1.2 Maximum quantum yield of PSII (Fv / Fm)

The maximum quantum yield of PSII (Fv/Fm) reflects the quantum efficiency of PSII open centers. The maximum state of excitability of PSII was measured after a resting phase, i.e. after placing the leaves in the dark for at least 30 minutes. Indeed, the dark phase allows PSII to fully charge in electron, and estimate the maximum quantum yield at issuance by the saturating flash meter. This type of measure provides an estimate of the actual state of PSII, because any reduction in the maximum quantum efficiency is irreversible and corresponds to a degradation of electron acceptor centers (Maxwell and Johnson 2000).

Fv / Fm = (Fm-F0) / Fm

Fm: maximal fluorescence yield

F0: minimum fluorescence yield

Maximum quantum yield of PSII (Fv/Fm) was measured at night with portable fluorometer (Hansatech Ltd., Kings Lynn. UK)

4.5.4.2 Chlorophyll and flavonoid content measurements

The chlorophyll content was measured using the SPAD-502 Chlorophyll Meter (Minolta, Japan) on mature leaves. Based on photoreceptor properties of chlorophyll, its quantity in the leaves was determined by measuring the absorbance at two wavelength ranges: blue (400-500nm) and red (600-700nm). The leaf phenolic content, mainly represented by flavonoids, was measured using an optical sensor Dualex3.3 ® start-up (Force-A ©).

4.5.4.3 Gas exchange measurements

Net photosynthetic rate (*A*) and stomatal conductance (gs) were determined with portable gas exchange fluorescence system (GFS-3000) (Heinz Walz GmBH, Germany with photosynthetically active radiation photon flux of 1000 µmol m^{-2} s^{-1}. Measurements were performed in the morning (8 to 11 AM) to avoid high external temperature and low humidity. Leaf temperature was 28 ± 2°C, leaf to air vapour pressure difference was 2.4 ± 0.4 kPa, and ambient CO_2 concentration was 370 ± 3 µmol mol^{-1} within the cuvette of the portable gas exchange fluoresence system.

4.5.4.4 Ionic strength

4.5.4.4.1 Sample preparation

The leaves were dried in an oven at 60°C for one week and ground using a ball mill (Retsch). 50 mg of powdered plant (leaves) was burnt for 4 hours at 400°C. The ash was dissolved in 100 ml of 0.5N nitric acid (HNO_3). Half of this solution was used to estimate the concentration of chloride, while the other half was used to assess the concentration of sodium (Mouhaya *et al*. 2010).

4.5.4.4.2 Measurement of chloride and sodium

The potential difference of each sample prepared was assessed using a specific chloride electrode (Thermo Orion). Chloride concentration was estimated by postponing the potential difference measured on the standard curve previously established from a standard range of NaCl. The dosage of sodium was performed by ICP-MS "Inductively Coupled Plasma-Mass Spectrometer at the U.R. 49 (CIRAD-PERSYST, Montpellier).

4.6 Methods related with gene mapping

4.6.1 Genotyping and scoring

The SSR markers (400 MEST, 79 BAC end and 100 genomics) were used for initial screening of both parents and their F1 hybrid. 444 markers either failed to amplify any DNA bands, or produced no heterozygosity for F1 hybrid. These primers were not used for further analysis. The rest of the markers (Total 135: 64 MEST, 50 BAC end and 21 genomics) produced consistent and obvious polymorphic DNA fragments, and were subsequently used for screening the genomic DNA of the 99 hybrids from the progeny population. For alleles existing in Cleopatra mandarin were named with “a” and Poncirus trifoliate were named with “b” and hybrid was named “h”. So-called generic genotypes were categorized by JoinMap as required for use in its locus data format.

4.6.2 Linkage analysis

JoinMap 3.0 (Stam and Van Ooijen 1995) was used for linkage analysis and comparison. Two heterozygous SSR alleles from either parent were expected to segregate at 1:2:1 ratio in all F2 progeny, and all loci were tested by $\chi 2$ ($\alpha = 0.05$) to detect distorted segregation ratios. A logarithm of the odds to the base 10 (LOD) score of 4.0, with 0.45 as the

maximum θ value, was used as tentative linkage threshold for JoinMap to group markers. Framework maps were established using a subset of evenly distributed loci ordered with the best likelihood. Map distances in centiMorgans (cM) were calculated using Kosambi's mapping function. All linkage groups were taken from JoinMap. The linkage maps were drawn using the MapChart Program (Voorrips 2002). The map length of the genome was estimated using only pairwise comparisons of all the framework loci that were anchored and had high LOD scores (Hulbert *et al.* 1988).

4.7 Methods for impact of zygotic and autotetraploid rootstocks

4.7.1 Fruit yield

Analysis of fruit production was done on already collected data for 11 years. From November to the end of December, the crop of each tree from each block was harvested and weighed. Measurements of annual yield were taken from 1979 to 1990. The harvest was performed in one or two times (usually after an interval of one or two weeks) according to fruit coloring.

4.7.2 Fruit quality analysis

Fruit quality analysis of clementine grown on the 32 different trifoliate orange genotypes was investigated for 5 consecutive years 1981 to 1985. In contrast to crop yield, for fruit quality measurements only four blocks among nine were selected and from each block 20 fruits per genotype, which had the same colour and the same medium diameter, were weighed and hand pressed. The juice retrieved was weighed to calculate the juice percentage (JP) and juice density.

Total soluble solids (TSS in °Brix) were measured using an ATC-1E ATAGO hand-held refractometer on the translucent part of the juice after decantation. 5 ml of the same translucent part were weighed to assess titratable acidity (TA) according to the AOAC method (NaOH, 0.1N and end pH=8.1) using a DL25 Mettler titrator. Results were expressed in g of anhydrous citric acid/100 g of juice. Incidence of maturity were calculated by ratio between TSS:TA.

4.7.3 Identification of tetraploid and zygotic seedlings

In 2008, ploidy status of trees of this field essay was determined by flow cytometry using bark samples or leaf samples from rootstock sprouts according to Froelicher *et al.* (2007) and DNA was also extracted from bark or leaves as described above.

4.7.4 Rootstocks and scion circumference and gas exchange measurements

Rootstocks and scion circumference was measured using a measuring tape in order to estimated the average scion/rootstock circumference ratio for the first eight years of the trial. Gas exchange measurements were performed during the summer season in 2009 only on fully expanded leaves of similar age, selected within branches homogeneous in terms of light exposition (oriented south-east and of a height of 1.8 meter). We make therefore state confidently that there was no bias in all our gas exchange measurements due to leaf age or light exposition on photosynthetic capacity. We moreover supplied water on an evapo-transpiration replacement basis to ensure that there were no limitations to stomatal conductance due to water availability. At least five leaves were selected within each tree per genotype (3 trees). Stomatal conductance (g_s) and the net photosynthetic rate (A) were determined using a portable gas exchange/chlorophyll fluorescence measurement system (GFS-3000) (Heinz Walz GmBH, Germany). The system was equipped with the LED-Array/PAM-Fluorometer 3055-FL which was used to impose a photosynthetically active radiation flux (Q) of 1000 µmol photons m^{-2} s^{-1} in all our measurement. This value is well-above the known threshold for light saturation for Citrus. All measurements were performed during the morning (08:00-11:00 AM) to prevent down regulation of photosynthesis by the accumulation of photosynthetates in leaves. During all measurements, leaf temperature was 30 ± 2°C, leaf vapour pressure deficit was 2.4 ± 0.4 kPa, and ambient CO_2 concentration was maintained at 370 ± 3 µmol CO_2 mol^{-1} air within the cuvette. In addition, we estimated the light-saturated rate of electron transport J_{max} (µmol electrons m^{-2} s^{-1}), as an indicator of photosynthetic capacity, following the method of (Urban *et al.et al.et al.et al.* 2004)

4.7.5 Chlorophyll content and Flavonoid content measurement

At least 30 leaves per tree (3 trees) were selected to measure chlorophyll content and flavonoid content. The leaf chlorophyll content was measured with the help of SPAD meter

(Minolta SPAD-502, Japan). The leaf phenolic contents, mainly represented by flavonoid, measured with the optical sensor Dualex3.3 ® start-up (Force-A ©).

4.7.6 Leaf nitrogen and non structural carbohydrates

Leaf nitrogen content per unit of mass (N_m) was determined with an elemental analyzer (Carlo Erba Instruments, Milan, Italy) following the method of Colombo *et al*. (1988). Glucose, fructose and sucrose in the leaves were determined with an enzyme based analyzer (YSI 2007, Yellow Springs Instruments, Yellow springs, OH). Starch was determined by enzymatic hydrolysis to glucose (Thivend *et al*. 1972). Dry mass was assessed by freeze-drying. The masses of starch and soluble sugars were deducted from the dry mass to obtain the structural dry mass from which mass-to-area ratio (M_a) and nitrogen content per area (N_a) ($N_a = M_a N_m$) were calculated.

4.7.7 Analysis of organic acids and sugars

In 2008, more elaborated fruit quality analysis was performed for the 4 genotypes. From each 2x and its corresponding 4x trifoliate orange rootstock, 15 fruits of same size and colour were collected. Fruits were peeled and their pulp was frozen in liquid nitrogen and stored at 20°C for further analysis. One hundred milligrams of lyophilized powdered pulp were dissolved in 5 ml of bidistilled water and centrifuged at 3000 g for 10 min. Supernatant was filtered through 25 mm syringe filters, 0.2 µm cellulose acetate membranes (VWR). Organic acids were analyzed using an analytical HPLC unit (Perkin-Elmer, Series 200, France) as previously described by Albertini *et al*. (2006). Sugar content was determined according to Gomez *et al*. (2002). Data were obtained using TotalChrom™ software for Windows version 6.2 (Perkin-Elmer Instruments, Shelton, U.S.A.). Concentrations of organic acids and sugars were expressed in mg g^{-1} dry weight.

4.7.8 Extraction and quantification of carotenoid content

From each kind of rootstock/scion association, 20 fruits of same size and colour were collected the same day according to Dhuique-Mayer *et al.et al.et al.et al.* (2005). Briefly, fruits samples were immediately squeezed and filtered through stainless steel sieves. Fruit juice was distributed in 30 ml amber vials under nitrogen gas. Three samples were prepared from each tree. Carotenoids were analyzed by HPLC using an Agilent 1100 system (Massy,

France). Carotenoids were extracted, identified and quantified (Dhuique-Mayer *et al*. 2005; Taungbodhitham *et al*. 1998). An aliquot (20 g) of orange juice was homogenized by magnetic stirrer with 120 mg of $MgCO_3$ and 35 ml of extraction solvent (ethanol/hexane, 4:3 v/v, containing 0.1% of BHT as antioxidant) for 5 min. Lycopene (750 µl of solution, equivalent to 90 µg) was added as an internal standard. The residue was separated from the liquid phase by filtration with a filter funnel (porosity no. 2) and re-extracted with 35 ml of ethanol/hexane as described previously. Ethyl alcohol (30 ml) and hexane (30 ml) were successively used to wash the residue. Organic phases were transferred to a separatory funnel and washed with 2 × 50 ml of 10% sodium chloride and 3 × 50 ml of distilled water. The aqueous layer was removed. The hexanic phase was dried using anhydrous sodium sulfate and filtered before evaporation to dryness under vacuum at 40°C. Carotenoid extracts were dissolved in 500 µl of dichloromethane and 500 µl of an 80:20 (v/v) mixture of MTBE and methanol. This solution was diluted 6-fold in the TBE/methanol mixture and stored in amber vials before HPLC analysis.

4.7.8.1 Saponification.

The hexanic extract was evaporated to dryness with a rotary evaporator, redissolved with 20 mL of hexane, and placed in a 50 ml amber vial to which was added 20 mlL of 10% methanolic KOH. Saponification was carried out overnight in the dark at room temperature. The sample was shaken under nitrogen in the sealed vial. The sample was transferred to a separatory funnel to which 50 ml of distilled water was added to separate the layers. The hexanic layer was rinsed until free of alkali. The methanolic KOH layer was extracted with 3 × 15 ml of dichloromethane. The extracts were pooled and washed to remove alkali. Aqueous traces from organic extracts were removed with anhydrous sodium sulfate; then the extracts were filtered and evaporated to dryness under vacuum. Carotenoid extracts were dissolved as described above. Analyses were conducted under red light to avoid carotenoid degradation during extraction and saponification. The coefficient of variation for extraction-saponification was <5%. The concentration of each carotenoid was expressed as mg l^{-1}.

4.7.9 Extraction and Quantification of hesperidine

Several different flavanone glycosides are present in citrus but i only quantify hesperidin content. The same juice samples as used for carotenoid analysis were used for hesperidin quantifications using the extraction procedure described by Mouly *et al.et al.et*

al.et al. (1997) through Agilent 1100 model HPLC analytical system (Massy, France). Separation of flavanones was performed by HPLC using an RP 18e Licrospher 100 (5 μm) column (250 mm × 4.6 mm i.d.) (Merck KgaA). The isocratic solvent system was water / acetonitrile / THF / acetic acid (80:16:3:1, v/v/v/v). Separation was performed at room temperature with a flow rate of 1 mL min^{-1} and quantification was carried out at 280 nm. Spectral data were obtained with a photodiode array detector. All data were analysed using Agilent Chemstation and related software. Hespridine concentrations were determined using an external calibration method. Hesperidin (HES) standards were diluted in DMF/water (2:1, v/v) to give 102 mg l^{-1}.

5 Results and discussion

5.1 Article No 1: Large changes of behaviors for salt stress tolerance exist among genotypes representing major citrus diversity but salt stress tolerance is always associated to low chloride accumulation

Sajjad Hussain[1,2], François Luro[1], Gille Costantino[1], Patrick Ollitrault[3], Raphaël Morillon[3*]

1. Unité de Recherche Génétique et Ecophysiologie de la Qualité des Agrumes, Inra. Centre Inra de Corse, F-20230 San Giuliano, France

2. Unité Propre de Recherche amélioration génétique des espèces à multiplication végétative, Cirad. Centre Inra de Corse, F-20230 San Giuliano, France

3. UPR amélioration génétique des espèces à multiplication végétative du Cirad. Instituto Valenciano de Investigaciones Agrarias 46113 - Moncada – Valencia, Spain.

* **Corresponding author:** Raphaël Morillon, UPR amélioration génétique des espèces à multiplication végétative du Cirad. Instituto Valenciano de Investigaciones Agrarias 46113 - Moncada – Valencia, Spain.

Key words: citrus, salinity tolerance, genetic diversity, chloride content, rootstock, Scion, photosynthesis.

Abstract

In this study 12 citrus genotypes including rootstocks and scion varieties Poncire commun citron (*C. medica*), Engedi pummelo (*C. maxima* (Burm.) Merr), Eureka lemon (*C. limon* (L.) Osb.), Mexican lime (*C. aurantifolia*), Cleopatra mandarin (*C. reshni* Hort. Ex Tan.), Sunki mandarin (*C. sunki* Hort. Ex Tan), Star Ruby grapefruit (*C. paradisi*), Carrizo citrange (*C. sinensis* X *Poncirus trifoliata*), Pomeroy poncirus (*Poncirus trifoliata* (L.) Raf), Australian Sour orange (*Citrus aurantium* (L.)), Combava (*C. hystrix* D.C.) and Marumi kumquat (*Fortunella japonica (Thunb.)* Swing) representing the major citrus diversity were subjected to moderate salt stress (75mM NaCl) to determine different physiological behavior. Plant material was propagated through seed for polyembryonic genotypes while monoembryonic genotypes were propagated through stem cuttings. Plant growth (shoot diameter), leaf chlorophyll content, leaf flavonoid content, maximum quantum yield of PSII, net photosynthesis, stomatal conductance and leaf Na^+ and Cl^- contents were measured during the 12 weeks of salt stress. A large diversity of Cl^- accumulation profiles in leaves was observed among genotypes. Poncire commun citron accumulated the highest Cl^- content while mandarin species accumulated lowest Cl^- content while *Fortunella*, lime, trifoliate orange, lemon and pummelo genotypes were in between of both extremes. In the meantime all the genotypes presented a Na^+ accumulation more or less similar than of Cl^-. Marumi kumquat was severely affected after 60 days of salt stress and accumulated high toxic ions and its PSII seemed to be severely damaged too. Engedi pummelo presented good salt tolerance by accumulating less toxic ions while maintaining high values of maximum quantum yield of PSII and very low gas exchanges. The behavior of pummelo probably inherited is a new form of original tolerance first observed in citrus. Mandarin and Pummelo species as well as Sour orange were the most tolerant species with the lowest toxic ion accumulations. On the contrary trifoliate orange and its citrange hybrid showed clear symptoms of salt stress that were associated to a maintained growth and high gas exchanges while they accumulated high toxic ion concentrations. The most salt stress sensitive genotypes accumulated high Na^+ and Cl^- concentrations and maintained fair growth and photosynthesis rate while salt tolerant genotypes accumulated less Na^+ and Cl^- contents but growth and gas exchange parameters were decreased too.

Introduction

Salinity is one of the major abiotic constraint that affects agriculture especially in countries where irrigation is required (Flowers 2004). The adverse effects of salinity on plant growth and development are associated with low osmotic potential, accumulation of ions to toxic level, and nutritional imbalance (Ashraf and Harris 2004; Byrt and Munns 2008). This usually leads to reduction in growth and fruit yields (Levy *et al*. 1979; Maas 1993; Storey and Walker 1998). The primary effect of high salt concentration to plants is stomatal closure which results in low transpiration rate and reduces CO_2 availability for photosynthesis while a limited mesophyll diffusion of CO_2 is observed (Flexas *et al*., 2007). In order to cope with primary effect of salinity, plants regulate their osmotic potential and compartment toxic ions. The regulation of osmotic potential that contributes to maintain the turgor pressure (despite of lower water potential) involves several processes such as the uptake of K^+, compartmentalization of Na^+ and Cl^- into the vacuole and synthesis of compatible solutes such as proline, glycinebetaine, polyol, sugar etc. (Ashraf 1994). At the molecular level, mechanisms involved in sodium compartmentation out of the cytoplasm are well documented in *Arabidopsis thaliana* and many genes coding for Na^+ co-transporters and regulators (e.g. *NHX1*, *SOS1*, *SOS2* and *SOS3*) have been characterized (Apse *et al.* 1999; Guo *et al.* 2004; Shi *et al.* 2000). However, chloride uptake and associated transporters are still poorly documented. Colmenero Flores *et al.* (2007) identified a chloride transporter that is presumably involved in long-distance transport and plant development process. A secondary effect of toxic ions of a salt stress but also other stresses such as drought (Chaves and Oliveira 2004) and temperature, is the triggering of oxidative stress processing leading to damages of the leaf photosynthetic machinery (Allen and Ort 2001).

Like many others crops, citrus species are classified as salt-sensitive (Maas 1993). Citrus include three major genera which are sexually compatible, *Citrus*, *Poncirus* and *Fortunella*. Most of the rootstocks used nowadays belong to the *Citrus* and *Poncirus* genera, or are hybrids obtained between these two related genera (Barrett 1985). However, the tolerance of citrus to salt stress has been investigated only through the characterization of the performance of using rootstock seedlings and grafted plants (Storey and Walker 1998). The adverse effects of salinity on commonly used genotypes have been extensively reported in the literature (Anjum 2008; Atmane *et al.et al.et al.et al.* 2003; Garcia-Sanchez and Syvertsen 2009; Lopez-Climent *et al*. 2008; Saleh *et al*. 2008) including symptoms of leaf injury, growth suppression and yield decline. Main effects of salinity in citrus are decreased stomatal

conductance which leads to reduce CO_2 diffusion and ultimately decrease net photosynthesis (García-Sánchez and Syvertsen 2006) and increase ion accumulation (Brumós *et al*. 2010). However, a large diversity of response within citrus towards salt stress exists. Rangpur lime (*Citrus limonia* Osbeck), Sunki mandarin (*Citrus sunki* Hort. ex Tan.) and Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan) are considered to be tolerant, while Trifoliate orange (*Poncirus trifoliata* (L.) Raf) and its hybrids such as Carrizo citrange (*Citrus sinensis* [L.] Osbeck × *Poncirus trifoliata* [L.] Raf.) are known to be salt sensitive (Maas 1993). Citrus damages caused by salinity are usually associated with chloride accumulation but not with sodium (Banuls *et al*. 1997; Moya *et al*. 2003). Trifoliate orange, and its hybrids are considered to be poor Cl^- excluder (Cooper 1961; Peynado and Young 1969) but have a great capacity to exclude sodium at low salinity level (Walker 1986). Citrus rootstock varieties such as Cleopatra mandarin and Rangpur lime are known to be chloride excluders, which explain why they are salt-tolerant rootstocks (Cooper *et al*. 1952; Zekri and Parsons 1992). Walker and Douglas (1983) observed significant differences between Rangpur lime, Kharna khatta (*Citrus karna*), and Etrog citron (*Citrus medica*) for Cl^- accumulation in the leaves but little difference was observed for Na^+ accumulation. According to Moya *et al.* (2003) if chloride absorption is not limited at the root level, those ions will be translocated to the leaves throughout the transpiration stream, where they will cause necrosis and even defoliation.

In Citrus, studies of salt tolerance are mostly confined to only seedlings, or rootstocks associated with sensitive scion varieties. In the literature it has been reported that both rootstocks and scion may influence Cl^- accumulation in leaves (Banuls *et al*. 1990; Garcia-Legaz *et al.* 1993; Nieves *et al.* 1991). Scion effects may be apparent when rootstocks are poor excluders (Lloyd *et al*. 1989). Although extensive analysis of the different responses of the currently used seedlings to salt stress are available at physiological and molecular level (Brumós *et al.* 2009), the global evaluation of rootstock seedlings as well as scion genotypes seedlings including a broad citrus diversity subjected to a same salt stress condition has never been studied. Hence, we then investigated the physiological and molecular responses of 12 different citrus genotypes representing the major citrus diversity under moderate salt stress condition.

Materials and methods

This study was carried out at the INRA-CIRAD station of Corsica, France. We have selected 12 different genotypes of citrus representative of the major diversity of citrus *genera* (Table 1). Plant materials were propagated in two ways: for polyembryonic genotypes, propagation was performed by sowing seeds in a neutral substrate (perlite), while for C. *medica* and *C. maxima* that are monoembryonic genotypes; stem cutting was required to produce true-to-type plants. Rhizopon (auxin 4%) was used for root induction. Seedlings and rooted cuttings were transplanted three months after germination in 2.5 liters pots in a mixture of river sand and soil both from Corsica (classified as Cambisol with 15–25% clay, 34% loam and 42% sand) and peat (1 : 1 : 1) at a pH of 6.5 for 6 months in a greenhouse. Plants were irrigated thrice a week with half diluted nutritive solution (fertilizer 28-14-14, ref 205, Fertil, France).

Verification of genetic status of plants

The genetic constitution of the seedlings was analyzed using four Inter-simple sequence repeat (ISSR) primers: $HVH(CA)_7T$, DBDA(CA)7, $BDB(CA)_7C$, $HVH(TCC)_5$ (Fang and Roose 1997) (Table 2). PCR products were analyzed using vertical denatured electrophoresis polyacrylamide gels (5% polyacrylamide, urea 7 M), or 1.5% agarose gel in buffer TBE 0.5X (Tris, acid boric 45mM and EDTA 0.5 M, pH 8) and silver stained according to (Beidler *et al*. 1982). Ploidy status of plants was determined by flow cytometry using leaf samples according to Froelicher *et al*. (2007).

Treatments

After the period of acclimation in greenhouse, plants were assigned at random into two blocks. Three to six plants per genotypes according to availability were assigned for salt treatment and three plants were assigned as non-saline control. The conditions of salt stress were applied from mid-May to 4th of August 2009 under natural photoperiod conditions, with a temperature regime of minimum 18°C and maximum 38°C, and the relative humidity kept between 50 and 60 %. Salt-treated plants were watered three times a week at the same hour with the nutrient solution supplemented with 75mM NaCl whereas control plants were irrigated only with the nutrient solution. Two leaves per plant at same height and age were tagged randomly for each different parameter.

Plant diameter and leaf symptoms

Plant diameter was measured at the start and at the end of the experiment. Plant diameter was measured using a vernier calliper in (mm). The diameter increase percentage

was calculated after 80 days for control and salt treated plants. Leaf symptoms were observed throughout the experiment and represented different physiological disorders: leaf necrosis, stem necrosis, leaf pointed depigmentation, green leaf fall down.

Gas exchange measurements

Net photosynthetic rate (*A*) and stomatal conductance (*gs*) were determined with portable gas exchange fluorescence system (GFS-3000) (Heinz Walz GmBH, Germany with photosynthetically active radiation photon flux of 1000 µmol m^{-2} s^{-1}. Measurements were performed in the morning (8 to 11 AM) to avoid high external temperature and low humidity. Leaf temperature was 28 ± 2°C, leaf to air vapour pressure difference was 2.4 ± 0.4 kPa, and ambient CO_2 concentration was 370 ± 3 µmol mol^{-1} within the cuvette of the portable gas exchange fluorescence system.

Chlorophyll fluorescence, Leaf greenness content and flavonoid content measurements

Chlorophyll fluorescence, leaf greenness and flavonoid content were measured on two leaves for each plant each week. Chlorophyll fluorescence was measured at night using a portable fluorometer (Hansatech Ltd., Kings Lynn. UK). Leaf greenness was measured using a SPAD meter (Minolta SPAD-502, Japan). Leaf phenolic compounds, mainly represented by flavonoids, were measured using an optical sensor Dualex3.3 ® start-up (Force-A ©).

Mineral analysis: sodium and chloride content

Control samples (one to three leaves depending on availability) and samples for salt treated plants (at least 3 replication) were collected after 10, 30, 50, 60 and 80 days of salt stress. Sodium and chloride analyses were carried out according to Saleh *et al*. (2008) and Mouhaya *et al*. (2010). Leaves, sodium and chloride contents were expressed in mg g^{-1} of dry weight.

Statistical analysis

We used SPSS software (Chicago; www.spss.com_software_science) to analyze the physiological data. Analysis of variance (ANOVA) test was used to detect the difference between the genotypes at the usual probability level P=0.05. SIGMAPLOT was used to create the graphs.

Results

Genetic conformity and plant ploidy status

Among the four ISSR markers tested, only the ISSR1 marker presented polymorphism among all studied genotypes, enabling to verify the genetic origin for all plants. All seedlings presented an identical genetic profile to those of their respective mother tree. Also, a selection based on the morphology of plants was made to remove a few plants presenting specific morphological characters. These plants were possibly either mutants or hybrids that were not detected by genetic analysis but the last hypothesis is rather improbable. Analysis of seedlings using flow cytometry did not reveal any spontaneous autotetraploid plant.

Plant growth and leaf symptoms

Investigation of the percentage of increase in plant diameter showed that control plants presented as expected a higher increase of diameter compared to the 80 days stressed plants (Fig. 1). In control condition, the highest increase in plant diameter was observed for Pomeroy poncirus (103.3 % ± 5.6) and comabava presented the lowest increase (36.0 % ± 10.3). For salt treated plants Pomeroy poncirus presented the greatest increase (58.9 % ± 3.3) while Marumi kumquat had the smallest increase in plant diameter (6.3 % ± 2.5) (Fig. 1).

Apparitions of leaf damage symptoms along the salt stress experiment were noted. Three types of leaf symptoms were observed: necrosis, yellowing and leaf fall down. The most affected genotypes were Poncire commun citron and Carrizo citrange, since symptoms of necrosis occurred earlier than for the rest of the genotypes. Leaf necrosis started from the borders of the leaves and then spread to the central vein. In Carrizo citrange symptoms first appeared in the lower leaves and then progressively spread to upper leaves. Star ruby grapefruit and Marumi kumquat showed sudden symptoms of leaf fall which happended concomitantly. Yellowing of leaf and then leaf fall was observed in Mexican lime. Leaves of Eureka lemon presented only yellowing while apparition of small yellow dots on Pomeroy poncirus leaves were observed on upper leaves. Australian sour orange, Cleopatra mandarin, Sunki mandarin, Engedi pummelo, and Cambava did not present any leaf symptom.

Mineral analysis: chloride and sodium leaf content

Salt treatment increased leaf chloride concentration in all tested genotypes. An illustration of the observed increases in presented on figure 2A. Poncire commun citron accumulated the highest concentration of chloride after 80 days (109.1 ± 15.6 mg g^{-1} DW) while Cleopatra mandarin accumulated the lowest concentration (12.1 ± 5.8 mg g^{-1} DW). After only ten days of salt stress, Poncire commun citron accumulated higher significant

concentration of chloride compared to the respective control. For all the genotypes investigated along the experiment, leaf chloride concentrations for control plants ranged from 1.0 to 2.1 mg g^{-1} DW. Therefore, we have chosen to rank all the investigated genotypes depending of the value of the ratio of the leaf concentration after 80 days of stress over the leaf concentration in control condition (Fig. 2B). A wide range of accumulation was observed, a group of low chloride accumulating genotypes (Cleopatra mandarin to combava) being clearly identified.

Salt treatment also increased the leaf sodium content in all genotypes as illustrated on figure 2C. Accumulation of sodium was lower compared to chloride in all genotypes, Poncire commun citron accumulated the highest concentration (40.9 ± 10.5 mg g^{-1} DW) while Cleopatra mandarin (11.6 ± 3.9) accumulated the lowest concentration. For all the genotypes investigated along the experiment, the leaf sodium concentrations for control plants ranged from 0.09 to 1.16 mg g^{-1} DW. The same ranking of the genotypes used in figure 2B is presented in figure 2D for leaf sodium content analysis. A wide range of leaf sodium accumulation was also observed among genotypes and the ranking of accumulation was quite similar to the one of chloride accumulation.

Leaf chlorophyll and phenolic compounds content

Leaf greenness which is reflecting the leaf chlorophyll content and the leaf phenolic compounds contents were monitored for all the genotypes. Illustrations of the changes of leaf chlorophyll and phenolic compounds contents for some genotypes are presented on figures 3A and 3C. Also, using the same ranking of genotypes used in figure 2B, the ratios of the leaf chlorophyll and phenolic content measured after 77 days of stress over the leaf chlorophyll and phenolic content measured in control condition are presented in figure 3B and 3D. Salt treated plant presented a decrease of leaf greenness in all genotypes, but a markable decrease was observed for Marumi kumquat after 50 days of salt stress (Fig. 3A). We observed significant differences between genotypes for decrease in leaf greenness. After 80days of salt stress, except for marumi kumquat, the acutest decrease in leaf chlorophyll content was observed for Citrange Carrizo (60%), Pomeroy poncirus (50%), and Poncire commun citron (49%); Cleopatra mandarin and Sunki mandarin indicating only little decreases in leaf chlorophyll content, 10% and 14% respectively.

Leaf phenolic compounds content was investigated concomitantly to leaf greenness (Fig 3C). For all genotypes, a clear increase of leaf phenolic compound content was observed along the stress; the highest increase being observed for Mexican lime and the lowest for Engedi pummelo (Fig. 3D).

Maximum quantum yield of PSII and gas exchange measurements

Maximum quantum yield of PSII [$(F_m-F_0)/F_m$], stomatal conductance (*gs*) and photosynthesis (*A*) were monitored for all the genotypes. Illustrations of the changes of $(F_m-F_0)/F_m$, *gs* and *A* for representative genotypes are presented on figure 4A, C and E. Using the same ranking of the genotypes used in figure 2B, the ratios of the value measured in stress condition over the control condition for $(F_m-F_0)/F_m$, *gs* and *A* after respectively 60, 80 and 80 days are presented on figures 4B, D and F. Marumi kumquat presented the highest decrease in $(F_m-F_0)/F_m$ (35 %) after only 43 days of stress. Three other genotypes: Carrizo citrange, Mexican lime, and poncire commun citron presented also drops of $(F_m-F_0)/F_m$ with respectively 24%, 19% and 16%, of their value in control condition. Interestingly Pomeroy poncirus presented only a small decrease in $(F_m-F_0)/F_m$ (3%) and genotypes such as Star ruby grapefruit, Cleopatra mandarin, Sunki mandarin, Engedi pummelo, and Eureka lemon did not show any significant decrease. Gas exchange measurements were measured for all genotypes except for Pomeroy poncirus and marumi kumquat because the size of the leaves of these genotypes was too small. Stomatal conductance decreased after only 10 days of salt treatment for nearly all genotypes. The most significant decrease in *gs* was observed for combava and Mexican lime; Australian sour orange, Carrizo citrange and Cleopatra mandarin being the less affected (Fig 4D). Whatever the genotype, *A* reflected the results obtained for *gs*. Also, it is interesting to note that eureka lemon and combava maintained a photosynthetic rate similar to sunki mandarin, star ruby grapefruit and poncire commun citron (Fig 4D).

Discussion

Most of the citrus investigated for abiotic tolerance are rootstocks belonging to *Citrus* and *Poncirus* genera, or are hybrids obtained between these two related genera. However, little is known concerning genotypes belonging to *Fortunella* genera or monoembryonic citrus genotypes such as Citron or Pummelo that might present high diversity of response with potential specific genotypes well adapted to salt stress. Then, through sexual or somatic hybridization it may be expected to recover the character of apomixis and to propose new rootstocks better adapted to salt stress.

In order to increase genetic gain through breeding schemes a wide range of citrus genotype covering the diversity was investigated expecting that different mechanisms of sensitivity /tolerance could be identified. In these plants several physiological parameters were studied to assess the variability in behaviour when facing a moderate salt stress (75mM) for 12 weeks. It has been shown that the rate of spontaneous autotetraploid seedlings of citrus rootstocks could vary from 0 to 7 % (Barrett and Hutchinson 1982; Saleh *et al.* 2008). Using flow cytometry, tetraploid plants were identified among diploid seedlings and were removed from the plot since such non true to type genotypes were shown to increase the tolerance to salt stress when compared to their respective diploid genotypes (Mouhaya 2008; Saleh *et al.* 2008). Non homogenous seedlings (smaller and bigger plants) were also discarded and the rest of the plants were investigated using SSRs markers in order to remove the potential zygotic plants since such genotypes were showed to affect fruit yields (Hussain *et al.*, submitted for publication) and could have affected the response to the salt stress. Also, monoembryonic genotypes, *i.e.* Poncire commun, citron and Engedi pummelo, were propagated through stem cutting in order to work with clonal material and avoid misleading results.

Physiological responses of citrus to alleviate salt stress are related to the plant capability to restrict Cl^- transport from root to shoot (Gómez-Cadenas *et al.* 2003; Iglesias *et al.* 2007; Moya *et al.* 2003; Moya *et al.* 2002; Romero-Aranda *et al.* 1998; Storey and Walker 1998). After 12 weeks of salt stress, a large diversity of Cl^- accumulation profiles in leaves was observed among genotypes (Fig. 1A). So, we arranged the order of genotypes according the amount of Cl^- accumulated in leaves (Fig.1 B). Interestingly, the range and the order of genotypes for Na^+ accumulation was more or less the same as for Cl^- (Fig. 1C, D). Poncirus pomeroy and Carrizo citrange were stand in the middle range of Cl^- accumulation genotypes.

After 12 weeks of salt stress, the percentage of increase in plant stem diameter for control and salt treated plants was significantly different (Fig. 2).

Under salt stress conditions Poncirus pomeroy, Carrizo citrange known to be salt sensitive genotypes exhibited the highest growth rate. In the meantime a high stress/control ratio for photosynthesis and stomatal conductance was observed for Carrizo citrange (Fig. 3D and 3F). Recently Brumos *et al.* (2009) showed that in Carrizo citrange, a high growth rate was associated to higher photosynthesis activity and stomatal conductance. For these authors, this phenotypic trait in Carrizo citrange but probably also in Poncirus would be due to the overexpression of genes related to carbon metabolism and energy under salt stress leading to a maintaining of growth rate associated to a higher susceptibility to salt stress. It is interesting to note that Cleopatra mandarin, Sunki mandarin and Star ruby grapefruit were the genotypes that presented the highest decrease in growth when comparing stressed and control plants (Fig. 2). In our conditions, most of the genotypes quickly reduced their stomatal conductance (Fig. 3C) and photosynthesis activity (Fig. 3D) even though wide range of Cl^- and Na^+ accumulation was observed (Fig 1B, D).

The ion accumulation in leaf has a great impact on different critical processes essential for plant survival such as *A*, *gs* and Fv/Fm. In literature, we find different observations regarding adverse affect of ion accumulation on CO_2 assimilation. Some studies have found a correlation between the decrease in CO_2 assimilation and the high Cl^- and Na^+ content in leaves (Anjum 2008; Garcia-Legaz *et al.* 1993; Walker and Douglas 1983). Contrastingly, some studies showed that Cl^- and Na^+ accumulation in leaves were not responsible for decrease in CO_2 assimilation (Banuls *et al.* 1997). Poncire commun citron presented decrease in *gs*, *A* and Fv/Fm. In both cleopatra mandarin and sour orange CO_2 assimilation seemed to be related with low chloride accumulation. However, in spite of high Cl^- and Na^+ accumulation, photosynthesis and PS II were not disturbed in Poncire commun citron which suggests that other mechanisms besides toxic ions avoidance could be involved in salt tolerance, such as intra cellular compartmentalization (Neumann *et al.* 1997) or increased oxygen radical-scavenging ability (Arbona *et al.* 2003; Yasar *et al.* 2006).

Results obtained by Brumos et al, (2009) suggested that important defensive mechanism are observed in Cleopatra mandarin in response to salinity, reflected by decreased photosynthesis, stomatal conductance and undamaged PS II associated with repressing of central metabolic processes such carbon utilization that may at the end favor to cope with oxidative stress. Indeed, in Cleopatra mandarin (Fig. 3A) and in most of low accumulating Cl^- and Na^+ genotypes (Fig 3B), the maximum quantum yield of PSII was shown to be

maintained even after 60 days of stress. Little is known about the behavior of salt stress tolerance of other citrus genotypes usually used as varieties. Eureka lemon is a hybrid between Sour orange and citron (Nicolosi *et al.*, 2000). Our results suggest that salt sensitivity of the citron was not transmitted to the Eureka lemon. Indeed Eureka lemon accumulated less toxic ions but *A* and *gs* were also lower. A lower value of the maximum quantum yield of PSII is usually a direct effect of oxidative stress and is correlated with the greatest decrease of *A* and *gs* conductance (Hernandez *et al.* 2000). Interestingly, the PS II did not seem to be damaged for most of the genotypes (Fig. 4A) and the percentage of increase in diameter was also very similar in control and salt stress conditions (Fig. 2) suggesting that Eureka lemon might decrease its metabolism higher than Cleopatra mandarin to cope with salt stress (Brumos et al 2009). Other genotypes such as Mexican lime, Marumi kumquat and Poncire commun citron presented a large decrease in the percentage of growth rate associated to large decrease of the maximum quantum yield of PSII and death for Marumi kumquat, clearly showing that those high accumulating Cl^- and Na^+ genotypes were the most affected genotypes. Indeed, Marumi kumquat belongs to *Fortunella* genera appears to be one of the most sensitive citrus genotype to salt stress with early and very pronounced symptoms of discoloration and defoliation (Fig. 4A, B) without leaf burn. Poncire commun citron and Carrizo citrange presented severe leaf symptoms also due to high Cl^- accumulation in leaves.

It is interesting to observe that Marumi kumquat and Mexican lime accumulated high chloride content and suffered leaf drops. Star ruby grapefruit and Marumi kumquat showed sudden symptoms of leaf fall which happened concomitantly. Star ruby grapefruit presented unique symptoms of sudden leaf fall while leaves were still green and subsequently new leaves emerged. These characteristics under stress are very specific and are probably related to specific molecular mechanisms since low leaf accumulation of the Cl^- and Na^+ occurred when leaf symptoms were observed for this genotypes. Indeed, this behavior could be characterized as defense mechanisms that can be described as evasive action to get rid of the leaves that have accumulated toxic ions and to produce new leaves to maintain photosynthesis and plant growth. We may assume that beside Cl^- ion content, there are other processes responsible for leaf drop e.g. changes of the hormonal balance leading to leaf abscission (Ghanem *et al.* 2008).

Salt tolerance of pummelos has never been studied because of their monoembryony. Our observations indicate that they have some good resistance to salt stress. Engedi pummelo accumulated less chloride and sodium ion content as compared to other salt sensitive

genotypes. In Engedi pummelo PS II system was not damaged, while it maintained fair flow of gas exchanges and chlorophyll content.

Conclusion

In conclusion, we propose that ability of genotypes to tolerant salt stress is related with their capacity to reduce central metabolic processes related with carbon utilization and toxic ions exclusion. The search for new sources of tolerance should be oriented towards mandarin grapefruit, and pummelo groups which contain the highest genetic diversity. Moreover, the behavior of pummelo probably inherited is a new form of original tolerance first observed in citrus.

Tables

Table 1: List of the genotypes representing the citrus diversity subjected to salt stress.

Common name	Tanaka system	ICVN[a] and SRA[b] no.	Code
Poncire commun citron	*C. medica*	SRA 601	**PCC**
Engedi pummelo	*C. maxima* (Burm.) Merr	SRA 610	**EP**
Eureka lemon	*C. limon* (L.) Osb.	SRA 4	**EL**
Mexican lime	*C. aurantifolia*	SRA 140	**ML**
Cleopatra mandarin	*C. reshni* Hort. Ex Tan.	SRA 948	**CM**
Sunki mandarin	*C. sunki* Hort. Ex Tan	ICVN 0110076	**SM**
Star Ruby grapefruit	*C. paradisi*	SRA 293	**SRG**
Carrizo citrange	*C. sinensis* X *Poncirus trifoliata*	SRA 796	**CC**
Pomeroy poncirus	*Poncirus trifoliata* (L.) Raf	ICVN 0110278	**PP**
Australian Sour orange	*Citrus aurantium* (L.)	SRA 851	**ASO**
Combava	*C. hystrix* D.C.	SRA 630	**Co**
Marumi kumquat	*Fortunella japonica(Thunb.) Swing.*	SRA 488	**MK**

[a], International Citrus Variety Numbering.

[b], Agronomical Research Station numbering

Table 2: ISSR primers used for the identification of the zygotic genotypes among the different *Citrus* genotypes (Fang and Roose 1997).

Name	Sequence	Hybridation T(°C)	Fragment length (bp)
ISSR 1	$HVH(CA)_7T$	55.5	300-1100
ISSR 4	DBDA(CA)7	55	200-1500
ISSR 5	$BDB(CA)_7C$	58	200-1000
ISSR 8	$HVH(TCC)_5$	55.5	450-1400

Legends of figures

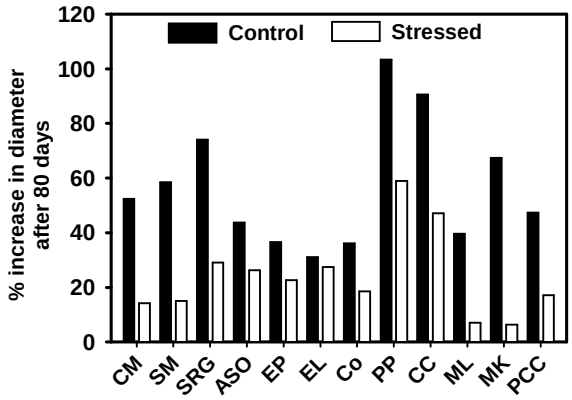


Fig. 1: % increase in stem diameter of Control and stressed plants after 80 days of salt stress (75 mM NaCl). Bar in black color represents control and white color represent stressed plant. ($n \geq 3$).

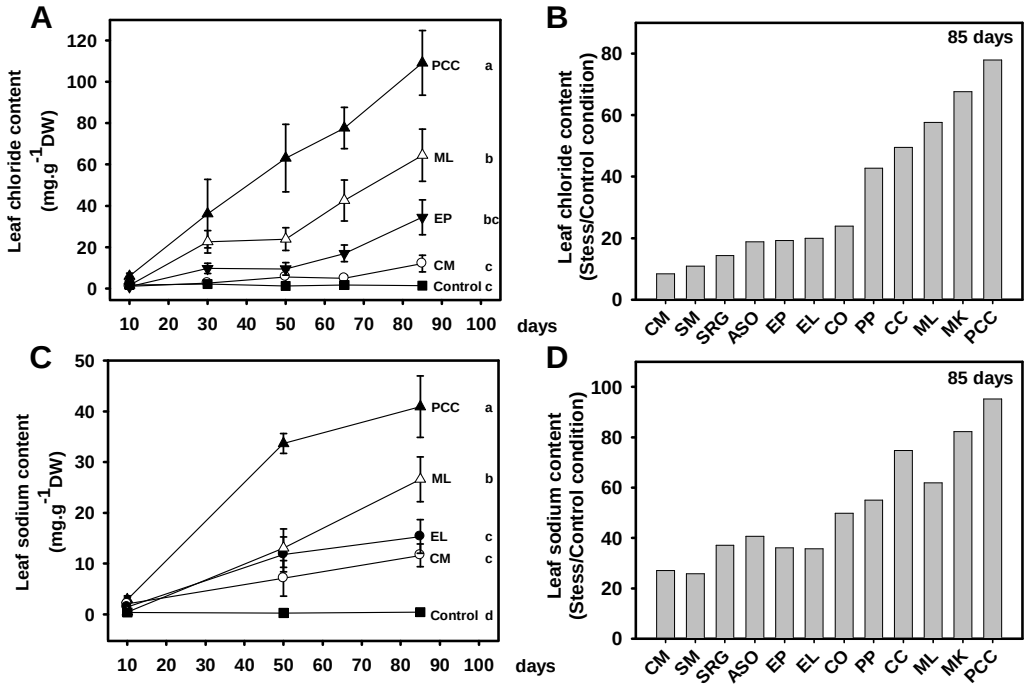


Fig. 2: (A) Leaf chloride content of four genotypes for 85 days. For each date, the control represents the mean value for the twelve genotypes. **(B)** Leaf chloride content based under the ratio of the chloride content after 85 days of salt stress over the leaf chloride content measured in control condition for the twelve investigated genotypes. **(C)** Leaf sodium content of four repetitive genotypes for 85 days. For each date, the control represents the mean value for the twelve genotypes. **(D)** Leaf sodium content based under the ratio of the sodium content after 85 days of salt stress over the leaf sodium content measured in control condition for the twelve investigated genotypes. Values (mean ± se) with different letters were significantly different ($P < 0.05$, $n \geq 3$). Names of the genotypes are presented in table 1.

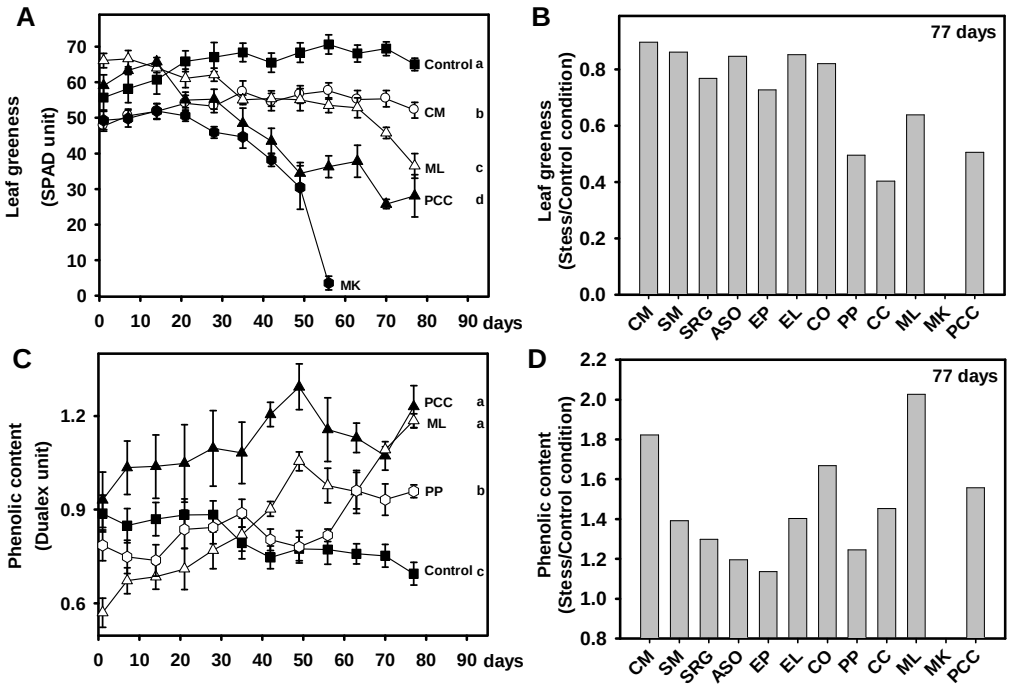


Fig. 3: (A) Leaf greenness of four repetitive genotypes for 77 days. For each date, the control represents the mean value for the twelve genotypes. **(B)** Leaf greenness based under the ratio of the leaf greenness after 77 days of salt stress over the leaf greenness measured in control condition for eleven. **(C)** Leaf phenolic content of three repetitive genotypes for 77 days. For each date, the control represents the mean value for the twelve genotypes. **(D)** Leaf phenolic content based under the ratio of the phenolic content after 85 days of stress over the phenolic content measured in control condition for eleven genotypes. Values (mean ± se) with different letters were significantly different ($P < 0.05$, $n \geq 3$). MK is not represented on graphs B and D because this genotype was not healthy after 60 days of stress. Names of the genotypes are presented in table 1.

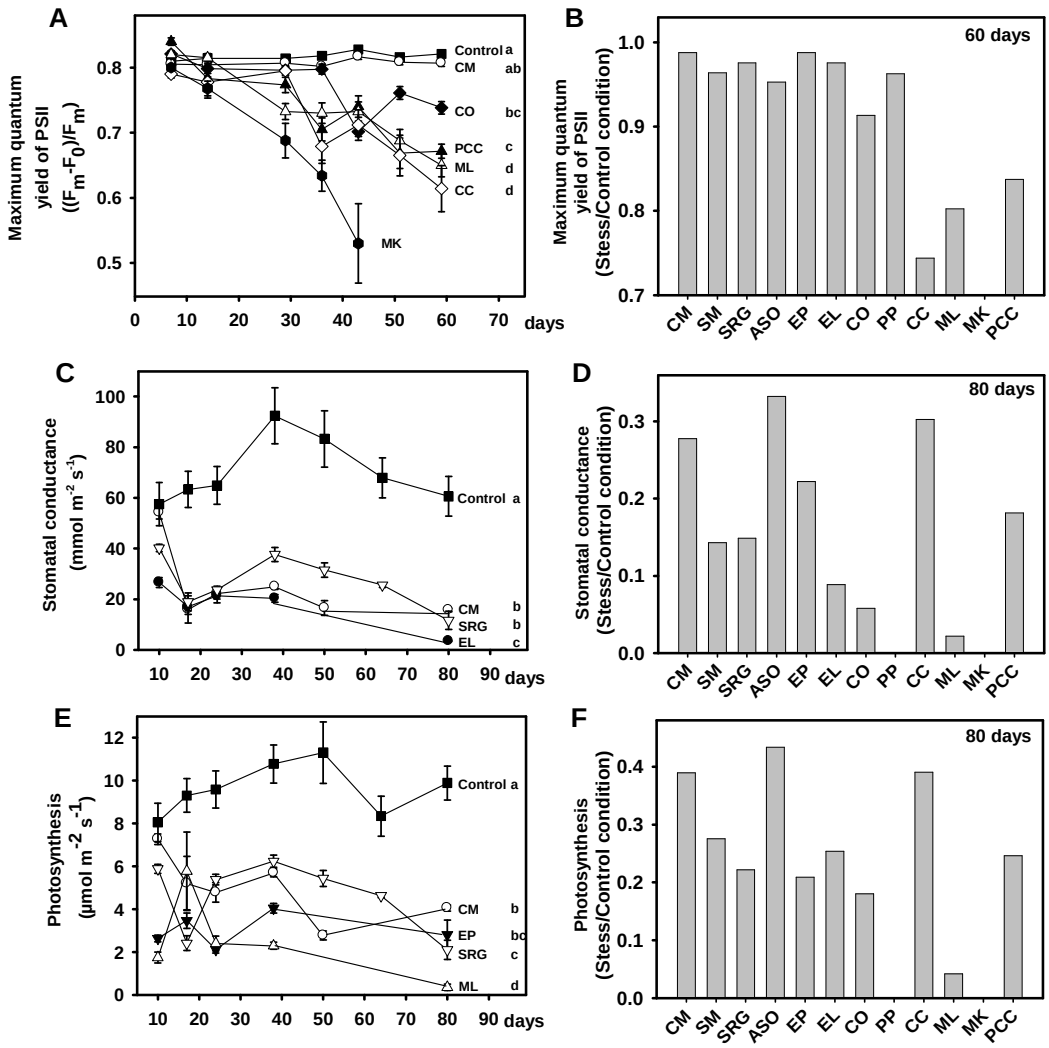


Fig. 4: (A) Maximum quantum yield of PSII of six repetitive genotypes for 60 days. For each date, the control represents the mean value for the twelve genotypes. **(B)** Maximum quantum yield of PSII based under the ratio of the maximum quantum yield of PSII after 60 days of salt stress over the maximum quantum yield of PSII measured in control condition for eleven genotypes. **(C)** Stomatal conductance of three repetitive genotypes for 80 days. For each date, the control represents the mean value for ten genotypes. **(D)** Stomatal conductance based under the ratio of the stomatal conductance after 80 days of stress over the stomatal conductance measured in control condition for ten genotypes. **(E)** Photosynthesis rate µmol m^{-2} s^{-1} of four repetitive genotypes for 80 days. For each date, the control represents the mean value for ten genotypes. (F) Photosynthesis based under the ratio of the photosynthesis rate after 80 days of stress over the photosynthesis rate measured in control condition for ten genotypes. Values (mean ± se) with different letters were significantly different ($P < 0.05$, $n \geq 3$). MK is not represented on graphs B, because this genotype was badly hurt after 60 days of stress. PP and MK were not represented on graphs D and F because the size of their leaves

was too small to measure the photosynthesis rate and the stomatal conductance. Names of the genotypes are presented in table 1.

5.2 Article No 2: Citrus diversity investigated by physiological behaviour of citrus plants under salt stress growth conditions.

Sajjad Hussain[1, 2], Raphaël Morillon[3*], Gille Costantino[1], Patrick Ollitrault[3], François Luro[1]

1. Unité de Recherche Génétique et Ecophysiologie de la Qualité des Agrumes, Inra. Centre Inra de Corse, F-20230 San Giuliano, France

2. Unité Propre de Recherche amélioration génétique des espèces à multiplication végétative, Cirad. Centre Inra de Corse, F-20230 San Giuliano, France

3. UPR amélioration génétique des espèces à multiplication végétative du Cirad. Instituto Valenciano de Investigaciones Agrarias 46113 - Moncada – Valencia, Spain.

*__Corresponding author:__ François Luro, Unité de Recherche Génétique et Ecophysiologie de la Qualité des Agrumes, Inra. Centre Inra de Corse, F-20230 San Giuliano, France

Key words: citrus diversity, salinity tolerance, chloride

Abstract

We analyzed the different physiological behaviors of a wide range of varieties and species belonging to the *Citrus* genus when subjected to salt stress with the aims to seek new sources of tolerance that might be species-specific. At the end, our goal was to use physiological parameters in order to reinvestigate the citrus genetic diversity. For that purpose, we have selected 20 different genotypes representing the major species on the basis of the genetic diversity of *Citrus* genus complemented with one intergeneric hybrid Carrizo citrange (*C. sinensis* x *P. trifoliata*). A moderate salt stress of 75mM of NaCl was applied for 12 weeks. For control plants the main parameters contributing for more than 25% to the diversity on the two axes of principal component analysis (PCA) were chlorophyll content, photosynthesis and Fv/Fm prime. However, the dispersal of species and varieties on the PCA did not show any particular structure. Under salt stress condition, four parameters (leaf chloride content, leaf chlorophyll content, photosynthesis and stomatal conductance) contributed more specifically to the dispersion on PCA representation with more than 15% of contribution for each parameter. Large differences were observed within three basic taxa of citrus: mandarin and pummelo presented good tolerance to salt stress while citron was very sensitive. Also it is interesting to note that all secondary genotypes that presented good tolerance to salt tolerance shared mandarin or pummelo as female parent. We may then suppose that the cytoplasm of mandarin and pummelo play an important role in tolerance of secondary species. We propose that the search for new sources of tolerance should be directed toward exploration of mandarins and pummelo groups which presented the highest genetic diversity.

Introduction

Like many others crops, citrus species are classified as salt-sensitive (Maas 1993). The adverse effects of salinity on plant growth and productivity are associated with low osmotic potential, accumulation of ions to toxic level, and nutritional imbalance (Ashraf and Harris 2004; Byrt and Munns 2008). In citrus, such phenomena have been extensively reported in the literature (Anjum 2008; Atmane *et al.* 2003; Garcia-Sanchez and Syvertsen 2009; Lopez-Climent *et al.* 2008; Saleh *et al.* 2008). The observed effects of salinity are various and one may cite symptoms of leaf injury, growth suppression and yield decline. Main effects of salinity in citrus are decreased stomatal conductance which leads to reduce CO_2 diffusion, ultimately decrease net photosynthesis (García-Sánchez and Syvertsen 2006) and ion accumulation (Sudhir *et al.* 2005). A large diversity of behavior within citrus towards salt stress does exit e.g. Rangpur lime, Sunki mandarin and Cleopatra mandarin are considered to be tolerant, while Carrizo citrange known to salt sensitive (Maas 1993). In citrus damages caused by salinity are usually associated with chloride accumulation but not with sodium (Banuls *et al.* 1997; Moya *et al.* 2002). Citrus rootstocks differ greatly in their ability to exclude Cl^- and Na^+, or both from the scions (Maas 1993). Walker and Douglas (1983) observed significant difference between Rangpur lime, Kharna khatta lime, and Etrog citron for Cl^- accumulation in the leaves but little difference was observed for Na^+ accumulation. Trifoliate orange (*Poncirus trifoliata*) is a poor Cl^- excluder (Cooper 1961) but an efficient Na^+ excluder at low salinity level (Walker 1986).

As the ability to exclude ions seems to be crucial in the evaluation of citrus tolerance status for salt stress, tests have been mainly performed with citrus used as rootstocks or with scion grafted on a rootstock. In citrus, rootstocks are propagated by seedlings of polyembryonic seeds. Some citrus produce in their seeds somatic embryos additional to the zygotic one. Those embryos are originated from nucellar cells, a maternal tissue, and therefore after regeneration reproduce the maternal characters. Citron (*C. medica*) and pummelo (*C. maxima*) are two basic species which are not able to produce polyembryonic seeds. This is the reason of their uselessness as rootstock in modern agriculture where homogeneity and reproducibility of plant phenotypes are the conditions of plant commercialization in citrus nursery. In breeding programs, many opportunities are offered by sexual or somatic crossings for combining favorable characters and create new improved rootstocks more adapted to environmental constraints (Grosser *et al.* 2007; Ollitrault *et al.* 1998; Ollitrault *et al.* 2000; Ollitrault *et al.* 2008; Ollitrault *et al.* 2007). This challenge may be achieved more efficiently if the genetic sources are widely evaluated for the selection of

new tolerances to salinity. Today all the citrus scientific community had adopted hypothesis about *Citrus* history and phylogeny proposed by Scora (1973) and Barett and Rhodes (1976) where a large diversity of *Citrus* genus is represented by three basic species such as mandarins, citrons and pummelos. They are at the origin of many secondary cultivated species such as orange, lemon, Sour orange, grapefruit, clementine by several sexual crosses which appeared spontaneously more or less recently during the citrus history. Several molecular studies have confirmed and specified sense and degree of relatedness of citrus phylogeny (Barkley *et al.* 2006; Luro *et al.* 2001; Nicolosi *et al.* 2000). The current assumption is for four main species citron (*C medica*), mandarin (*C. reticulata*), pummelo (*C. maxima* (Burm.) Merr.) and micrantha (*C. micrantha* Wester). From these four main species all other species were derived by hybridization. This hypothesis is supported by the results of studies using biochemical such as isozymes (Herrero *et al.* 1996), and molecular markers such as Restriction Fragment Length Polymorphism, (RFLP) (Federici *et al.* 1998), Random Amplified Polymorphic DNA (RAPD) (Corazza-Nunes *et al.* 2002; Nicolosi *et al.* 2000), Inter Simple Sequence Repeat ISSR (Fang *et al.* 1998), Simple Sequence Repeat (SSR) (Corazza-Nunes *et al.* 2002; Luro *et al.* 2001). This concept gained further support from various studies using cytoplasmic DNA markers (Froelicher *et al.* 2010; Nicolosi *et al.* 2000). Therefore analysis of physiological behavior of a range of varieties and species of the *Citrus* genus subjected to salt stress aims to seek new sources of tolerance but also to detail the mechanisms of tolerance that might be species-specific.

Materials and methods

Plant material

This study was carried out at INRA-CIRAD station of Corsica, France. We have selected 20 different genotypes of citrus belong to major diversity of Citrus genera complemented with one intergeneric hybrid Carrizo citrange (*C. sinensis* x *P. trifoliata*) and Marumi kumquat (*Fortunella japonica* (Thunb.) Swing.). The different varieties that were selected represent the major species on the basis of the genetic diversity of *Citrus*, we assume that the mechanisms of tolerance acquired during the evolution should be shared by several varieties of the same species and specificities should be observed in larger taxa or their descendants. Thus three of the basic taxa, covering most of the genetic diversity of the genus *Citrus*: pummelo, mandarin and citron, were more represented than secondary species. In addition many species have only a secondary low genetic diversity linked to phenotypic diversification gained by accumulation of somatic mutations in contrast to the ancestral species where sexual crossing has been the main mechanism of evolution. A total of 8 species were represented (Table 1). Six trees per genotype were subjected to salt stress while only three plants were selected for each control and salt stress condition.

Plant materials were propagated in two ways: for polyembryonic genotypes, propagation was done by sowing seeds in a neutral substrate (perlite), while for monoembryonic genotypes C. *medica* and *C. maxima* stem cutting was used to produce true-to-type plants. Synthetic auxin (Rhizopon 4%) was used for root induction. Seedlings were transplanted three months after germination in 2.5 liters pots with filter media, comprising 2/3 river sand and 1/3 silt, and grown under greenhouse. Plants were irrigated thrice a week with half diluted nutritive solution (fertilizer 28-14-14, ref 205, Fertil, France).

Verification of genetic status of plants

To verify genetic material propagated from seed, total DNA was extracted on 0.5 cm^2 of leaf of each plant seedlings plus mother tree according to (Doyle and Doyle 1987) and adapted to citrus (Cabasson *et al*. 2001). The genetic constitution of the seedlings was analyzed using four Inter-simple sequence repeat (ISSR) primers: $HVH(CA)_7T$, DBDA(CA)7, $BDB(CA)_7C$, $HVH(TCC)_5$ (Fang and Roose 1997). The conditions and separation of PCR products were done by means of vertical denaturalized electrophoresis polyacrylamide (5%, urea 7 M), or 1.5% agarose gel in buffer TBE 0.5X (Tris, acid boric 45mM and EDTA 0.5 M, pH 8) and silver stained according to (Beidler *et al*. 1982).

Ploidy status of plants was determined by flow cytometry using leaf samples according to Froelicher *et al.* (2007). Approximately 0.5 cm^2 of leaf was chopped in 250 µl of nuclei

extraction buffer (Partec Cystain UV Precise P). The solution obtained was filtered (pore size of 30 µm) to eliminate cell debris. After filtration, 400 µl of staining buffer (Partec, Cystain UV Precise P Staining Buffer) was added for DNA staining. The samples were incubated for 5 min and analyzed by flow cytometer PA1 (PARTEC, Germany). The nuclear DNA contents were evaluated according to (Seker *et al*. 2003). A tetraploid citrus plant was used as internal control. Only true to type plants were selected for further measurements.

Salt treatments

After the period of acclimation in greenhouse, plants were divided as a non-saline control and salt treated. Three plants per genotypes were used for salt treatment and three plants were used as non-saline control. The conditions of salt stress were applied during 12 weeks (from mid-May to 10^{th} of August). Salt-treated plants were watered three times a week with the nutrient solution plus 75mM NaCl. The frequency of watering controls plants was identical to that of stressed plants. Growing conditions were 18°C min 38°C max and a relative humidity of 55% on average.

Plant diameter and leaf symptoms

Plant diameter was measured at start and at the end of experiment for each non-saline control and salt treated plant. Plant diameter was measured with the help of vernier calliper in (mm). The diameter increase percentage was calculated after 80 days of salt stress for non-saline and salt treated plants. Leaf symptoms were observed throughout the experiments representing different physiological disorders: leaf necrosis, stem necrosis, leaf pointed depigmentation, green leaf fall down. The symptoms were recorded and transposed in different classes representing the severity, the extension on leaves and on trees and the delay of apparition.

Physiological parameters

Two leaves per plant at same height and age were selected randomly for each different parameter. The measurements were done at the beginning of the experiment and after 80 days of salt stress for non saline control and salt treated plants.

Gas Exchange measurements

Net photosynthetic rate (*A*) and stomatal conductance (*gs*) were determined with portable gas exchange fluorescence system (GFS-3000) (Heinz Walz GmBH, Germany with photosynthetically active radiation photon flux of 1000 µmol m^{-2} s^{-1}. Measurements were performed in the morning (8 to 11 AM) to avoid high external temperature and low humidity. Leaf temperature was 28 ± 2°C, leaf to air vapour pressure difference was 2.4 ± 0.4 kPa, and

ambient CO_2 concentration was 370 ± 3 µmol mol^{-1} within the cuvette of the portable gas exchange fluorescence system.

Chlorophyll fluorescence, Chlorophyll content and Flavonoid content measurements

Chlorophyll fluorescence was measured during the night to evaluate the maximum fluorescence capacity on dark adapted leaves with portable fluorometer (Hansatech Ltd., Kings Lynn. UK). The chlorophyll content or leaf greenness was measured for each plant with the help of SPAD meter (Minolta SPAD-502, Japan). The leaf phenolic compounds, mainly represented by flavonoids, was measured with the optical sensor Dualex3.3 ® start-up (Force-A ©).

Mineral analysis: sodium and chloride content

One leaf for non-saline control and 3 leaves for salt treated were collected after 80 days of stress. Mineral analysis was carried out according to Mouhaya *et al.* (2010) and Saleh *et al.* (2008). Leaves were oven dried at 60°C for one week and used for chloride and sodium content analysis. Leaves were weighed and crushed in hammer-mill and stored at room temperature. 50 mg of powder was burnt at 400°C for 4 hours. The resulting ashes were dissolved in 100mL of 0.5N concentrated nitric acid. The solution was divided into two parts for chloride and sodium analysis. The chloride concentration was determined using a specific chloride electrode (Orion, 9417BN). Chloride content was expressed in mg g^{-1} of dry weight of plant material. Leaves, sodium and chloride contents were expressed in mg g^{-1} of dry weight. For sodium analysis inductively coupled plasma mass spectrometer (ICP-MS) assays were performed at the Unité de Service 'Analyse des eaux, sols et végétaux', Département performances des systèmes de production et de transformation tropicaux of CIRAD in Montpellier, France.

Statistical analysis

Average values were calculated for each tree from leaf repetition measurements. All the data were analysed with R statistical software to represent physiological diversity under normal growth conditions (plant controls) and by plant behaviours under salt stress pressure. Data were normalized and used in Principal Component Analysis (PCA) or to construct a dendrograme by Ward aggregation method.

Results and discussion

Salt stress leaf symptoms

The leaf necrosis symptoms first appeared after 15 days of stress in citron (*C. medica* L.), particularly in variety Etrog. Leaf necrosis were developed from the tip of the blade and then spread throughout the leaves and reached the ramifications (Fig. 1). Most of the trees of citron species were strongly affected with only a small amount of leaves which were not affected by necrosis. Theses leaves were used to measure the photosynthetic parameters. Marumi kumquats (*Fortunella japonica* (Thunb.) Swing.) lost most of their leaves at once after a little over 55 days of stress. A dark brown color of the leaves appeared very quickly after their detachment from the tree. Without plant material for physiological investigations at 80 days, we were unable to integrate Marumi kumquat in our study. A progressive loss of leaves was observed in Mexican lime (*C. aurantifolia* (Christm.) Swing.) after 65 days to onwards. Some leaves were still present on trees after 80 days of salt stress which allow us measuring physiological parameters. Beyond this period trees eventually lost all their leaves. Carrizo citrange showed leaf necrosis from the ends of leaves and first observed on lower leaves while Poncirus pomeroy (*Poncirus trifoliata* (L.) Raf.) presented small yellow dots of necrosis and first observed were observed on upper leaves. These symptoms appeared after about 60 days of salt stress treatment. No significant symptom was observed for all other genotypes during the 80 days of salt stress. Only a few points of small discoloration (less than one mm) were observed on some leaves of pummelo but we did not consider them as salt symptoms. However, we may expect that if the stress duration would have been prolonged, we may have observed stronger symptoms. A very unique behavior of sudden drop of all the leaves while they were still green was observed in the two varieties of grapefruit (*C. paradisi* Macf.) after 80 days of salt stress. Unlike the Mexican lime, this leaf fall was followed by an emergence of new leaves. We observed this behavior after 90 days of salt stress. To our knowledge such a behavior in response to salt stress has never been reported in citrus. Other symptoms associated to the salt stress such as the reduction of leaf or branch growth have been observed in few varieties. Nevertheless this phenomena was particularly visible when the control plants were vigorous by growing but less or not observable when the control varieties did not present a strong vegetative flush. The most expressed difference between control and salt stressed variety was observed for Australian sour orange (Fig. 1).

Principal component analysis of genotypes under control condition

All the result concerning the photosynthetic parameters is presented in Table 2. The results obtained from different parameters for non saline control showed a wide dispersion of

physiological behavior within species (Fig. 2). We observed considerable differences in growth rate or photosynthesis among the investigated genotypes. Within one variety, all trees presented the same behavior showing little difference between plant reps which proved that differences were due to genetic diversity and not caused by a possible change in growing conditions. Therefore for control plants, we were able to obtain an average value of each parameter for all varieties and used this average value to calculate the relative values for trees when subjected to salt stress. Pummelo species presented good photosynthetic efficiency and the highest rate of polyphenols, while the Buddha's Hand citron, grapefruits, Willow leaf mandarin and Carrizo citrange had the highest growth rates with high content of sodium in leaves. This result reinforces the proposition that the behavior of trees under stress must first be compared to control trees and then, relative values can be used to compare different varieties. The parameters (chlorophyll content, photosynthesis and Fv/Fm prime under light) contributing to more than 25% to the diversity were represented onto two axes of a PCA (Fig. 3). Dispersal of species and varieties on the PCA did not have a particular structure.

Principal component analysis of genotypes under salt stress

When genotypes were subjected to salt stress, responses of each variety was more heterogeneous than that ones observed for the control trees (Fig. 4). The most heterogeneous behavior was observed for the Australian sour orange (*C. aurantium* L.), Willow Leaf mandarin (*C. deliciosa* Ten.) grapefruits (*C. paradisi* Macf.) and Poncire commun citron (*C. medica* L.). For all other varieties, behavior of the 3 trees was almost identical. The principal component analysis of diversity in behavior of genotypes relative to control under salt stress showed two clearly distinguishable varietal groups (Fig. 4). The first group corresponds to varieties or species that accumulated high Cl^- and Na^+ contents in leaves as well as higher rate of phenolic compounds. In that group we found all the citrons, the Mexican and Brazil Sweet limes and Carrizo citrange. In the second group we found lemons, mandarins, pummelos, Rangpur lime, combava, sour orange and grapefruit. This group was distinguished mainly by a lower accumulation of toxic ions but also by a higher photosynthetic activity than for varieties of the first group. Four parameters contributed more specifically to the dispersion of varieties in PCA (Fig. 5). These parameters were leaf chloride content, leaf chlorophyll content, photosynthesis and stomatal conductance with more than 15% of contribution for each parameter. The growth rate measured as the increase in stem diameter relatively to the value measured for control plants did not appear to be a good indicator of the differentiation between the two groups and therefore, is probably not a good indicator of sensitivity or tolerance for any variety subjected to salt stress. For example, Diamante citron and Carrizo

citrange showed relatively high growth but also presented the characteristics of sensitivity to salt stress such as high Cl^- content.

Our results also reflect the degree of relatedness of some minor species. For example, the sour orange presented a behavior quite close to both assumed parents (Pummelo and mandarin). The lemon probably derived from a cross between sour orange and citron inherited a behavior from sour orange while Mexican and Brazil Sweet lime inherited the behavior from citron recognized as their paternal parent. Unlike other limes, Rangpur lime is related with the mandarin (Barkley *et al.* 2006) and, consequently, its behavior is normally equated with varieties of the second group.

Furthermore if we look at the varietal dispersion of species with symptoms, it is interesting to note that the first group except the Brazil Sweet lime presented symptoms of leaf necrosis, leaf fall and for citron genotypes leaded ultimately to the death of plants. The second group of varieties is characterized by an absence of symptoms as observed in the first group. However we can note that grapefruit species presented a unique behavior with sudden leaf drops after 90 days of salt stress followed by the emergence of new leaves. This behavior could be a defensive mechanism, possibly in order to get rid of the leaves that accumulated high contents of toxic ions (Cl^-) and at the end produced new leaves to maintain photosynthesis and plant growth.

Detoxification mechanisms involved in tolerance to salt stress allow reducing the level of intracellular reactive oxygen species (ROS) produced consecutively to oxidative stress (Jacoby *et al.* 2010; Mittler 2006). These mechanisms are often controlled by cytoplasmic and mitochondrial genes (Addabbo *et al.* 2009; Asada 2006). Our results may suggest that the cytoplasm of mandarin and pummelo could bring effective detoxification mechanisms contributing to better tolerance of these genotypes. We know that cytoplasm in *Citrus* is maternally inherited (Green *et al.* 1986; Masashi Yamamoto 1993). All the supposed hybrids with pummelo or mandarin cytoplasm such as sour orange, grapefruit, lemon and Rangpur lime were found in the group of varieties tolerant to salt stress. The Mexican lime inherited the cytoplasm of *C. micrantha* (Nicolosi *et al.* 2000). We did not have any information about the tolerance to salin stress of *C. micrantha.* The Brazil sweet lime inherited a cytoplasm similar to that of Mexican lime and was also found in the group of the susceptible varieties. Of course, one cannot only consider the ability to detoxify ROS as the only way to cope with salt stress. Indeed, there are other mechanisms of adaptation (vacuolar compartmentation) but our results do not confirm that assumption. It would be then required to analyze the genetics of tolerance to salt stress and extend the experiment to other basic genotypes and varieties but

also to hybrids with favorable nucleo-cytoplasmic combinations to confirm our hypothesis of the strong involvement of cytoplasmic in inheritance of salt stress tolerance. Undoubtedly the analysis of nuclear inheritance provides insights into the mechanisms of adaptation to salt stress which is also under the control of nuclear genes. To do so, two segregating populations from two reverse crosses between a sensitive and tolerant variety would be required. Thus cytoplasm with cytoplasmic inheritance we could study the segregation of tolerance genes in two opposites nuclear cytoplasmic environments in terms of effectiveness of detoxification. But the establishment of such experimental tests will be relatively long and measurements should be limited to only the most informative parameters.

Conclusion

In conclusion, we propose that the search for new sources of tolerance could be directed toward exploration of mandarins and pummelo groups which presented the highest genetic diversity. We may suppose that the cytoplasm of mandarin and pummelo play an important role in tolerance of secondary species. Then, more emphasis should be given to the behavior of mandarins and pummelos groups since those genotypes seem to be at the origin of new traits of salt stress tolerance.

Table 1: List of genotypes used for salt stress experiment

Common name	Tanaka system	Code	ICVN[a] and SRA[b] no.
Diamante citron	*C. medica* L.	CiD	SRA 540
Digite citron	*C. medica* L.	CiB	SRA 640
Etrog citron	*C. limonimedica* Lush.	CiE	SRA 709
Poncire commun citron	*C. medica* L.	CiP	SRA 601
Engedi pummelo	*C. maxima* (Burm.) Merr	PuE	SRA 610
Kao Pan pummelo	*C. maxima* (Burm.) Merr	PuK	SRA 321
Timor pummelo	*C. maxima* (Burm.) Merr	PuT	SRA 707
Eureka lemon	*C. limon* (L.) Burm.	LeE	SRA 4
Lisbon lemon	*C. limon* (L.) Burm.	LeL	SRA 16
Brazilian sweet lime	*C. limettioïdes* Tan.	LiB	SRA 697
Mexican lime	*C. aurantifolia* (Christm.) Swing.	LiM	SRA 140
Rangpur lime	*C. limonia* Osb.	LiR	SRA 777
Cleopatra mandarin	*C. reshni* Hort. Ex Tan.	MaC	SRA 948
Sunki mandarin	*C. sunki* Hort. Ex Tan	MaS	ICVN 0110076
Willow leaf mandarin	*C. deliciosa* Ten.	MaW	SRA 133
Duncan grapefruit	*C. paradisi* Macf.	GrD	SRA 470
Star Ruby grapefruit	*C. paradisi* Macf.	GrS	SRA 293
Carrizo citrange	*C. sinensis* x *Poncirus trifoliata*	CaC	SRA 796
Australian Sour orange	*Citrus aurantium* L.	SoA	SRA 851
Combava	*C. hystrix* D.C.	CoK	SRA 630
Marumi kumquat	*Fortunella japonica*(Thunb.) Swing.	MK	SRA 488

[a] International Citrus variety numbering.

[b] station de research agronomic

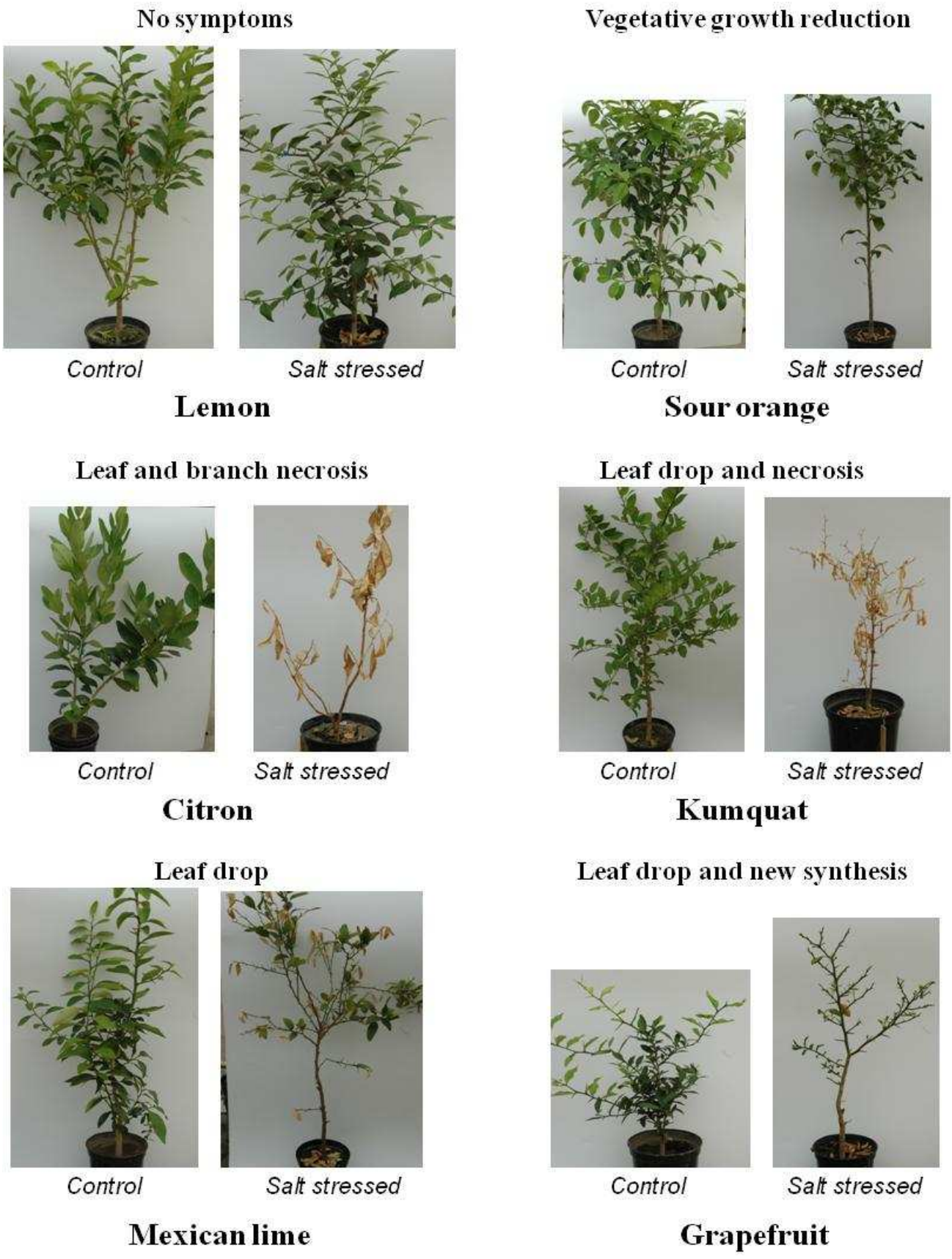


Fig 1: Different symptoms presented by different genotypes under salt stress condition compared to their control plants.

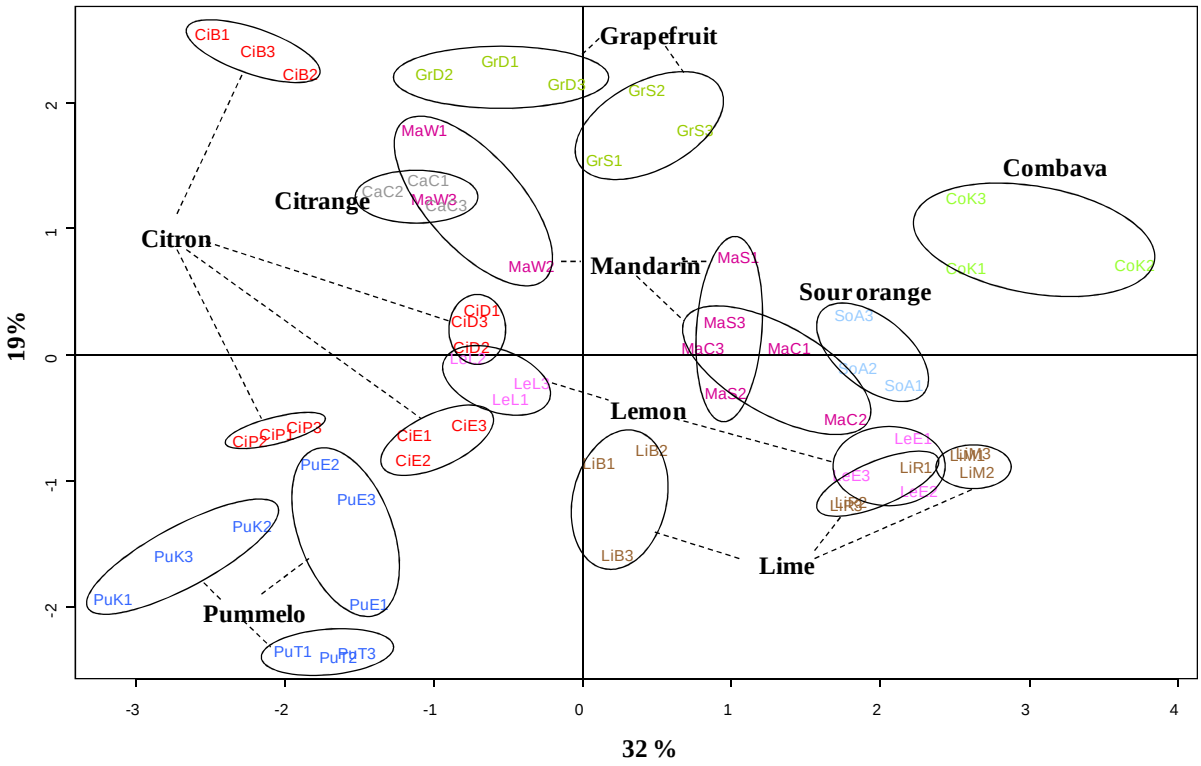


Fig 2: PCA of citrus varieties and trees repartition on the two first axes representing 51% of the population diversity calculated from physiological parameters estimated on the unstressed plants. Circles group the 3 repetitions of each genotype.

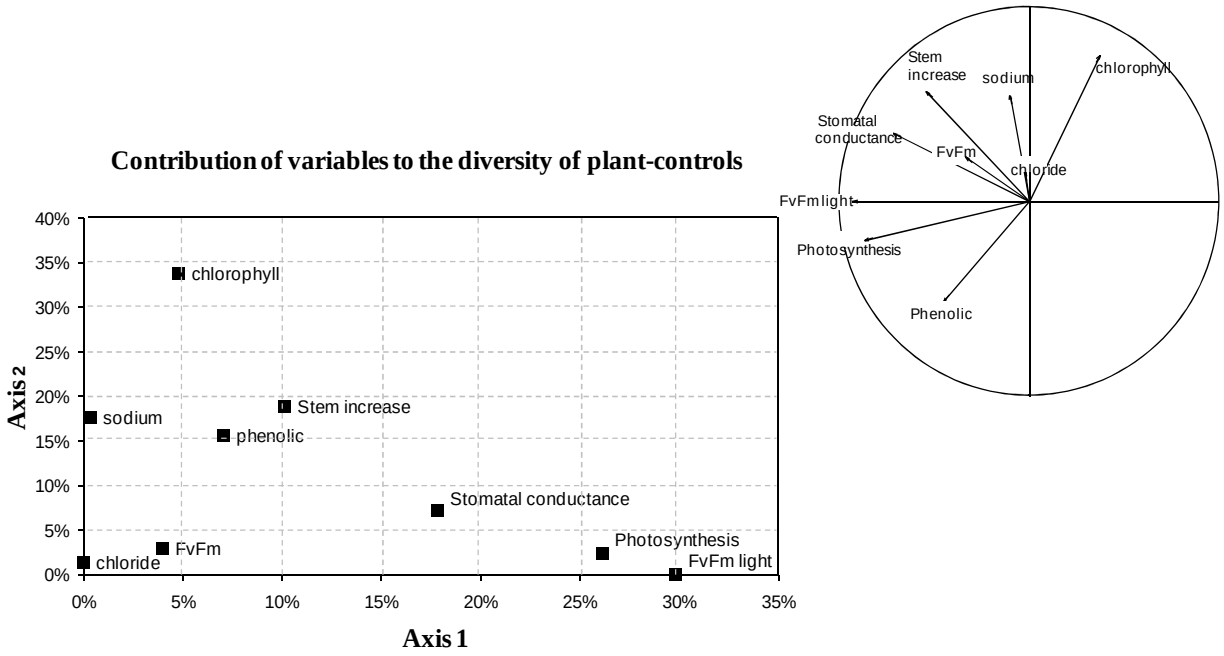


Fig 3: Contribution of each physiological factor to the two first axes of PCA explains the diversity under control condition.

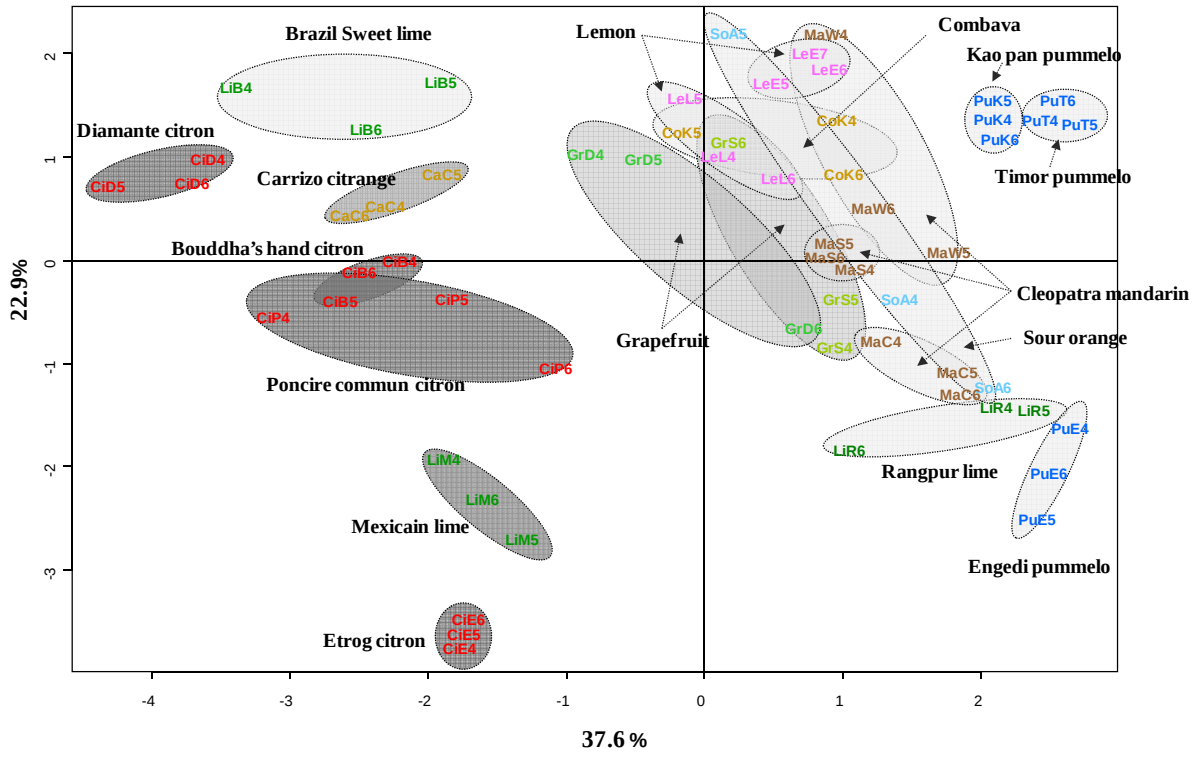


Fig 4: PCA of citrus varieties and trees repartition on the two first axes representing 61% of the population diversity calculated from physiological parameters estimated on the stressed plants. Circles group the 3 repetitions of each genotype.

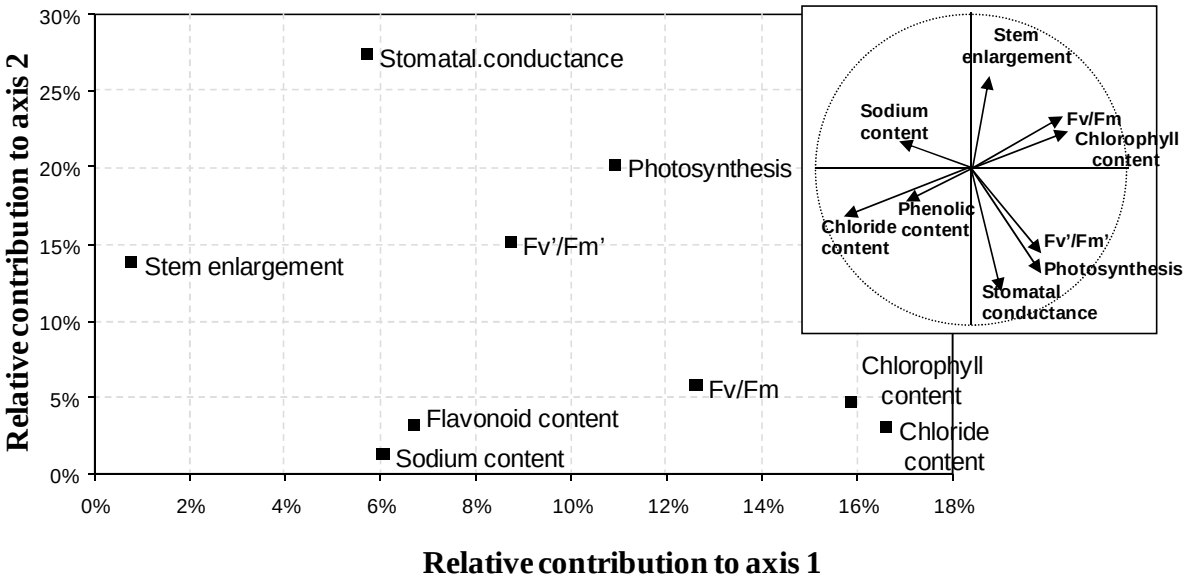


Fig 5: contribution of each physiological factor to first two axes of PCA explaining the diversity of stressed plant compared relatively to control.

5.3 Construction of a genetic map of an inter-generic hybrid (Cleopatra mandarin x *Poncirus trifoliata*) and positioning of candidates salt tolerance genes

The environmental factors have a major influence on plant salt stress responses and hindered the direct selection of salt-tolerant genotypes under field conditions (Richards 1996). It has been reported that salt tolerance is a complex trait involving the function of many genes (Foolad 2004; Lindsay *et al.* 2004). One of recently used ways of studying the genetic determinants of phenotypic traits is the production of genetic maps. This involves determining the relative position of loci (genes or sequences) on chromosomes but also the effect of these loci on the quantitative character expression. The scheduling and distance separating the loci are estimated from the frequency of recombination occurring in meiosis. A segregating population is necessary to estimate the frequency of recombination. Linkage group, in genetics means all of the genes on a single chromosome. They are inherited as a group and during cell division move as unit rather than independently. So, studying the segregation of phenotypic characteristics in this lineage made possible to identify chromosomal region, containing genes putatively involved in the expression of desired trait. If the character is a quantitative value as salt tolerance chromosomal region are name by the term "QTL" for Quantitative Trait Loci. We can count, localize but also measure the QTL effect (positive or negative) about the cast. Then from this array of markup (genetic map) we can try to identify the genes putatively involved in the QTL by a series of technical approaches involving the cloning of large fragments of DNA, sequencing and verification of involved genes by plant transformation. Another possibility for exploitation of genetic maps and the use of markers near to the QTL regions is to predict their presence in hybrids of lineage in an established pattern of recurrent selection. These markers are useful for reducing the size of the population at each generation but also for reducing the number of generations necessary for the obtention of desired hybrid combining different suitable characters. Generally an efficient selection scheme is time consuming. Molecular markers mapped near genes or QTLs allow accelerating this process. This approach also helps to control the hybrid containing the desired traits. Recent development in the field of molecular biology has enabled the development of DNA markers that can be used to identify potential QTLs (Yamaguchi and Blumwald 2005). The use of QTLs has improved the efficiency of selection, in particular for those traits that are controlled by several genes and are highly influenced by environmental factors (Flowers

2004). QTLs and marker-assisted selection provide several advantages over direct phenotypic screening, particularly because the PCR-based methodologies used to detect the markers reduce the time needed to screen individuals and reduce the impact of environmental effects on the trait under study. There is considerable evidence to support the view that salt tolerance and its sub-traits are determined by multiple QTLs and that both additive and dominance effects are important in the inheritance of many of the traits associated with salt tolerance (Flowers 2004; Foolad 2004; Yamaguchi and Blumwald 2005). Recent advances in molecular genetics techniques, including genetic transformation, marker mapping and quantitative trait loci (QTLs) analysis, have contributed significantly to a better understanding of the genetic, physiological and biochemical bases of plant salt tolerance and have facilitated the development of plants with enhanced salt tolerance (Foolad 2004).

In citrus, many whole or partial genetic maps have been developed over the past decade (Table 5). Each has a different mapping population type and size, genome coverage, and marker systems. Most of these maps were covered by a majority of randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and/or inter-simple sequence repeat (ISSR) markers, and a limited number of restriction fragment length polymorphism (RFLP), SSR, sequence characterized amplified region (SCAR), cleaved amplified polymorphic sequence (CAPS), sequence tagged site (STS), or single/simple nucleotide polymorphism (SNP) markers. The linkage groups of the current maps are not all uniform in length and marker saturation (Roose 2007), whereas the cytogenetic evidence suggests that the chromosomes of citrus are similar in length or show a continuous gradation in size (Yamamoto and Tominaga 2004). The reason is that map distance strictly depends on level of polymorphism between two parents. However, an increased number of polymorphic markers and progenies would be a distinct advantage for marker-assisted selection and for cloning quantitative trait loci (QTLs) and other loci responsible for traits of horticultural importance (Gulsen *et al.* 2010). Further saturation of the map is especially desirable to resolve ambiguities in the correlation of cytogenetic evidence and linkage group characteristics.

Table 5: Developed citrus genome maps

Crosses name	Crosses type	Marker types	Markers	Linkage	cM	References
C. grandis x *C. jambhiri*	Citrus F1	Isozymes	5	2		(Torres *et al.* 1985)
C. grandis x *P. trifoliata*	Intergeneric F1					
(*C. reticulata* x *C. paradisi*) x *C. reticulata*	Citrus BC1	RFLP, isozymes	35	8	314	(Liou 1990)
C. grandis x *P. trifoliata*	Intergeneric BC1	RFLP, isozymes	52	11	533	(Durham *et al.* 1992)
Sacaton (*C. paradisi* x *P. trifoliata*) x Troyer (*C. sinensis x P. trifoliata*)	Intergeneric F1	RFLP, isozymes	38	10	351	(Jarrell *et al.* 1992)
C. grandis x *P. trifoliata*	Intergeneric BC1	RAPD, RFLP, isozymes	189	9	1,192	(Cai *et al.* 1994)
C. grandis x *C. grandis*	Citrus self F1		34 for *C. grandis*	7	600	(Luro *et al.* 1996)
C. reshni x *P. trifoliata*	Intergeneric F1		95 for *Poncirus*	12	1503	
Sacaton (*C. paradisi* x *P. trifoliata*) x Troyer (*C. sinensis x P. trifoliata*)	Intergeneric F1	RFLP, ISSR	48	12	410	(Kijas *et al.* 1997)
C. aurantium x *C. latipes*	Citrus F1	AFLP, RAPD, RFLP	247 for *C. aurantium*	20	1,000	(De Simone *et al.* 1998)
C. aurantium x *C. latipes*	Citrus F1		92 for *C. latipes*	12	600	
Sacaton (C. paradisi x P. trifoliata) x Troyer (*C. sinensis x P. trifoliata*)	Intergeneric F1	RFLP, ISSR, RAPD	156	16	701	(Roose *et al.* 1998)
C. grandis x (*C. grandis* x*P. trifoliata*)	Intergeneric BC1	AFLP, RFLP, isozymes	337	11	1,026	(Ling *et al.* 1999)
C. volkameriana x *P. trifoliata* cv Rubidoux	Intergeneric F1	RAPD, RFLP, CAPS, isozyme	69	3		(Garcia *et al.* 1999)
C. sunki x *P. trifoliata*	Intergeneric F1	RAPD	63 for *C. sunki*	10	732	(Cristofani *et al.* 1999)
			62 for *P. trifoliata*	8	866	
(*C. unshiu* x *C. sinensis*) x *C. unshiu*	Citrus BC1	CAPS	120	9	801	(Omura *et al.* 2000)
C. grandis x (*C. grandis* x*P. trifoliata*)	Intergeneric F1	ISSR, RAPD, RFLP, isozymes	310	9	874	(Sankar and Moore 2001)
C. aurantium x *P. trifoliata*	Intergeneric F1,	RAPD, SSR, IRAP	48 for *Poncirus*	10		(Ruiz and Asins 2003)
C. volkameriana x *P. trifoliata*	Intergeneric F1 and Poncirus		120 for *C. aurantium*	17		
P. trifoliata cv Flying Dragon	self F1					
C. sinensis X *P. trifoliata*	Intergeneric F1	EST-SSR	141	11	775.8 425.7	(Chen *et al.* 2008)
Clementine mandarin xOrlando tangelo	F1	SRAP, SSR, ISSR, POGP, RGA and RAPD	609	9	858 886	(Gulsen *et al.* 2010)

Modified from (Chen *et al.* 2008)

5.3.1 Generating a F2 population

As part of the genetic study of tolerance to salt stress we undertook the study of genetic determinants by producing genetic map. Firstly we have produced a F2 population suitable for genetic map construction. To do this, we created an F2 population by self pollination of F1 hybrid obtained from cross between (Cleopatra mandarin x *Poncirus trifoliata*). The choice of this hybrid has been governed by the salt stress tolerance of mandarin and sensitivity of Poncirus ensuring the segregation of the tolerance trait in the progeny.

Self pollination of hybrid F1 (Cleopatra mandarin x *Poncirus trifoliata*) was performed to produce F2 segregating population. Hybrid F1 has characteristic of polyembryony produces the seed with more than one embryo. Only zygotic individuals of F2 population were useful to study the segregating characters from both parents. So screening for zygotic individual was done on F2 population. Initially from a population of 800 seedlings 110 zygotic genotypes

were selected. Five heterozygous SSR markers were used to identify zygotic genotype with gel electrophoresis. Before the start of genotyping total segregation population of 110 plants were reanalyzed by with new extraction of DNA on polyacrylamide gel electrophoresis and only 60 genotypes were reconfirmed as zygotic. So there were 50 plants that finally were identified as false zygotic. It could be due to wrong identification of plantlets because seeds were sown randomly into the box. Indeed seedlings were tagged but as the density of seedling was high, we may assume that it could generate identification errors. We did not find any other reason for this event.

Genotyping was started on this population (60 hybrids) with the help of SSR markers by using the M13 tailing method for amplification (Steffens *et al.* 1993). Our results of markers segregation for 60 hybrids showed that half of individuals presented a lack of homozygosity not showed a balanced percentage of heterozygous and homozygous loci as suggested by the theory of Mendelian segregation (Fig 7). In extreme cases this defect is materialized by a percentage of minimal homozygous loci (only 1 for 20 heterozygous loci). The rest of 30 hybrids followed the normal distribution according to Mendelian segregation. The large shift of Gaussian curve was observed for no of loci (~ 0.35 instead of 0.5) (Fig. 5A & B). This result suggests putative malfunctioning of meiosis in the inter-generic hybrid or viability problems for certain allele combinations in F2 hybrids. A technical disorder during marker amplification could be also suggested as explanation for this segregation shift (Arruda *et al.* 2010).

To resolve this incertitude, the 30 genotypes previously identified as zygotic with abnormal allelic constitution were reanalyzed by polyacrylamide gel electrophoresis with the help of SSR markers suspected to be critical in the genotyping results. The amplification method has been changed avoiding the tailing with M13 extension. These individuals found finally to be clones of the F1 hybrid (nucellar origin) because all used markers gave an identical profile to the mother tree. These results were probably related to the use of primers for PCR with an extension of an 'M13' oligonucleotide that would generate a competition between alleles of one locus leading to a false homozygosity or/and incorrect identification of zygotic seedlings (Arruda *et al.* 2010). M13 tailling method is usually performed in genetic programs based on automatic sequencer analysis for population genotyping. It reduces the cost of genotyping and simplifies the analysis of amplicons. This putative technical troubleshooting has never been referenced before in the literature. We have not estimated the impact of this mistake on mapping construction and marker linkage analysis but it depends on its frequency. This badly effects the number of population which reduces to 30 plants

insufficient to construct a genetic map. Therefore it was necessary to increase the hybrid population.

A second series of self pollination of the F1 hybrid was performed to create F2 segregating population in spring 2008 to increase the population size. To avoid any false identification of zygotic genotypes, seeds were sown individually with large sowing distance (3-4 cm) in lines. After germination seedlings were tagged and also separated by thin string in small squares before leaf collection for DNA extraction (Fig. 5). About 700 seedlings were tested using 4 microsatellite markers without M13 tailing to identify zygotic genotypes. 69 additional hybrid seedlings were selected and then genotyped. Total population of 99 zygotic genotypes was finally used to study the marker segregation (Fig. 6).

5.3.2 Segregation analysis

Mapping can be conducted with a variety of markers types. In general there are two types of markers co-dominant markers (SSRs, RFLPs, CAPS etc.) and dominant markers (RAPDs, ISSRs, AFLPs). For a cross in which most markers are expected to have 1:1 segregating ratio, dominance does not affect the mapping. However, for an F2 type population co-dominant markers are more informative. Among the SSR's markers (Total 579: 400 MEST, 79 BAC end and 100 genomics) used for initial screening of both parents and their hybrid F1, 444 markers either failed to amplify any DNA bands, or produced no heterozygosity for hybrid F1. These primers were not used for further analysis. The rest of the markers (Total 135: 64 MEST, 50 BAC end and 21 genomic) which is about 23% of all random marker tested) produced consistent and obvious polymorphic DNA fragments, and were subsequently used for screening the genomic DNA of the 99 hybrids from the progeny population for segregation.

From 135 SSR markers and four candidate genes used, 52 SSR markers and 2 candidate genes have followed the normal theory of Mendelian segregation (Fig 5: C and D). Distorted segregation ratios departing from the expected 1:2:1 Mendelian ratio (α=0.05) were detected in 83 SSR markers and 2 candidate genes. These distortions are manifested by an excess frequency of one allele compared to the other or excess of heterozygote as compared to homozygote allele combinations (Table 6). From 83 markers presenting distorted segregations, 38 markers (46%) were distorted by an excess of Cleopatra mandarin alleles in population, 22 markers (26%) presented an excess of Poncirus trifoliate alleles, 16 markers (19%) showed an excess of heterozygote loci and 7 markers (8%) were distorted by excess of

both heterozygote loci and one of allele from Cleopatra mandarin or Poncirus trifoliata. Marker CiBE1324 illustrates the extreme situation it can be observed in distorted segregation where 90% of the F2 hybrids are heterozygous and 10 % homozygous for poncirus allele. In this situation no homozygous loci sharing a mandarin allele was detected. The heterozygous locus seems to be the most favorable allelic combination for this marker. Two candidate genes, *LaPXc* and *CAx4c*, putatively involved in salt tolerance were also distorted due to excess of heterozygote loci.

5.3.3 Linkage group analysis

Linkage relationships of the segregating markers were compared for independence of segregation at LOD 4. Overall, linkages were robust at LOD scores ranging from 3.0 to 4.0. For example, there were 10 linkage groups at LOD score 3 while at LOD score 4 there were 15 linkage groups. Generally, the LOD scores determined the agglomeration or fragmentation of some linkage groups; larger LOD scores tended to increase the number of linkage groups. Only 132 SSR's markers were positioned on genetic chart. Four candidate genes putatively involved in salt tolerance were also positioned on this genetic map.

The total map length of hybrid F1 is 888.709 cM (139 markers). The average distance (cM/marker) between markers is 6.73 cM. Two gaps of 25 cM between markers are located in linkage group 3 (1) and linkage group 15 (1) and eight more gaps (≥ 15 cM) are located on linkage groups 1 (1), 2 (2), 3 (1), 5 (2), 7 (1) and 8 (1). The smallest gap between markers is located in linkage group 1 between CIBE 5866 and MEST 287. *ADc* and *CAx4c* was positioned at linkage group 1 the distance between two genes was 77.754 cM. Gene *Adc* is located at very dense place in hybrid 1 where average distance is 1.3 cM between markers (from 50 cM to 59 cM: with 6 markers), which could potentially inherited together. Gene *LaPXc* was found on linkage group 12 and *NHX1c* was positioned on linkage group 3.

We also compared the genetic map of the F1 hybrid with the international reference genetic map of the clementine (Ollitrault *et al.* 2008) (Fig. 8). All F1 linkage groups have at least one marker in common with clementine linkage groups except linkage groups 6, 7, 9 and 11. All of SSR markers in these groups were more or less distorted except in linkage group 11 where 2 markers followed the Mendelian segregation. F1 linkage group 1 had 7 markers common with clementine group 9. Also, most of the markers in hybrid linkage group 1 were highly distorted. Same was the case for common markers between hybrid group 1 and clementine group 9. On other hand hybrid linkage group 2 showed 8 SSR markers common

with clementine group 4. In hybrid group 2, all markers were segregated according to Mendelian law except CIBE 5055. This SSR marker in clementine group 4 is also highly distorted. Apart from CIBE 5055 four markers common between both groups were normally segregated. Both hybrid group 10 and 4 have markers common with clementine group 5. Markers on these two groups were not distorted except for Ci04H01b and CIBE1098 on group 4. Hybrid linkage group 13 presented similarities with clementine group 6, 2 SSR markers were common in both groups. Clementine group1 showed only one marker common with hybrid group 15.

We found a total 35 markers (20 MEST, 7 BAC end and 8 genomics) common in both genetic maps and in most of them co-linearity is preserved. Nevertheless, some reversals of order are also observed (Fig. 8). The inversion of some shared loci on Clementine and Hybrid F1 maps might be due to some chromosomal rearrangement events in F1 hybrid, such as translocations or inversions, involving these specific loci (Omura *et al.* 2000; Ruiz and Asins 2003). Maybe also the effect of segregation distortion can be impacts the distance and arrangement between markers. In our linkage analysis we have 15 group instead of 9 linkage groups same as chromosomes number.

5.3.4 Distribution on genetic map of markers showing a skewed segregation

The markers showing a skewed segregation are not randomly distributed on the genetic map. They are concentrated on several linkage groups. Moreover, considering the parental origin of the allele in excess at these loci their repartition is organized by grouping the markers characterized by a type of allelic combination in excess. Over-represented mandarin homozygous loci are located on linkage groups 8, 5, 3, 15, 7 and 6 while poncirus homozygous loci in excess are located on linkage groups 1 and 11. Markers with abnormal low frequency of both parental homozygous forms are grouped in one extremity of the linkage group 1 and in the linkage group 9.

The high percentage of markers with distorted segregation (57%) and their organized distribution along the genetic map suggest a default of chromosome pairing during meiosis that could arise from a lack of complementarities between few mandarin and Poncirus homologue chromosomes or by a selection of more favorable alleles during gametogenesis. Such situations are frequently encountered in the literature where parent's pedigrees are genetically distant (Chen *et al.* 2008). The literature reports skewed segregation markers in

genetic linkage maps at several levels at intergeneric as well as intraspecific hybrids with frequencies between 3.9% (Grattapaglia and Sederoff 1994) and 100% (Nienhuis *et al.*). Abnormal mendelian segregation does not depend from the type of markers as demonstrated by different studies e.g. RFLP markers showed 37% segregation distortion (Durham *et al.* 1992), RAPD markers exhibited 40% (Cai *et al.* 1994), a combination of RFLP and isozymes demonstrated 20% (Jarrell *et al.* 1992) and microsatellite markers displayed 22% segregation distortion (Kijas *et al.* 1997). These distorted segregations are probably linked to genetic disorders modifying the allelic segregation. Also it is interesting to know that most of the previous genetic map studies on citrus, or generally on fruit trees, involve the construction of genetic map of both parents by exploiting the F1 hybrids population and the heterozygous loci of parental genomes. But we constructed the F1 hybrid linkage map based on the segregation of SSR markers by exploiting into a F2 progeny. It supposed that the meiosis of an intergeneric hybrid could display chromosome miss-pairing, recombination disorders or allelic selection during gametogenesis more frequently than in other segregating populations.

Genetic map construction is more than a tool for genetic studies of phenotypic characters by the information about the genome structure and the segregation of markers or linkage groups. It allows to study the efficiency and the feasibility of genetic improvement based on intergeneric sexual crosses in citrus. The negative selection of linkage group containing alleles from one parent can be impact the obtaining of suitable hybrid if the researched allele is carried by this parental genome. To complete our work, additional segregating SSR markers, with a more even and broad distribution across the genomes will certainly be needed for an unbiased coverage of all the chromosomes. The present genetic map is a first step towards the QTL,s analysis of salt tolerance trait in F2 segregating population. In future more SSR markers will be added to present map to a large markup of genome. Also, an experiment on the F2 population of the physiological response of trees to salt stress treatment will be conducted.

Fig. 5: Separation and tagging of seedlings before DNA extraction.

Marker CiBE5866

Fig. 6: Genotyping of F2 population.

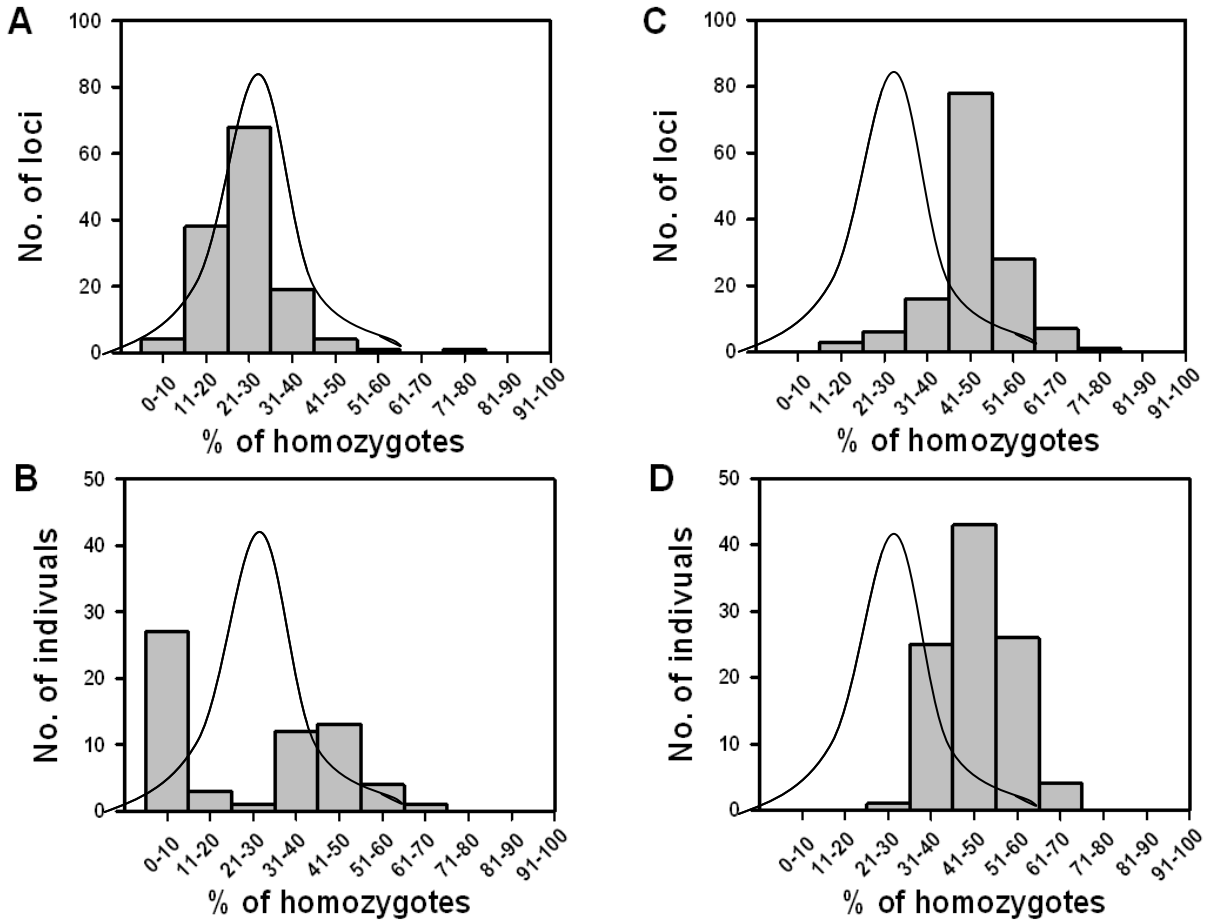


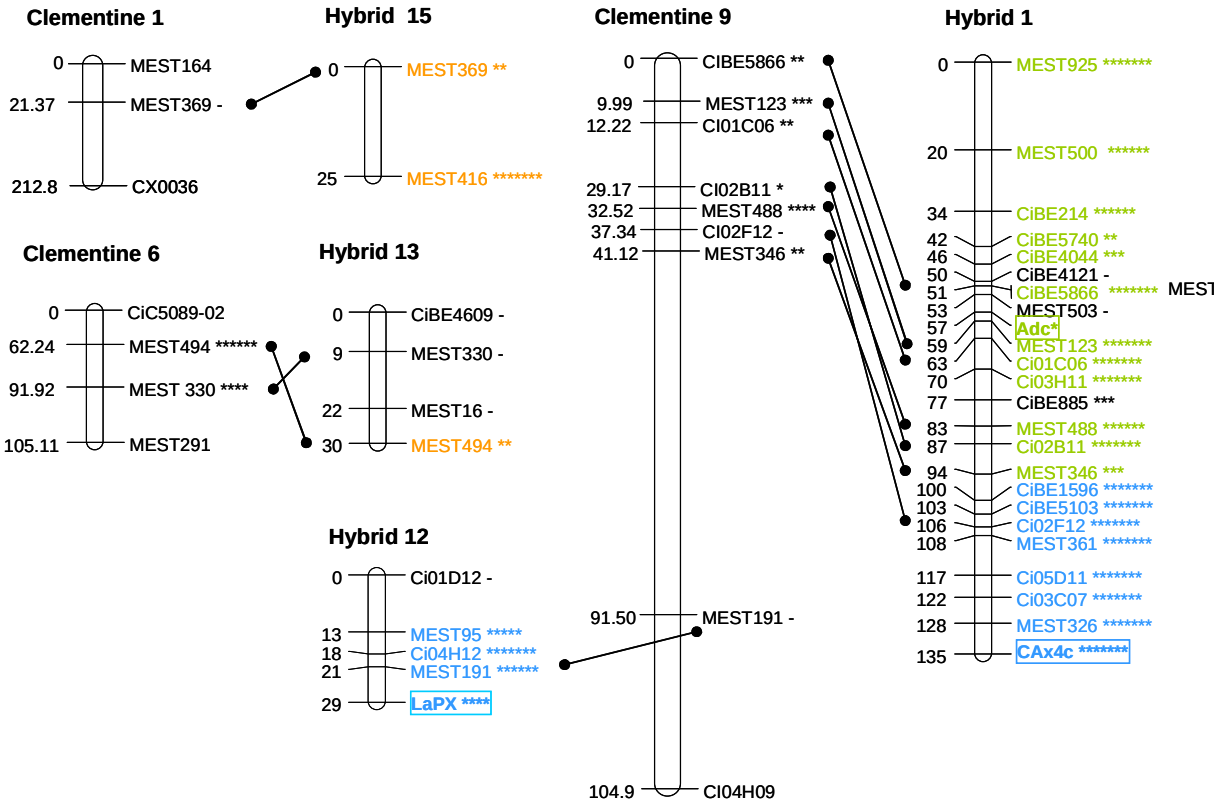
Fig. 7: Skewed frequencies of homozygous locus and individual in segregating F2 progeny

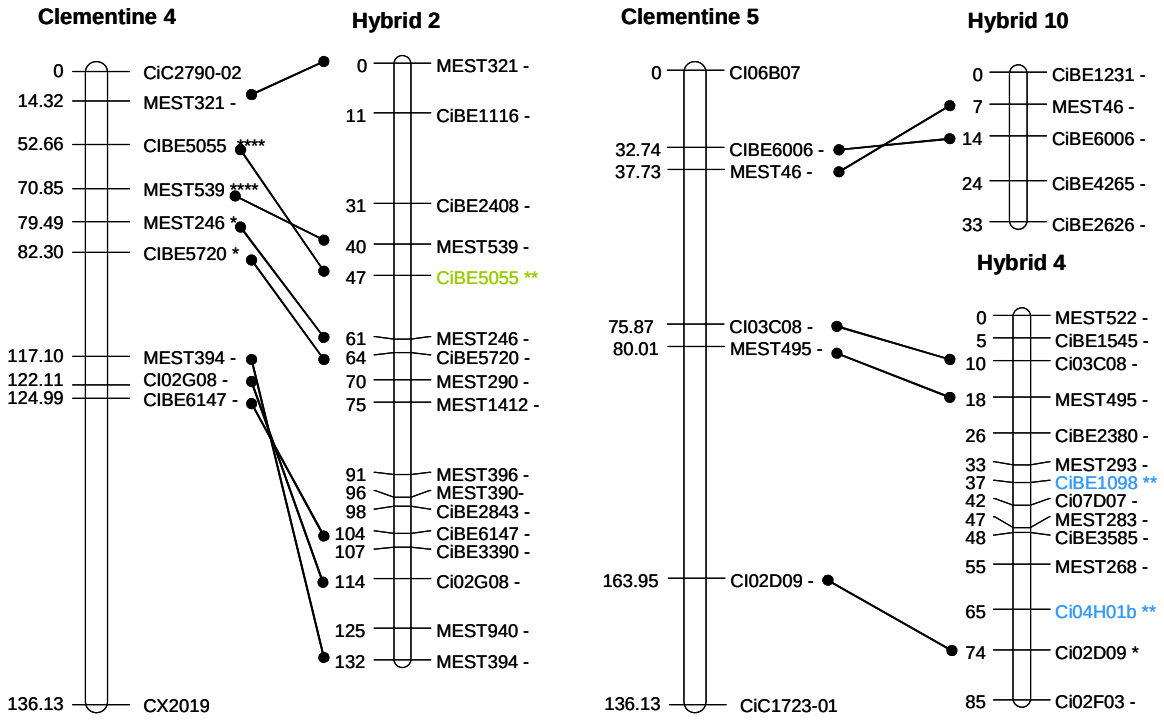
Graph (A and B) presented Skewed frequencies of homozygotes for No. of loci and no. of individuals respectively for first series of 60 hybrids. Also we can clearly observe the shift of Gaussian curve for no of loci and no of individuals ~ 0.35 and 0.45 instead of 0.5 respectively. Graph (C and D) present the % of homozygote for no. of loci and individuals of F2 segregating population of 99 hybrids. Gaussian curve for no of loci and no of individuals is not shifted from (~ 0.5).

Table 6: Name of locus presenting the distorted segregation ratios departing from the expected 1:2:1 Mendelian ratio (α=0.05). a= Cleopatra mandarin, b= poncirus trifoliata and h= Heterozygous (hybrid F1)

	Locus	a	h	b	X2	Si
1	CI02D09	16	53	3	4.	*
2	CiBE230	31	54	1	5.	*
3	Mest104	31	53	1	4.	*
4	Mest120	25	60	1	5.	*
5	Mest417	31	54	1	5.	*
6	Mest404	33	49	1	5.	*
7	CiBE442	35	47	1	5.	*
8	Mest309	24	60	1	5.	*
9	ADc	16	51	3	5.	*
10	CiBE116	19	64	1	7.	**
11	CiBE109	14	63	2	8	**
12	CiBE469	35	51	1	8.	**
13	CiBE505	17	46	3	7.	**
14	CiBE574	25	38	3	7.	**
15	Mest149	24	61	1	6.	**
16	Mest369	30	56	1	6.	**
17	Mest540	13	53	3	9.	**
18	CI01F08	36	47	1	7	**
19	CiBE496	22	62	1	6.	**
20	Mest232	19	44	3	7.	**
21	Mest494	31	57	1	8.	**
22	CI04H01	18	65	1	8.	**
23	Mest504	33	55	1	9.	**
24	CiBE404	19	42	3	9.	***
25	CiBE508	27	62	1	10	***
26	Mest346	11	59	3	10	***
27	Mest389	31	58	1	10	***
28	CiBE885	11	61	2	9.	***
29	CiBE216	10	57	3	12	***
30	CiBE422	32	58	1	12	***
31	CiBE753	28	63	9	13	***
32	Mest102	39	37	2	11	***
33	Mest367	12	52	3	11	***
34	LaPXc	12	67	2	13	***
35	Mest444	33	57	9	13	***
36	CiBE39	37	53	1	15	***
37	Mest506	39	48	1	14	***
38	Mest95	12	69	1	14	***
39	CiBE206	31	61	8	14	***
40	Mest419	35	56	9	14	***
41	Ci02A04	42	40	1	16	***
42	CiBE422	9%	53	3	17	***
43	CiBE477	43	39	1	18	***
44	Mest191	11	71	1	18	***
45	Mest422	39	49	1	15	***
46	Mest425	37	54	9	16	***
47	Mest488	8%	57	3	16	***
48	CiBE105	37	55	8	17	***

49	Mest500	13	45	4	17	***
50	CiBE214	14	43	4	16	***
51	CiBE325	43	39	1	17	***
52	Ci02B11	5%	55	4	25	***
53	Ci02D04	44	38	1	20	***
54	Ci02E08	19	25	5	50	***
55	Ci03C07	9%	81	1	37	***
56	Ci03H11	6%	47	4	32	***
57	Ci05D11	7%	80	1	35	***
58	CiBE132	0%	90	1	65	***
59	CiBE159	4%	69	2	24	***
60	CiBE246	41	47	1	18	***
61	CiBE479	41	51	8	22	***
62	CiBE572	38	57	5	23	***
63	CiBE633	45	38	1	22	***
64	Mest297	55	33	1	46	***
65	Mest326	8%	75	1	25	***
66	Mest72	35	59	6	19	***
67	Mest998	39	57	4	26	***
68	Ci03F09	39	55	6	21	***
69	CiBE510	6%	71	2	23	***
70	Mest123	6%	48	4	31	***
71	Mest146	46	43	1	25	***
72	Mest361	7%	72	2	23	***
73	Mest511	35	60	5	21	***
74	Ci02F12	7%	69	2	19	***
75	CiBE529	6%	49	4	28	***
76	Mest925	24	31	4	23	***
77	CAx4c	5%	81	1	39	***
78	Ci01C06	6%	49	4	28	***
79	Ci04H12	10	75	1	24	***
80	Mest474	40	58	2	29	***
81	CiBE494	44	38	1	18	***
82	CiBE586	8%	52	4	19	***
83	Mest416	34	64	2	26	***


Clementine 1
0 MEST164
21.37 MEST369 -
212.8 CX0036
Hybrid 15
0 MEST369 **
25 MEST416 *******
Clementine 6
0 CiC5089-02
62.24 MEST494 ******
91.92 MEST 330 ****
105.11 MEST291
Hybrid 13
0 CiBE4609 -
9 MEST330 -
22 MEST16 -
30 MEST494 **
Hybrid 12
0 Ci01D12 -
13 MEST95 *****
18 Ci04H12 *******
21 MEST191 ******
29 LaPX ****
Clementine 9
0 CIBE5866 **
9.99 MEST123 ***
12.22 CI01C06 **
29.17 CI02B11 *
32.52 MEST488 ****
37.34 CI02F12 -
41.12 MEST346 **
91.50 MEST191 -
104.9 CI04H09
Hybrid 1
0 MEST925 *******
20 MEST500 ******
34 CiBE214 ******
42 CiBE5740 **
46 CiBE4044 ***
50 CiBE4121 -
51 CiBE5866 ******* MEST
53 MEST503 -
57 Adc*
59 MEST123 *******
63 Ci01C06 *******
70 Ci03H11 *******
77 CiBE885 ***
83 MEST488 ******
87 Ci02B11 *******
94 MEST346 ***
100 CiBE1596 *******
103 CiBE5103 *******
106 Ci02F12 *******
108 MEST361 *******
117 Ci05D11 *******
122 Ci03C07 *******
128 MEST326 *******
135 CAx4c *******


Clementine 4
0 CiC2790-02
14.32 MEST321 -
52.66 CIBE5055 ****
70.85 MEST539 ****
79.49 MEST246 *
82.30 CIBE5720 *
117.10 MEST394 -
122.11 CI02G08 -
124.99 CIBE6147 -
136.13 CX2019
Hybrid 2
0 MEST321 -
11 CiBE1116 -
31 CiBE2408 -
40 MEST539 -
47 CiBE5055 **
61 MEST246 -
64 CiBE5720 -
70 MEST290 -
75 MEST1412 -
91 MEST396 -
96 MEST390-
98 CiBE2843 -
104 CiBE6147 -
107 CiBE3390 -
114 Ci02G08 -
125 MEST940 -
132 MEST394 -
Clementine 5
0 CI06B07
32.74 CIBE6006 -
37.73 MEST46 -
75.87 CI03C08 -
80.01 MEST495 -
163.95 CI02D09 -
136.13 CiC1723-01
Hybrid 10
0 CiBE1231 -
7 MEST46 -
14 CiBE6006 -
24 CiBE4265 -
33 CiBE2626 -
Hybrid 4
0 MEST522 -
5 CiBE1545 -
10 Ci03C08 -
18 MEST495 -
26 CiBE2380 -
33 MEST293 -
37 CiBE1098 **
42 Ci07D07 -
47 MEST283 -
48 CiBE3585 -
55 MEST268 -
65 Ci04H01b **
74 Ci02D09 *
85 Ci02F03 -

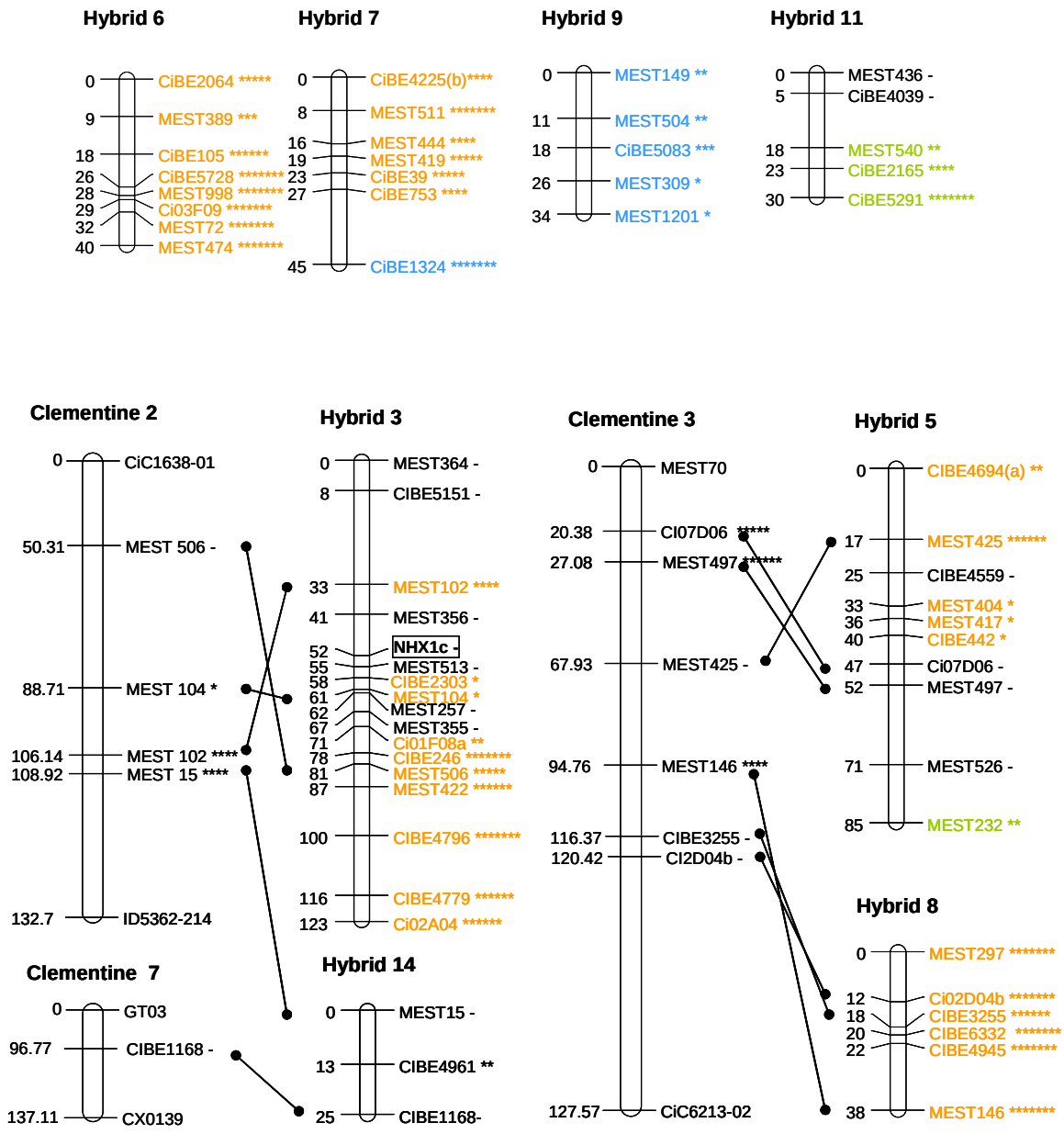


Fig. 8: Linkage group for hybrid F1 (Cleopatra mandarin X Poncirus trifoliata) and their comparison with reference map of clementine

Marker in orange colour presented the excess of mandarin allel, marker in green color presented the excess of poncirus aller and blue color represent the excess of heterzygote

5.4 Article No 3: Facultative apomixis and chromosome doubling are heterogeneity sources in citrus rootstock trials: impact on clementine production and breeding selection

(Submitted in: *Euphytica*)

Sajjad Hussain[1,2], Franck Curk[1], Patrick Ollitrault[3], Raphaël Morillon[3], François Luro[1*].

1. Unité de Recherche Génétique et Ecophysiologie de la Qualité des Agrumes, Inra. Centre Inra de Corse, F-20230 San Giuliano, France.

2. Unité Propre de Recherche amélioration génétique des espèces à multiplication végétative, Cirad. Centre Inra de Corse, F-20230 San Giuliano, France.

3. UPR amélioration génétique des espèces à multiplication végétative du Cirad. Instituto Valenciano de Investigaciones Agrarias 46113 - Moncada – Valencia, Spain.

*__Corresponding author:__ François Luro. Unité de Recherche Génétique et Ecophysiologie de la Qualité des Agrumes, Centre Inra de Corse, 20213 San Giuliano, France.

Phone: (00 33) 495 59 59 46

Fax: (00 33) 495 59 59 37

E-mail: luro@corse.inra.fr

Key words: Polyembryony, Clementine, fruit production, *Poncirus trifoliata*, tetraploid, zygotic rootstock, nucellar rootstock.

Abstract

All commercial citrus rootstocks are polyembryonic and are propagated by seeds allowing, most of the time, the propagation of conform to type plant material. However, in citrus seedlings, zygotic or polyploid plants may arise. The aim of our study was to understand how the presence of zygotic or tetraploid rootstocks can affect a field trial for rootstock selection. A trifoliate orange selection field trial planted in 1974 grafted with clementine was newly investigated taking account of the presence of not conform rootstocks. Using flow cytometry, 2.4 % of the rootstocks were shown to be tetraploid and 6.6 % of zygotic rootstocks were identified using SSR markers among 288 trees. Yield data showed that the presence of tetraploid rootstocks in the field dramatically decreased (about 45%) clementine fruit production. The presence of zygotic did not affect always fruit production with a wide range of behavior (slight increase to 24% decrease). Concerning results of rootstocks selection, by removing the non true-to-type genotypes, the better candidate was a clone previously ranged at the middle score among all the 32 rootstocks in evaluation. However, analyze of fruit quality performed during the first years of the plot independently of the presence of zygotic rootstocks did not reveal any significant changes suggesting that not true-to-type plants masked changes of fruit quality parameters. According to our results, tetraploid and zygotic rootstocks have a strong impact on fruit production in orchards and rouging of off-type seedlings prior planting any agronomical trial is then required.

Introduction

All citrus trees cultivated for commercial use are nowadays grown grafted on rootstocks. Grafting technique has the advantage of maintaining true-to-type varieties and the use of rootstocks reduce to some extent the adverse effect of abiotic constraints such as water logging, drought, salinity, alkalinity or may confer tolerance to *Phytophthora, nematodes* and tristeza virus (Soost *et al.* 1975). The first citrus rootstock known to be used is sour orange (*Citrus aurantium* L.) which is resistant to *Phytophthora* but sensitive to tristeza when grafted (Bar-Joseph and Lee 1989; Louzada *et al.* 2008). The damaging effect of tristeza worldwide constrained citrus producing countries to run new rootstock selection trials to replace sour orange. *Poncirus*, trifoliate orange, and its hybrids have been selected because of their tolerance to tristeza (Garnsey *et al.* 1987). Selection of new rootstocks is nowadays based on production and tolerance towards biotic and abiotic stresses. In Corsica, trifoliate orange has been also selected, not only because of its resistance to tristeza but also because of its capacity to improve clementine yield and quality in acidic soil conditions (Jacquemond *et al.* 2004). The effect of rootstocks on scion citrus fruit production and quality has been extensively reported in the literature (Wheaton *et al.* 1991; Fallahi and Rodney 1992; Economides and Gregoriou 1993; Zekri 1996; Jacquemond *et al.* 2004; Zekri and Al-Jaleel 2004). All citrus rootstocks are polyembryonic and propagated by seeds. A polyembryonic seed produces two or more embryos; one has a sexual origin (zygotic) and all the other embryos have a somatic origin (nucellar) and consequently mother tree clones. Nucellar seedlings therefore are actually genetically identical to their maternal parent. On the other hand the production of a zygotic embryo arises from the fusion of the male and female parent gametes. The occurrence of zygotic in citrus seedlings varies both with the genotype (Cameron and Frost 1968; Khan and Roose 1988) and environmental factors such as the tree age, tree nutrient status, seasonal effects or crop load (Khan and Roose 1988). In polyembryonic citrus seedlings several studies using isozyme analysis show that there may be from 1 to 50% zygotic plants depending of the rootstock genotype (Ashari *et al.* 1988; Khan and Roose 1988; Moore and Castle 1988; Roose and Kupper 1992; Xiang and Roose 1988). Analysis of the frequency of zygotic seedlings among genotypes of three cultivars of trifoliate orange showed a percentage ranging from 0 to 76 (Khan and Roose 1988) with in some cases large changes in phenotypical traits.

The occurrence of zygotic genotypes for 24 selections of trifoliate orange in a rootstock trial was previously investigated (Roose and Traugh 1988). Analyze of the bark of the rootstocks using isozymes revealed a percentage of zygotics below to 4% among trifoliate

orange rootstocks, the production of those off-type rootstocks being most of the time lower compared to nucellar genotypes.

In citrus, doubling of the chromosome number may occur during seed formation which leads to tetraploid seedlings. These tetraploids arise from chromosome set doubling of nucellar cells (Cameron and Soost 1969). Thus, spontaneous doubled diploid clones obtained from specific diploid citrus species are considered to be genetically identical, with the same genome expression profile. Spontaneous tetraploid plants arise in seedlings with a frequency varying from 1 to 11% depending of the rootstock analysed (Barrett and Hutchison 1978; Saleh *et al.* 2008) and is supposed to be dependant on the environment (Barrett and Hutchinson 1982). The effect of citrus tetraploid rootstocks on fruit production and quality is poorly documented. *Poncirus trifoliata* "tetraploid N° 1" showed more dwarfing effect and lower fruit quality than diploid form of *Poncirus* trifoliate orange (Tutberidze and Kalandarishvili 1975).

Observations of important changes in citrus tetraploid morphology compared to diploid date back a long time. These include thicker and greener leaves, larger fruit and depressed growth compared to diploids (Cameron and Frost 1968; Ollitrault and Jacquemond 1994). Additionally, tetraploid citrus seedlings have lower growth rates than their respective diploid parents, which are associated with lower rates of whole-plant transpiration (Syvertsen *et al.* 2000).

In nurseries, citrus seedlings are not 100% true-to-type even through visual screenings is preformed in order to try to rogue off-type seedlings. If both zygotic and tetraploid rootstocks are not identified with certitude, those none true-to-type genotypes may lead to errors about performances of varieties and rootstocks. Nowadays, numerous molecular tools useful for detection of genetic variants are making this task easier and much more efficient than visual phenotyping. The objective of this study was to clear up the impact of off-type genotypes rootstocks on citrus fruit quality and production. For that purpose, a trial planted in 1974 was reanalyzed by using rapid and low cost molecular biology tools for the selection of genetically homogeneous rootstocks.

Materials and methods

Experimental trial

From seedlings, one year old trifoliate orange plants (*Poncirus trifoliata* (L.) Raf.) were selected visually according to their homogeneous phenotype before being grafted with clementine "Commune" buds (*Citrus reticulata* Blanco x *Citrus sinensis* (L.) Osb.). 32 trifoliate orange genotypes were tested on this trial. Trees have been randomly planted in nine blocks at a planting distance of 6 m x 4 m at the INRA-CIRAD Research Station in Corsica, France in 1974. After five years of growing first fruit yield were harvested.

From November to the end of December, corresponding to the clementine fruit maturity period, fruit of each tree from each block was harvested and weighed. Measurements of annual yield were taken from 1979 to 1990 (11 years). The harvest was performed in one or two times (usually after an interval of two weeks) according to fruit coloring.

Fruit quality analysis of clementine was investigated for 5 consecutive years 1981 to 1985. In contrast to fruit yield, for fruit quality measurements only 4 blocks were selected and from each block 20 fruits per genotype, which had the same color and the same medium diameter, were weighed and hand pressed. The juice retrieved was weighed to calculate the juice percentage (JP) and juice density.

Total soluble solids (TSS in °Brix) were then measured using an ATC-1E ATAGO hand-held refractometer on the translucent part of the juice after decantation. 5 ml of the same translucent part were weighed to assess titratable acidity (TA) according to the AOAC method (NaOH, 0.1N and end pH=8.1) using a DL25 Mettler titrator. Results were expressed in g. of anhydrous citric acid/100g of juice. Incidence of maturity was calculated by ratio between TSS/TA.

Identification of tetraploid and zygotic seedlings

Ploidy status of trees was determined by flow cytometry using bark samplings or leaf samples from rootstock sprouts according to Froelicher *et al.* (2007).

For identification of zygotic seedlings, DNA were extracted also from bark (1 cm^2 cut below the bud union) or leaves samples picked on rootstock buds of all rootstocks according to Doyle and Doyle (1987) and adapted to citrus (Cabasson *et al.* 2001). For bark, DNA samples were extracted by using the inner layer of tissue of the bark that is composed of alive tissues. DNA was then amplified by PCR using five Sequence Tagged Microsatellite sites (STMS) primers mCrCIR08A03, mCrCIR07D05, mCrCIR01E02, Ci07C09 and Ci01H05 (Froelicher *et al.* 2007). The conditions and separation of PCR products were done by means of vertical

denaturalized electrophoresis polyacrylamide (5%, urea 7 M), in buffer TBE 0.5X (Tris, acid boric 45mM and EDTA 0.5 M, pH 8) and silver stained according to Beidler *et al.* (1982).

Statistical analysis

Data were expressed by the mean value ± SE. We used SIGMASTAT from SPSS (Chicago; www.spss.com_software_science) to analyze the data. ANOVA test was used to detect differences of fruit production between clementine grown on diploid zygotic and tetraploid rootstocks at the usual probability level of P= 0.05.

Results

Ploidy status and nucellar or zygotic status of the rootstocks

Inner bark tissues and trifoliate leaf samples from sprouts from all the rootstocks allowed checking ploidy status using flow cytometry. Only 2.4% of the rootstocks were tetraploid, all the rest were being diploid (Table 2). Investigation of the nucellar or zygotic status of rootstocks using 5 SSRs markers showed that among the 288 trees that were analyzed, 19 trees were shown to be zygotic (6.6% not true-to-type genotypes). Four genotypes (N° 4, 17, 19, and 31) presented two zygotic individuals among 9 reps. Two genotypes presented one tetraploid plus one hybrid off-type (N°3 and 8). Also, two hybrids and one tetraploid were detected among the nine reps of rootstock N°19. Then, 91% of clementine scions were grafted on diploid rootstocks of nucellar origin. Even after precocious visual screening of seedlings in order to remove off-types seedlings from the trial, 9% of the selected rootstocks were still not true-to-type rootstocks.

Effect of tetraploid and zygotic rootstocks on clementine fruit production

Analysis of the clementine fruit production when grafted on tetraploid and diploid rootstocks was then performed (Fig. 1). For the six trifoliate orange genotypes presenting one or two tetraploid representatives (rootstock N°: 3, 7, 8, 11, 18, 19), clementine fruit production was significantly reduced by 30 to 50%. The impact on the presence in the plot of zygotic rootstocks when compared to diploid rootstocks on clementine fruit production was also investigated (Fig. 2). In some cases, a tendency to higher fruit production was observed (N° 6, 19, 24, 31) but for other rootstocks, no impact or a much reduced clementine production (N° 4, 5, 9 with a maximum of 24% decrease) was observed.

Effect of off-type genotypes on rootstock selection

The cumulative fruit production for all the trifoliate orange genotypes independently of the presence of tetraploid and zygotic rootstocks for a period of 11 years is presented on figure 3A. For each combination, genotypes were ranked depending on their cumulative fruit production. This rank of fruit production was used in table 1 to present all the investigated genotypes. Morocco trifoliate orange (N° 32) and Pomeroy trifoliate orange (N°31) were identified as the most productive rootstocks. Their cumulative fruit productions were about 20% higher than for trifoliate oranges N° 1, 2 and 3. Statistical analysis showed significant differences for clementine fruit production between few rootstock genotypes. The breeders that managed this trial had selected these two more productive genotypes and recommended it to the local producers. Nowadays, taking account of the presence of tetraploid and zygotic

rootstocks in the plot, the clementine fruit production was reanalysed only for true-to-type rootstocks. On figure 3B is presented the cumulative fruit production keeping the same order of the genotypes than used in figure 1A. Removing tetraploid and zygotic rootstocks showed that the most productive rootstocks were not the same, the most productive rootstock being now Argentine (N° 18), that was previously ranked at the middle of the set, since among reps of this genotype, 2 tetraploids were present (Statistical analysis is presented in supplementary table 1).

Fruit quality analysis of clementine grown on the 32 different trifoliate orange genotypes was investigated for 5 consecutive years 1981 to 1985. Whatever the year and the criterion investigated, we did not observe any significant change due to the rootstock genotype used (data not shown).

Discussion

Analysis of the number of tetraploid present in the field trial showed that 2.4% of tetraploid were still present even after roguing of nursery seedlings. In a previous study, we obtained 5.4% of tetraploid among seedling of trifoliate orange Pomeroy (SRA 1074) taking account of all germinated seedlings (Saleh *et al.* 2008). Tetraploids are characterized by slower growth, typically broader, thicker and darker leaves (Barrett and Hutchison 1978; Cameron and Soost 1969). Nevertheless, these changes to diploid phenotype could be considered as a tendency and not a systematic expressed difference. Like many quantitative characters, the value distribution of morphological traits can be represented by a Gaussian curve with variation around the average value. For any specific character, overlapping of the Gaussian curves may occur in between diploids and tetraploids. Then, this is a valid assumption to consider that many unidentified tetraploid rootstocks remain unidentified in trials.

Concerning the 19 hybrids (6.6%) we detected, their presence is due to the partial apomixis and the relative low polyembryony level of *Poncirus trifoliata* seeds favorable for zygotic embryo growing. In the trial, hybrids are much more numerous than tetraploids suggesting that zygotics were initially present at a greatest rate among seedlings than tetraploids or were more difficult to identify. Different studies (Ashari *et al.* 1988; Khan and Roose 1988; Moore and Castle 1988) revealed a 2.6 to 21.6 percentage of zygotic plants depending of the trifoliate orange genotype analyzed.

Screening based on phenotype may be not totally efficient for tetraploid selection because for some crosses, morphological modifications at the early stage of vegetative growth before grafting may not be visible. This may be particularly true for self crosses that are supposed to be highly frequent in *Poncirus* since this genotype is flowering in Corsica from March to mid April before all other *Citrus* species. Then it is expected that the encountered zygotics are the result of a self pollination or a cross between different *Poncirus trifoliata* genotypes which limits genetic diversity and therefore limits the apparition of large changes of phenotype as previously proposed by (Khan and Roose 1988). Hence, molecular screening and cytometry analysis are required since any visual screening in order to remove non true-to-type are not enough selective.

Screening of tetraploid and zygotic trees showed that even after strong nursery visual screening, many seedlings were still off-type genotypes and consecutively leaded to misinterpretation about the best genotypes for fruit production. Indeed, analysis of the cumulative fruit clementine production showed that removing tetraploid and zygotic

genotypes from the plot significantly changed the fruit production of several genotypes. The use of tetraploid rootstocks dramatically decreased fruit production while presence of zygotic genotypes in the field trial was without significant impact. Then, if the presence of not true-to-type rootstocks in the plot is the origin of considerable changes of fruit production, the possibility that fruit quality properties could also be affected can not be excluded.

Depending of the trifoliate orange genotype, large changes in cumulative clementine production were observed. However, unlike fruit production, clementine fruit quality analysis performed on young trees whatever the trifoliate orange rootstocks, did not reveal any significant change for all of the criteria we investigated.

Analyses of the nucellar or zygotic status of the rootstocks showed that zygotic rootstocks may lead, depending of the genotype, to lower fruit production as previously reported by (Roose and Traugh 1988). Indeed in our trial, only 4 zygotic rootstocks over 19 presented considerable decrease in fruit production. By comparing the effect of each off-type on scion fruit yield, tetraploid rootstocks have a larger impact than hybrid rootstocks: the average percentage of yield reduction was 45.4 % for tetraploid rootstock while it was only 2.7% for zygotic rootstocks. This is not in agreement with the hypothesis that hybridization has lager effect on phenotype diversity than chromosome doubling. These results suggest that in spontaneous doubled diploid, genome regulation modifications may impact of scion fruit production. These results suggest that chromosome doubling at the origin of tetraploid citrus rootstocks impacts the regulation of the genome expression modifying the physiological relation between rootstock and scion.

In citrus trial, the number of reps is usually limited which requires a strong homogeneity among seedlings. If not, the impact of non true-to-type on fruit production would be increased by the reduced number of reps. In this trial the more productive rootstock (N°18) was not identified due to the presence of 2 tetraploids over 9 reps. A reduction of 50% of the production due to these 2 tetraploids is at the origin of a reduction of 11% for the 9 trees analyzed together. 2.4% of tetraploid trees have been detected in the trial suggesting that a much higher frequency is encountered in orchards. Indeed Schwarz (2001) observed that the rate of tetraploid Carrizo citrange rootstock plant ready to be planted in Spanish nurseries was greater than 10%. Consecutively, we can estimate the decrease of Clementine production yield to 5%. Thus, the removing of tetraploid genotypes among diploid seedlings should be highly profitable for the citrus industry.

The impact of non true-to-type hybrid rootstock is not always significant on fruit production and therefore might be considered as a negligible factor for growers. However,

the requirement to use Tristeza resistant rootstocks constitutes an essential reason for seedling screening. Indeed, trifoliate orange genotypes are resistant to citrus tristeza virus (CTV), and at genomic level, this resistance is conferred by a unique heterozygous gene (Mestre *et al.* 1997). Thus, for trifoliate orange zygotic seedlings originating from self pollination, 25% of the seedlings are expected to be susceptible to CTV and 50% in the situations of cross-pollinations. Considering, that at least 6% of the poncirus seedlings are zygotic, meaning that 1.5 to 3% of the rootstocks are susceptible to CTV and may increase risks of CTV dissemination.

For any field trials but also for orchards, the screening of zygotic and tetraploids among diploid seedlings using any codominant molecular markers and flow cytometry is absolutely required since visual screening is not enough efficient to remove all non conform types.

Acknowledgments

Sajjad Hussain was granted by the High Education Commission of Pakistan and the work was supported by the “Bureau des Resources Génétiques” (2007, project n° 34).

This paper is dedicated to Camille Jacquemond on the occasion of his retirement.

Tables

Table 1: List of the 32 trifoliate orange genotypes studied in the field trial

#.	Common Name	Latin name	ICVN	Origin
1	Rubidoux	*Poncirus trifoliata* (L.) Raf.	110126	South africa
2	Boufarik	*Poncirus trifoliata* (L.) Raf.	110507	Algeria
3	Menager	*Poncirus trifoliata* (L.) Raf.	110116	France
4	Rusk	*Poncirus trifoliata* (L.) Raf.	110437	USA(Texas)
5	Ferme blanche	*Poncirus trifoliata* (L.) Raf.	110435	Algeria
6	Jacobsen	*Poncirus trifoliata* (L.) Raf.	110107	USA(California)
7	English	*Poncirus trifoliata* (L.) Raf.	110097	South africa
8	SEAB	*Poncirus trifoliata* (L.) Raf.	110438	Algeria
9	Beneke	*Poncirus trifoliata* (L.) Raf.	110436	USA
10	Pomeroy	*Poncirus trifoliata* (L.) Raf.	110081	USA(California)
11	Rusk	*Poncirus trifoliata* (L.) Raf.	110443	USA(Texas)
12	Christian	*Poncirus trifoliata* (L.) Raf.	110338	South africa
13	Ferme blanche	*Poncirus trifoliata* (L.) Raf.	110087	Algeria
14	Kryder	*Poncirus trifoliata* (L.) Raf.	110446	USA(Florida)
15	Luisi	*Poncirus trifoliata* (L.) Raf.	110088	France
16	Rusk	*Poncirus trifoliata* (L.) Raf.	110082	USA(Texas)
17	Brazil	*Poncirus trifoliata* (L.) Raf.	110510	Brazil
18	Argentine	*Poncirus trifoliata* (L.) Raf.	110505	Argentine
19	Morocco	*Poncirus trifoliata* (L.) Raf.	110511	Morocco
20	Boufarik	*Poncirus trifoliata* (L.) Raf.	110506	Algeria
21	Morocco	*Poncirus trifoliata* (L.) Raf.		Morocco
22	Beneke	*Poncirus trifoliata* (L.) Raf.	110083	USA
23	Kryder	*Poncirus trifoliata* (L.) Raf.	110110	South africa
24	Christian	*Poncirus trifoliata* (L.) Raf.	110084	South africa
25	Boufarik	*Poncirus trifoliata* (L.) Raf.	110508	Algeria
26	Luisi	*Poncirus trifoliata* (L.) Raf.	110448	France
27	Christian	*Poncirus trifoliata* (L.) Raf.	110085	USA
28	Menager	*Poncirus trifoliata* (L.) Raf.	110449	France
29	Kryder	*Poncirus trifoliata* (L.) Raf.	110108	USA(Florida)
30	Christian	*Poncirus trifoliata* (L.) Raf.	110447	USA
31	Pomeroy	*Poncirus trifoliata* (L.) Raf.	110442	USA(California)
32	Morocco	*Poncirus trifoliata* (L.) Raf.	110512	Morocco

Table 2: Percentage of nucellar, zygotic and tetraploid plants identified among the 288 trifoliate orange rootstocks investigated.

Plant genetic status	Number of plants	Percentage
Nucellar	262	91
Zygotic	19	6.6
Tetraploid	7	2.4
Total	288	100

Figure legends

Figure 1: Comparison of cumulative clementine fruit production of the 5 trifoliate orange genotypes presenting spontaneous tetraploid rootstocks among reps. Numbers refers to Table 1 where are listed the different trifoliate orange genotypes that were investigated (Mean ± SE).

Figure 2: Comparison of cumulative clementine fruit production grown on the diploid nucellar genotypes presenting spontaneous diploid zygotic trifoliate orange rootstocks. Each zygotic being genetically different, fruit production is individually represented. Numbers refers to Table 1 where are listed the different trifoliate orange genotypes that were investigated (Mean ± SE).

Figure 3: Comparison of cumulative clementine production grown on 32 different trifoliate orange rootstocks for a period of 11 years. **A**) Fruit production, taking account of the presence of spontaneous tetraploid and zygotic rootstocks among diploid nucellar genotypes. For each combination, genotypes were ranked form the lowest to the highest cumulative fruit which has been used to order the genotypes presented in table 1 (Mean ± SE). **B**) For each combination, fruit production, taking account only of the diploid nucellar rootstocks (Mean ± SE).

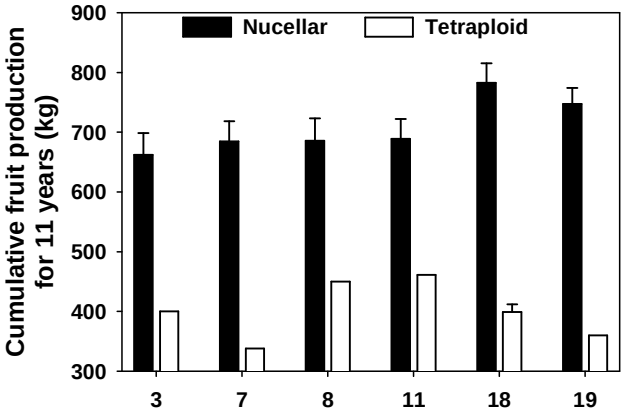

Nucellar
Tetraploid
Cumulative fruit production for 11 years (kg)
900
800
700
600
500
400
300
3
7
8
11
18
19

Fig. 1

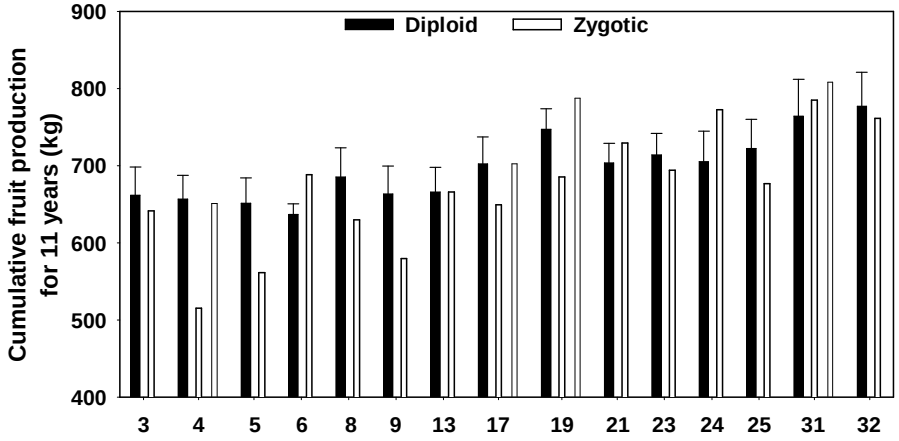

Diploid
Zygotic
Cumulative fruit production for 11 years (kg)
900
800
700
600
500
400
3
4
5
6
8
9
13
17
19
21
23
24
25
31
32

Fig. 2

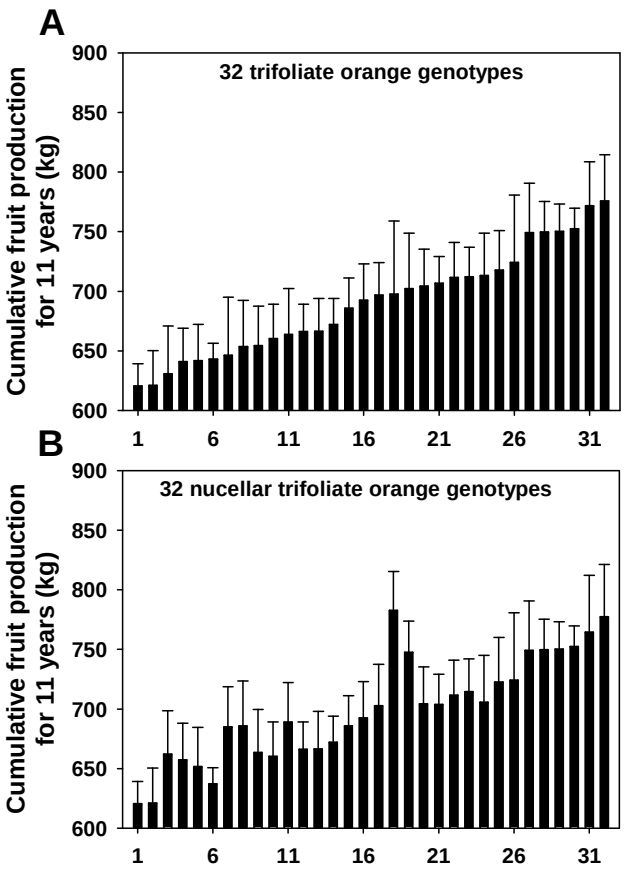

A
32 trifoliate orange genotypes
Cumulative fruit production for 11 years (kg)
900
850
800
750
700
650
600
1
6
11
16
21
26
31
B
32 nucellar trifoliate orange genotypes
Cumulative fruit production for 11 years (kg)
900
850
800
750
700
650
600
1
6
11
16
21
26
31

Fig.3

5.5 Use of autotetraploid trifoliate orange citrus rootstocks does not impact on Clementine quality but reduces yields and highly modifies the scion / rootstock physiology

(Submitted in: The European Journal of Agronomy**)**

Sajjad Hussain[1,4], Claudie Dhuique-Mayer[2], Frank Curk[1], Laurent Urban[3], Patrick Ollitrault[4], François Luro[1], Raphaël Morillon[4,] *

1. Unité de Recherche Génétique et Ecophysiologie de la Qualité des Agrumes, Inra. Centre Inra de Corse, F-20230 San Giuliano, France.

2. Département Systèmes de Production et Transformation, UMR Qualisud, TA B-95/16
73, Av. J. François Breton, 34398 Montpellier cedex 5, France.

3. Laboratoire de Physiologie des Fruits et Légumes, Université d'Avignon et des Pays de Vaucluse, 84000 Avignon, France.

4. UPR amélioration génétique des espèces à multiplication végétative du Cirad. Instituto Valenciano de Investigaciones Agrarias, 46113 - Moncada – Valencia, Spain.

* **Corresponding author:** Raphaël Morillon, Unité Propre de Recherche amélioration génétique des espèces à multiplication végétative du Cirad, Centro de Genomica, Instituto Valenciano de Investigaciones Agrarias (Ivia) 46113 - Moncada - Valencia Spain.

Phone: (00 34) 963 424 143

Fax: (00 34) 963 424 106

E-mail: raphael.morillon@cirad.fr

Key words: Citrus, Clementine, *Poncirus trifoliata*, autotetraploid rootstocks, fruit yield,

Abstract

Two autotetraploid (4x) poncirus (*Poncirus trifoliata* (L.) Raf.) rootstocks and their corresponding diploid (2x) rootstock were compared for their effect on clementine "Commune" (*Citrus clementina* Hort. ex Tan) fruit yield, fruit quality and impacts on rootstock/scion physiology. Cumulative fruit yield, fruit quality (sugar, organic acids, carotenoids, hesperidin), and clementine growth (stem diameter), leaf starch, sugar, chlorophyll content, phenolic content and gas exchange parameters were investigated. Cumulative fruit yield for 11 years was lower in clementine/4x rootstock associations and clementine vigour was also lower when using 4x rootstocks. Fruit quality was not affected by the ploidy level of the rootstocks except for the hesperidin content that was higher in clementine grown on 4x rootstocks. Leaf chlorophyll and phenolic contents were higher for clementine when grown on 4x rootstocks. If the rate of photosynthesis and stomatal conductance was much higher when using 2x rootstocks then the maximum electron transport rate of leaves was however lower when compared to the use of 4x rootstocks. Similarly leaf starch and sugar contents were much higher in clementine/4x rootstock associations when compared to 2x rootstocks. Taking together our results demonstrate that the use of 4x rootstocks dramatically change the tree physiology and fruit yield without promoting large changes in fruit quality criteria.

Introduction

Most of citrus are diploid (2x) species with a basic chromosome number x= 9 (Krug 1943) but some spontaneous triploid (3x) and tetraploid (4x), have been identified among seedlings (Lee 1988). Tetraploid plants arise in seedlings with a frequency varying from 1 to 11% (Barrett and Hutchinson 1982; Barrett and Hutchison 1978) and in Spanish nurseries, the rate of spontaneous tetraploid Carrizo citrange (*Citrus sinensis* [L.] Osbeck ×*Poncirus trifoliata* [L.] Raf.) rootstock among plants ready to be planted ranged from 11 to 19 % (Schwarz 2001). The occurrence of spontaneous 4x seedlings is under genetic and environmental controls. Indeed, it has been showed that 4x arise from the doubling of the chromosome set of nucellar cells (Cameron and Soost 1969). Moreover, a negative correlation has been observed between high temperatures during the flowering period and low rates of 4x seedlings (Aleza et al 2011 accepted).

Interest of the use of 4x rootstocks has been emphasized this last decade with the making of somatic hybrids allowing combining traits of two parents (Grosser and Gmitter, 2010, Dambier *et al.,* 2011). For example, by combining a genome of *Poncirus trifoliata* with a *Citrus* genome such as Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) it is possible to generate plants resistant to biotic traits such as *Citrus tristeza virus* and *Phytophthora* and abiotic stress such as alkalinity, salinity and drought. Interestingly, allopolyploidization doesn't seem to be the only way to improve trait of tolerance in rootstocks through the change of ploidy level. Indeed, autotetraploid citrus seedlings were shown to present a better tolerance to salt stress and water stress than their parental 2x (Saleh *et al.* 2008; Allario 2009), probably because of a higher abscisic acid constitutive biosynthesis in 4x (Allario 2009). Recent work in 4x *Dioscorea zingiberensis* indicated that 4x plants possessed a stronger antioxidant defence system and increased heat tolerance (Zhang *et al.* 2010). Altogether, this suggests that polyploidization event independently of the allelic combinations is also a way to

get increased traits of tolerance to biotic and abiotic stress. In autopolyploid series in potato (*Solanum tuberosum* L.) or in maize (*Zea mays* L.), it has been showed that gene expression changes were very limited (Stupar *et al.* 2007; Riddle *et al.* 2010) on the contrary to allotetraploids which are considered to be strongly affected by genome hybridization resulting to subfunctionalization phenomena (Ward and Durrett 2004; Auger *et al.* 2005) and large genome expression changes when compared to the parents. Therefore, according to Stupar *et al.*, (2007), gene expression changes observed in between 2x and autotetraploid genotypes could be attributed to nuclear dosage or epigenetic regulations. Indeed, in a recent study, we showed that the large phenotypic differentiation in 4x Rangpur lime (*Citrus limonia*, Osbeck) compared to 2x was not associated with large changes in leaf genome expression suggesting that subtle changes in gene expression could be at the origin of the phenotypic differentiation (Allario *et al.* 2011).

Tetraploidy *per se* is known to affect phenotypic traits such as stomata density, cell size, division rate, organellar composition, root and leaf morphology, growth and fruit quality, among others (Cameron and Frost 1968; Mouhaya *et al.* 2010; Ollitrault *et al.* 2008; Romero-Aranda *et al.* 1997) which will dramatically affect tree physiology. Citrus 4x were showed to present lower stomatal conductance, lower photosynthesis and by consequence lower rates of whole-plant transpiration and lower growth leading to lower fruit yields when compared to 2x rootstocks (Soost *et al.* 1975; Perez-Perez *et al.* 2007). Kyoho grapevines grafted on 4x rootstocks presented slower growth, thicker leaves and lower fruit quality (Motosugi *et al.* 2007). In 2x, 4x and 6x wheat species, carotenoid content (lutein) were significantly decreased with increase of ploidy level (Leenhardt *et al.* 2006). Investigations of carotenoid contents in juice sacs of mandarin, lemon and their allotetraploid hybrid showed a 60 fold greater content in mandarin than that in lemon, the allotetraploid hybrid producing all the same compounds as mandarin but at very low levels (Bassene *et al.* 2010). Rootstocks

genotypes such as *Poncirus trifoliata* were showed to give lower fruit production with thick and irregular albedo (Jacquemond and Rocca Serra 1992; Soost *et al.* 1975; Tutberidze and Kalandarishvili 1975). However, the effect of the use of citrus 4x rootstocks on fruit quality criteria of any given 2x varieties has to our knowledge never been investigated.

In this article we investigated the effects of the use of citrus 4x trifoliate orange (*Poncirus trifoliata* [L.] Raf.) rootstocks when grafted with clementine "Commune" (*Citrus clementina* Hort. ex Tan), compared to 2x rootstock / Clementine associations on tree growth and clementine production and quality.

Materials and Methods

A trifoliate orange rootstock trial composed of 36 different Poncirus (*Poncirus trifoliata* (L.) Raf.) genotypes grafted with clementine "Commune" (*Citrus clementina* Hort. ex Tan) was established in 1974 at the INRA-CIRAD Research Station in Corsica, France (Jacquemond and Rocca Serra 1992). Among all 36 genotypes, two trifoliate orange genotypes were previously showed to be 4x. All the trees were grafted with SRA 64 clementine (9 reps).

Identification of the 2x parental genotypes putatively corresponding to the 4x trifoliate orange rootstocks and plant ploidy status.

Five molecular markers were used to identify the two 2x genotypes presenting identical profile and putatively being the parental diploid of the 4x genotypes. Using flow cytometry (Froelicher *et al.* 2007), all trees belonging to the set of parental 2x genotypes rootstocks were confirmed to be diploid and all the trees belonging to the set of 4x genotypes rootstocks were confirmed to be tetraploid.

Circumference of rootstock and scion and gas exchange analysis

Rootstocks and scion circumference was measured using a measuring tape in order to estimated the average scion/rootstock circumference ratio for the first eight years of the trial. Gas exchange measurements were performed during the summer season in 2009 only on fully expanded leaves of similar age, selected within branches homogeneous in terms of light exposition (oriented south-east and of a height of 1.8 meter). We make therefore state confidently that there was no bias in all our gas exchange measurements due to leaf age or light exposition on photosynthetic capacity. We moreover supplied water on an evapo-transpiration replacement basis to ensure that there were no limitations to stomatal conductance due to water availability. At least five leaves were selected within each tree per genotype (3 trees). Stomatal conductance (g_s) and the net photosynthetic rate (A) were determined using a portable gas exchange/chlorophyll fluorescence measurement system

(GFS-3000) (Heinz Walz GmBH, Germany). The system was equipped with the LED-Array/PAM-Fluorometer 3055-FL which was used to impose a photosynthetically active radiation flux (Q) of 1000 µmol photons m^{-2} s^{-1} in all our measurement. This value is well-above the known threshold for light saturation for Citrus. All measurements were performed during the morning (08:00-11:00) to prevent down regulation of photosynthesis by the accumulation of photosynthetates in leaves. During all measurements, leaf temperature was 30 ± 2°C, leaf vapour pressure deficit was 2.4 ± 0.4 kPa, and ambient CO_2 concentration was maintained at 370 ± 3 µmol CO_2 mol^{-1} air within the cuvette. In addition, we estimated the light-saturated rate of electron transport J_{max} (µmol electrons m^{-2} s^{-1}), as an indicator of photosynthetic capacity, following the method of (Urban *et al.* 2004)

Chlorophyll content and Flavonoid content measurement

At least 30 leaves from each three tree per genotype were selected to measure the chlorophyll and the flavonoid concentrations, expressed on a leaf surface basis. The leaf chlorophyll concentration was measured with a SPAD meter (Minolta SPAD-502, Japan). The content in flavonoids was measured using the non-destructive method developed by (Cerovic *et al.* 2002). Measurements were performed with a Dualex 3.3 ® (Force-A © ; (Goulas *et al.* 2004) on the same leaves than the ones used for gas exchange and fluorescence measurements, following the recommendations of (Cartelat *et al.* 2005).

Leaf nitrogen and non structural carbohydrates

Leaf nitrogen content per unit of mass (N_m) was determined with an elemental analyzer (Carlo Erba Instruments, Milan, Italy) following the method of (Colombo *et al.* 1988). Glucose, fructose and sucrose in the leaves were determined with an enzyme based analyzer (YSI 2007, Yellow Springs Instruments, Yellow springs, OH). Starch was determined by enzymatic hydrolysis to glucose (Thivend *et al.* 1972). Dry mass was assessed by freeze-drying. The

masses of starch and soluble sugars were deducted from the dry mass to obtain the structural dry mass from which mass-to-area ratio (M_a) and N_a ($N_a = M_a N_m$) were calculated.

Fruit yield, fruit quality analysis

From November to the end of December, the crop of each tree was harvested and weighed. Measurements of annual yield were taken for 11 years from 1979-1990. Fruit quality analysis was performed according the previous article .

Analysis of organic acids and sugars.

In 2008, more elaborated fruit quality analysis was performed for the 4 associations. From each 2x and its corresponding 4x trifoliate orange rootstock, 15 fruits of same size and colour were collected. Fruits were peeled and their pulp was frozen in liquid nitrogen and stored at -20°C for further analysis. One hundred milligrams of lyophilized powdered pulp were dissolved in 5 mL of bidistilled water and centrifuged at 3000 g for 10 min. Supernatant was filtered through 25 mm syringe filters, 0.2 µm cellulose acetate membranes (VWR). Organic acids were analyzed using an analytical HPLC unit (Perkin-Elmer, Series 200, France) as previously described by (Albertini *et al.* 2006). Sugar content was determined according to (Gomez *et al.* 2002). Data obtained using TotalChrom™ software for Windows version 6.2 (Perkin-Elmer Instruments, Shelton, U.S.A.). Concentrations of organic acids and sugars were expressed in mg g^{-1} dry weight.

Extraction and quantification of carotenoid content

Sample size of 20 fruits of same size and colour from each kind of rootstock/scion association were collected the same day according to (Dhuique-Mayer *et al.* 2005). Three fruits samples from each tree were immediately squeezed and the juice was filtered through stainless steel sieves and distributed in 30 ml amber vials under nitrogen and kept frozen (-20°C). Carotenoid were extracted and analyzed according to the methods of Dhuique-Mayer *et al.* 2009. Briefly, 20 ml of juice were extracted with ethanol/hexane 4:3 v/v and saponified

overnight with 10 % methanolic KOH at room temperature. Carotenoid extracts were dissolved and diluted in MTBE/Methanol (80/20; v/v) before injection in HPLC. Analysis of carotenoids was performed by HPLC system Agilent 1100 system (Massy, France). Carotenoids were separated along a C_{30} column (250 x 4.6 mm i.d., 5 µm YMC EUROP GMBH, Germany), mobiles phases were H_2O as eluent A, methanol as eluent B, and MTBE as eluent C. Flow rate was fixed at 1m $Lmin^{-1}$, column temperature was set at 25 °C, and injection volume was 20 μL. Quantification of carotenoids was achieved using calibration curves with β-carotene, β-cryptoxanthin and lutein with five concentration levels. Identification was carried out according previous studies (Dhuique-Mayer *et al.* 2007; Fanciullino *et al.* 2006)

Determination *of hesperidin*

Hesperidin from sample juices (previously described for carotenoids) was determined by HPLC according to Dhuique-Mayer *et al.* (2005). HPLC system was Agilent 1100 model (Massy, France) using a RP 18e Licrospher® 100 (5 µm) column (250 mm x 4.6 mm id) (Merck KgaA, Darmstadt, Germany). The isocratic solvent system was water/acetonitrile/THF/acetic acid (80:16:3:1;v/v/v/v). Quantification was carried out at 280 nm. Flow rate was fixed at 1 $mL.min^{-1}$. Hesperidin concentrations were determined using an external calibration method. Standards HES was diluted in DMF/water (2:1, v/v) to give 102 mgl^{-1}.

Statistical analysis

Data are expressed by the mean value ± SE. We used SIGMASTAT from SPSS (Chicago; www.spss.com_software_science) to analyze the data. ANOVA test and T test were used to detect differences between diploid and tetraploid rootstocks at the usual probability level of $P < 0.05$.

Result

Identification of the2x parents of both 4x tetraploids

Among the 36 rootstocks of the trial, thirty-four 2x genotypes were analyzed using SSRs markers in order to identify the putative 2x parents of both 4x genotypes. Two 2x genotypes presenting identical patterns for all the markers for each of the 4x genotypes were identified (Table 1). No one spontaneous zygotic individual was detected among the 2x genotypes. Also, no one diploid and autotetraploid individuals was found respectively in the putative autotetraploid and diploid genotypes.

Circumference of rootstock and scion

The average scion/rootstock circumference ratio of both 2x and 4x trifoliate orange/clementine associations was measured annually for eight years (Fig. 1). Ratio values were all the time greater when using 2x rootstocks compared to the respective 4x, differences being significant the second year for both 2x/4x couples, the fifth and sixth years for the first couple (0110086/0110509) and the seventh and eighth years for the second couple (0110125/0110440).

SPAD values, Dualex units and gas exchange measurements

The concentration in chlorophylls given by the SPAD was 5.5 to 7.7 %, higher in the 4X (Fig. 2). Similarly, the concentration in flavonoids given by the Dualex was 7.9 to 8.3 % higher in the 4X when compared to the 2X. Globally the relationship between A and g_s appears well maintained in each combination (Fig. 3A). However, values of both A and g_s were 70 % higher in the 2X than in the 4X. J_{max} was 15 to 41 % higher in the 4X combinations, when compared to their two corresponding 2X combinations (Fig. 3B).

Leaf nitrogen and non structural carbohydrates

Starch and soluble sugars of leaves of clementine scions grafted onto 4x rootstocks were higher in the leaves of their corresponding 2X combinations (Table 2). N_m was not affected by

ploïdy, on the contrary to M_a which was 30.5 to 30.9 % lower in the leaves of the scions grafted onto the two 4x rootstocks. Logically N_a which is product of N_m and M_a was lower in the leaves of the scions grafted onto the two 4x rootstocks, but the difference was significant only for the 0110086 – 0110509 couple.

Fruit yield and classical fruit quality analysis

Cumulative clementine fruit production for 11 years grafted on both 2x trifoliate orange rootstocks compared to the use of their corresponding 4x trifoliate orange rootstocks is presented in Table 3. Whatever the couple, 2x trifoliate orange rootstocks were more productive with almost one third higher fruit production than their corresponding 4x rootstocks. Analysis of clementine fruit quality for two consecutive years (1982-1983) was also investigated: sugar content, acidity content, incidence of maturity and the percentage of juice were not affected whatever the level of ploidy of the rootstock (Table 3).

Sugars, organic acids, carotenoid and hesperidin content analysis

A more elaborated analysis of fruit quality was carried out in 2008. Three major sugars (sucrose, glucose and fructose) were identified in fruit pulp of clementine variety grafted on 2x and 4x rootstocks. The major identified sugar was sucrose but no difference in sucrose, glucose or fructose was observed in clementine pulp whatever the level of ploidy of the rootstock (Table 4). The content of different organic acids and carotenoids was monitored as well in fruit for both clementine/2x and clementine/4x rootstock associations. Organic acids contents was higher in clementine grown on 4x rootstock but this was significant for only one 4x genotype. Changes in fruit carotenoid contents were not significantly different in clementine / 2x and 4x associations except for lutein where significant change was identified. To end, investigation of hesperidin contents in fruit juice showed much higher values when clementine scion was grafted on 4x trifoliate orange rootstocks compared to 2x trifoliate orange.

Discussion

In the rootstock field trial analyzed by Jacquemond and Rocca Serra (1992), thirty-four 2x and two 4x trifoliate orange grafted with clementine were analyzed for fruit quality parameters. More recently the presence of zygotic and spontaneous 4x genotypes among thirty-two over the thirty-four 2x trifoliate orange was reanalyzed Hussain *et al.* (2011). Among the 288 trees, 2.4 % and 6.6 % of the rootstocks were shown to be respectively 4x and zygotic. If zygotic individuals did not seem to affect the mean fruit production, all spontaneous 4x were shown to strongly reduce fruit yields. However, fruit quality for any zygotic or 4x genotype did not seem to be changed. Since zygotic and 4x individuals were spontaneous, no more than one repetition was present in the trial. Therefore, a specific analyze of 4x rootstocks clones grafted with clementine was required to understand why yields were lesser and if fruit quality criteria were really not affected. Using SSRs markers, we attempt to identify the putative 2x parents of both 4x genotypes among the thirty-four 2x clone and only two 2x genotypes presenting profiles identical to each of the 4x genotype were identified (Table 1). Moreover, each 2x identified parent was originating from same geographic area than its associated 4x strengthen the results obtained with microsatellites markers.

Fruit production and physiology of the clementine/ 4x rootstock association

The 4x rootstock associations proved less vigorous than the 2x rootstock associations in our trial (Fig. 1). This is consistent with the lower growth rates of 4x seedlings and rootstocks already reported in the literature (Saleh *et al.* 2008), although the mechanisms by which rootstock regulates scion vigor is still unclear (Basile *et al.* 2003). The lower performance in terms of growth of the 4x rootstock associations appears also consistent with the lower *A* values measured in leaves (Fig. 3A). Syvertsen *et al.* (2000) had already observed that *A* and g_s were lower in the 4x rootstock associations (Syvertsen *et al.* 2000). Because proteins of the

Calvin cycle and thylakoids represent the majority of leaf nitrogen, photosynthetic capacity, which determines gas exchanges, is strongly related to leaf nitrogen on an area (N_a) or mass (N_m) basis (Evans 1989; Field and Mooney 1986; Kellomäki and Wang 1997; Walcroft *et al.* 1997). We observed indeed that both A and N_a were lower in the 4x rootstock associations (Table 2). These lower values are attributable to lower values in M_a, not N_m ($N_a = N_m.M_a$). Differences in photosynthetic capacity due to differences in M_a have been reported previously in several species, including fruit trees, as the consequence of the presence of fruits (Proietti 2001; Urban *et al.* 2003), water stress (Bacelar *et al.* 2006; Damour *et al.* 2008; Diaz-Espejo *et al.* 2006) or light acclimation (Niinemets 2007; Terashima *et al.* 2006) .

While data about vigour, photosynthesis and N_a are positively correlated, as one would expect, this data appear to be conflicting with the J_{max} and the data about leaf chlorophyll concentration. It comes indeed as a surprise that differences in photosynthetic or N_a are not mirrored in differences in J_{max} and chlorophyll concentration.

It is reminded that leaf chlorophyll concentration is related to the capacity to harvest light, and J_{max} to the bioenergetic pool of the photosynthetic machinery (Niinemets and Tenhunen 1997). Because a decrease in J_{max} indicates downregulation of electron transport, it is commonly associated with limited RuBP regeneration and activity of soluble enzymes of the stroma such as sedo-heptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase (Flexas *et al.* 2004; Harrison *et al.* 2001). Our observations suggest that the decrease in photosynthesis and N_a we observed in leaves of the 4x rootstock association was not driven by a decrease in the allocation of leaf nitrogen either to the light harvesting or the bioenergetic pools. We are thus left with the hypothesis that this decrease came at the expense of the third major nitrogen pool in the leaves, i.e. the carboxylation pool (the Rubisco).

Shifts in N allocation have been much studied over a large range of species, especially in response to light exposure. A decrease in M_a and an increase in N allocation to the light

capture pool are common features among leaves under low light levels. A decrease in M_a maximizes the leaf surface exposed to the sun while reducing leaf respiration (Terashima *et al.* 2006), and an increase in N allocation to chlorophylls and other pigments increases light capture (Niinemets 2007). Leaves of the 4x rootstock associations clearly seem to share some common traits with leaves acclimated to low light levels. Interestingly, lower N allocation to carboxylation (i.e. less Rubisco) has also been associated with low light conditions (Niinemets and Tenhunen 1997; Ogren 1993).

Leaf starch and total soluble sugar concentrations were higher in leaves of the 4x rootstock associations (Table 2). Transitory starch reserves serve two functions in plants: i) as a carbon overflow, allowing photosynthesis to go faster than sucrose synthesis which is useful to prevent downregulation of photosynthesis when sink limitation reduces sucrose export from the leaf, ii) as a continuous supply of carbon at night when CO_2 cannot be fixed by photosynthesis (Farquhar *et al.* 1980; Huber and Hanson 1992; Manter and Kerrigan 2004; Stitt and Quick 1989). Considering that the lower growth observed in 4X rootstock associations reflects lower sink activity, it can be hypothesized that 4X reacted to sink limitation of photosynthesis by increasing starch synthesis.

The higher concentrations in total soluble sugar concentrations in the leaves may in turn explain why leaf N allocation to rubisco was reduced in the 4X rootstocks associations. High concentrations of carbohydrates are indeed known to repress the expression of genes coding for several photosynthetic enzymes (Drake *et al.* 1997; Krapp and Stitt 1995). Alternatively, it has been observed that ABA concentration increases in 4X rootstock associations (Allario 2009). ABA has been shown to repress photosynthetic genes in several plant species (Rook *et al.* 2006). Eventually, it has been showed that scion genes expression related to photosynthesis, transcription/translation, cell division and stress tolerance may depend upon the rootstock used (Jensen *et al.* 2003). In summary, we are left with three

hypothesis to account for the observed negative effect of 4X rootstocks on photosynthesis: i) there was an indirect effect attributable to reduced sink activity, ii) there was a direct effect due to increased production of ABA by the roots, iii) there was a direct effect due to a specific, unknown effect of rootstocks. These hypotheses are not mutually exclusive. For instance, there is evidence that expression of photosynthetic genes is controlled by signaling networks involving both sugars and ABA (Rook *et al.* 2006).

Leaf flavonoid and chlorophyll content

High dualex values in the 4X rootstocks associations when compared to the 2X are surprising (Fig. 2A). One would indeed expect the concentrations in flavonoids and in chlorophylls to be negatively correlated according to the Protein Competition Model (PCM) (Jones and Hartley 1999). However, such negative correlations are only observed when concentrations are expressed on a mass basis. When expressed on an area basis, which is the case here, the concentrations in flavonoids and chlorophylls can be positively correlated (Meyer *et al.* 2006).

Yield and fruit quality

In our experiment clementine fruit yield obtained from 4x rootstocks was very low compared to the use of 2x rootstocks enlighten the physiological impact of the use of 4x rootstocks. However, if the use of any given 4x rootstock confers better tolerance to salt stress (Saleh *et al.* 2008) and water deficit (Allario 2009) which are very important criteria for the citrus industry, it would be possible to compensate the more limited yields by increasing the tree density as already proposed by (Wheaton *et al.* 1995). Also, we may expect to increase the profitability of any orchard planted with 4x rootstocks since it will be less water consuming and will require more limited pruning.

Concerning fruit quality, despite of decreasing fruit yields, 4x rootstocks did not change fruit quality parameters such as sugar content, acidity and fruit weight when compared to the use

of 2x rootstocks. This suggests that in absence of stress, the much greater leaf sugar and starch contents we measured when using 4x rootstock did not lead to large changes in the source /sink relation between leaves and fruits and consequently to large changes of fruit quality criteria. Carotenoid contents are highly influenced by genetic factor (Fanciullino *et al.* 2006). In 2x, 4x and 6x wheat species, carotenoid content (lutein) were significantly decreased with the increase of ploidy level (Leenhardt *et al.* 2006). We did not observe any difference in carotenoid content when using 2x compared to 4x rootstocks. Recently attempts have been made to elaborate the inheritance of fruit quality traits in allotetraploid citrus hybrid. The allotetraploid hybrid (*C. reticulata* Blanco x *C. limon* (L.) Burm.) produced the same carotenoid compounds as mandarin but at very low levels. The total carotenoid content was about 10-fold lower in the allotetraploid somatic hybrid juice sacs than in mandarin, leading to global quantitative dominance of lemon at the phenotypic level (Bassene *et al.* 2009). However this allotetraploid somatic hybrid presented higher ABA content in juice sacs than its two parents. In our hands, the use of 4x rootstocks did not seem to change carotenoid contents in fruit. This suggests that even through greater ABA contents can be measured in leaves of 2x scion grafted onto 4x rootstock because of a transfer of ABA from 4x roots to scion leading to limited gas exchanges and a more limited growth of the clementine /4x associations (Allario *et al.* 2009), this will not lead to any change carotenoid biosynthesis in fruit juice sacs.

Interestingly we found the higher hesperidin contents in clementine grown on 4x rootstocks. Hesperidin is the predominant flavonoid in most citrus fruits but its content depends upon the cultivar, environmental growing conditions and maturity stage (Mouly *et al.* 1997). Hesperidin is believed to play a role in plant defense. Also hesperidin was showed to be involved in defense mechanism of Valencia Late orange (*Citrus sinensis* (L.) Osb.) against infection with *P. citrophthora* (Del Río *et al.* 2004). It was also shown that hesperidin

provides strong cellular antioxidant protection against the damaging effects of hydrogen peroxide (Wilmsen and Salvador 2005). Tetraploid rootstocks induced a better protection to adverse effect of environment. As mentioned above 4x enhanced the electron flow rate under saturated light in leaves which is a way to overcome stress. Hence hesperidin found in fruit could help to nullify the stress effect of environment when used as a rootstock. To confirm these results it would be required to investigate leaf hesperidin contents of clementine grown onto 4x and 2x rootstocks but also in leaf of 2x and 4x seedlings.

Conclusions

Our work demonstrated that the use of 4x rootstocks dramatically changed the physiology of the rootstock/scion association. However even through fruit yields were much lower, clementine fruit quality criteria did not seem to be affected by the use of 4x root stock. Taking account of previous results we obtained (Allario et al, 2009), the better adaptation to abiotic constraint when using 4x rootstock would be favorable for the citrus industry.

Tables

Table 1: Show plant material used in this experiment (Rootstocks name, ICVN, Ploidy level and origin).

Name	ICVN	Ploidy level	Origin
Poncirus SEAB	0110086	2x	Algeria
Poncirus Boufarik	0110509	4x	Algeria
Poncirus Rubidoux	0110125	2x	USA (California)
Poncirus Rubidoux	0110440	4x	USA (California)

Table 2: Total starch, total sugar, total nitrogen, structural dry mass, mass to area ratio (M_a), leaf nitrogen content per unit mass (N_m), nitrogen per unit leaf area (N_a) for the two couples of 2x and 4x poncirus rootstocks grafted with clementine. Values (mean value ± se) with a same letter were not significantly different ($P < 0.05$, $n = 8$).

	ICVN.0110086 (2x)	**ICVN.0110509 (4x)**	**ICVN.0110125 (2x)**	**ICVN.0110440 (4x)**
Total starch (mg)	2.5 ± 1.0 a	27.4 ± 3.2 b	0.8 ± 1.2 a	19.5 ± 1.8 b
Total sugar (mg)	2.9 ± 0.4 a	6.7 ± 0.9 b	2.8 ± 0.6 a	4.5 ± 0.3 b
Structural dry mass (g)	0.11 ± 0.01 a	0.07 ± 0.01b	0.11 ± 0.02 a	0.06 ± 0.01 b
M_a (g m^{-2})	40.6 ± 4.6 a	28.2 ± 1.1 b	37.2 ± 3.5 a	25.7 ± 2.8 b
Total nitrogen (mg)	0.28 ± 0.03 a	0.19 ± 0.02 a	0.29 ± 0.05 a	0.17 ± 0.01 a
N_m (g N g^{-1})	2.42 ± 0.09 a	2.52 ± 0.04 a	2.54 ± 0.10 a	2.76 ± 0.05 a
N_a (g N m^{-2})	0.10 ± 0.01 a	0.07 ± 0.002 b	0.09 ± 0.01 a	0.07 ± 0.01 a

Table 3: Cumulative clementine fruit production (kg) for 11 years for the two couples of 2x and 4x poncirus rootstocks grafted with clementine. Average fruit weight, total sugar content, acidity content, incidence of maturity and % of juice is presented for two consecutive years. Values (mean value ± se) with a same letter were not significantly different ($P < 0.05$, $n \geq 4$).

	ICVN.0110086 (2x)	**ICVN.0110509 (4x)**	**ICVN.0110125 (2x)**	**ICVN.0110440 (4x)**
Cumulative fruit productior	689 ± 36.7 a	476.4 ± 44 b	665.8 ± 43 a	487.8 ± 20.6
Data (1982)				
Fruit weight (g)	61.75 ± 0.92 a	62.81 ± 3.26 a	59.87 ± 5.29 a	64.0 ± 2.39 a
Total sugar content (Brix)	8.85 ± 0.05 a	8.85 ± 0.10 a	8.92 ± 0.13 a	8.95 ± 0.02 a
Acidity content (g/100g)	1.35 ± 0.01 a	1.38 ± 0.05 a	1.30 ± 0.04 a	1.35 ± 0.02 a
Incidence of maturity	6.82 ± 0.06 a	6.68 ± 0.29 a	7.32 ± 0.16 a	6.87 ± 0.09 a
% of juice	50.59 ± 0.45 a	48.50 ± 0.85 a	46.48 ± 2.02 a	49.22 ± 0.22 a
Data (1983)				
Fruit weight (g)	55.98 ± 2.32 a	63.43 ± 1.18 b	60.8 ± 3.11 a	58.7 ± 3.98 a
Sugar content (Brix)	9.67 ± 0.21 a	9.92 ± 0.04 a	9.73 ± 0.35 a	9.62 ± 0.19 a
Acidity content (g/100g)	0.99 ± 0.04 a	0.95 ± 0.02 a	0.99 ± 0.04 a	0.94 ± 0.04 a
Incidence of maturity	10.12 ± 0.24 a	10.86 ± 0.27 a	10.22 ± 0.13 a	10.61 ± 0.29 a
% of juice	50.27 ± 0.42 a	48.94 ± 0.73 a	47.74 ± 1.28 a	48.82 ± 0.73 a

Table 4: Clementine fruit content of sugars (mg g^{-1} DW), Organic acids (mg g^{-1} DW), Carotenoid content (mg L^{-1}) and Flavanone Glycosides (mg L^{-1}) for the two couples of 2x and 4x poncirus rootstocks grafted with clementine. Values (mean value ± SE) with a same letter were not significantly different ($P < 0.05$, $n \geq 3$). All fruit quality parameters were analyzed in 2009.

	ICVN.0110086 (2x)	**ICVN.0110509 (4x)**	**ICVN.0110125 (2x)**	**ICVN.0110440 (4x)**
Sugar content (mg g^{-1} DW)				
Sucrose	346.3 ± 12.4 a	361.6 ± 7.6 a	354.7 ± 10.0 a	333.1 ± 15.0 a
Glucose	75.9 ± 3.3 a	75.7 ± 1.9 a	75.5 ± 1.4 a	72.2 ± 3.8 a
Fructose	105.6 ± 3.9 a	104.2 ± 2.2 a	105.6 ± 1.7 a	101.8 ± 4.7 a
Organic acids content (mg g^{-1} DW)				
Oxalique acid	3.7 ± 0.5 a	3.3 ± 0.2 a	3.3 ± 0.3 a	3.5 ± 0.5 a
Malic aicd	43.7 ± 2.0 a	46.1 ± 1.6 a	42.3 ± 1.4 a	49.0 ± 1.5 b
Ascorbic acid	6.8 ± 0.5 a	8.0 ± 0.3 b	7.3 ± 0.3 a	8.1 ± 0.4 a
Citric acid	68.4 ± 2.5 a	82.4 ± 2.1 b	67.0 ± 3.7 a	76.1 ± 5.9 a
Succinic acid	65.3 ± 3.8 a	72.2 ± 2.6 a	60.1 ± 3.2 a	85.4 ± 4.0 b
Carotenoid content (mg L^{-1})				
Phytoene	0.9 ± 0.1 a	0.9 ± 0.1 a	1.0 ± 0.2 a	0.9 ± 0.1 a
Phytofluene	0.9 ± 0.0 a	0.8 ± 0.1 a	0.9 ± 0.2 a	0.9 ± 0.1 a
Beta-carotene	0.8 ± 0.1 a	0.7 ± 0.1 a	0.9 ± 0.1 a	0.8 ± 0.1 a
Beta-cryptoxanthin	11.9 ± 0.4 a	11.8 ± 1.3 a	11.2 ± 1.4 a	12.3 ± 0.6 a
Zeaxanthin	0.8 ± 0.1 a	0.7 ± 0.0 a	0.7 ± 0.0 a	0.8 ± 0.0 a
Zeinoxanthin	0.2 ± 0.0 a	0.2 ± 0.0 a	0.2 ± 0.0 a	0.2 ± 0.0 a
Cis-antheraxanthin	2.0 ± 0.1 a	1.8 ± 0.1 a	1.8 ± 0.1 a	2.0 ± 0.1 a
Cis-violaxanthin	5.8 ± 0.3 a	5.6 ± 0.4 a	5.5 ± 0.7 a	5.9 ± 0.2 a
Neoxanthin	1.6 ± 0.1 a	1.6 ± 0.1 a	1.6 ± 0.2 a	1.7 ± 0.1 a
Luteine	0.9 ± 0.0 a	0.7 ± 0.0 b	0.7 ± 0.0 a	0.9 ± 0.0 b
Flavanone Glycosides (mg L^{-1})				
Hesperidin	435.4 ± 2.8 a	348.8 ± 2.0 b	532.8 ± 3.3 a	408.8 ± 1.8 b

Legends of Figures:

Fig. 1: Average scion/rootstock circumference ratio for two couples of 2x and 4x poncirus rootstocks grafted with clementine for eight years. Symbols in black color represent 2x rootstocks and white color represents 4x rootstocks. Values (Scion/rootstock ratio) with different letters were significant ($P < 0.05$, $n = 9$. Bar presented in the graphs follow this order 0110086 (2x), 0110509 (4x), 0110125 (2x) 0110440 (4x) for each year.

Fig. 2: (A) Relationship between the Photosynthesis rate (µmol m^{-2} s^{-1}) and the stomatal conductance *gs* (mmol m^{-2} s^{-1}) for the two couples of 2x and 4x poncirus rootstocks grafted with clementine. **(B)** Maximum electron transport rate J_{max} (µmol m^{-2} s^{-1}). Symbols and vertical bars in black color represent diploid and white color represent tetraploid rootstocks. Values (mean ± se) with different letter were significantly different ($P < 0.05$, $n \geq 3$).

Fig. 3: (A) Leaf phenolic content and **(B)** leaf chlorophyll content of 2x and their respective 4x genotypes. The vertical bars represent mean values with standard error. The values with different letter were significantly different ($P < 0.05$, $n \geq 25$).

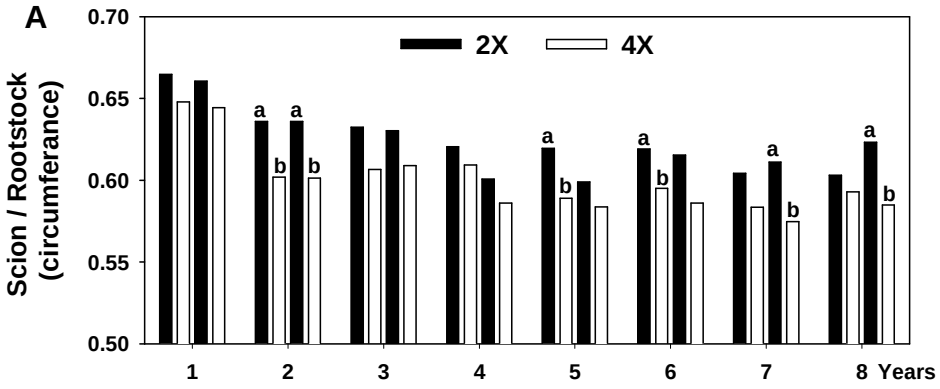


Fig. 1: Average scion/rootstock circumference ratio for two couples of 2x and 4x poncirus rootstocks grafted with clementine for eight years. Symbols in black color represent 2x rootstocks and white color represents 4X rootstocks. Values (Scion/rootstock ratio) with different letters were significantly ($P < 0.05$, $n = 9$). Bar presented in the graphs follow this order 0110086 (2x), 0110509 (4x), 0110125 (2x) 0110440 (4x) for each year.

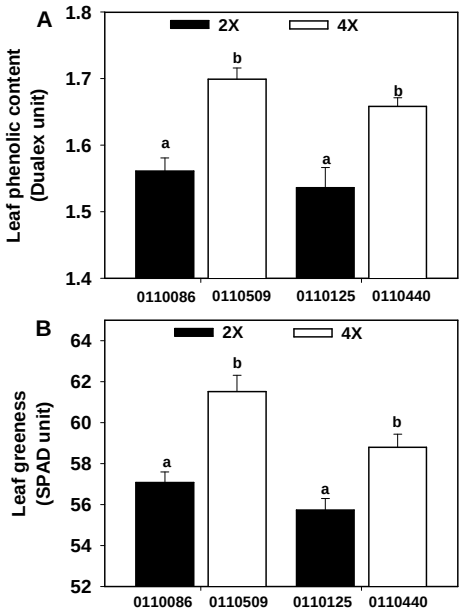


Fig. 2: (A) Leaf phenolic content and **(B)** leaf chlorophyll content of 2X and their respective 4X genotypes. The vertical bars represent mean values with standard error. The values with different letter were significantly different ($P < 0.05$, $n \geq 25$).

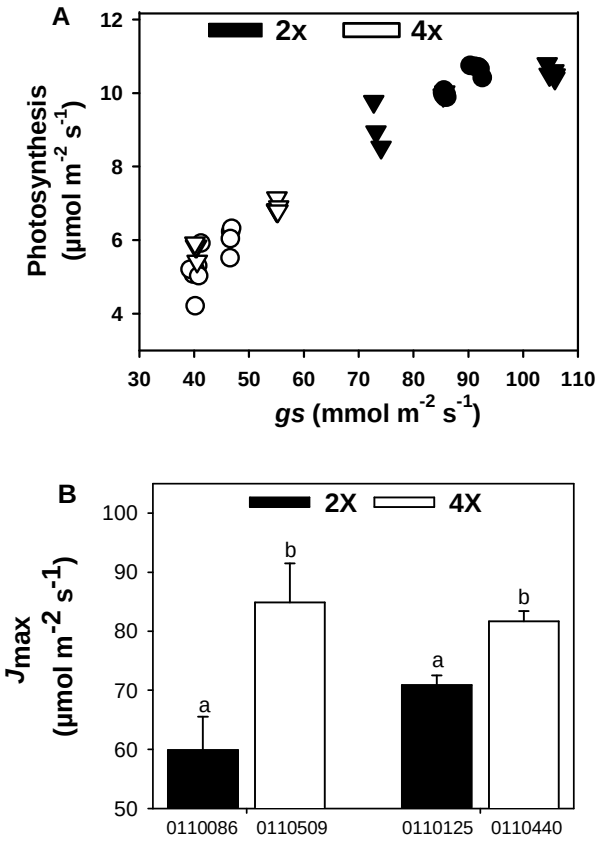


Fig. 3: (A) Relationship between the Photosynthesis rate (µmol m^{-2} s^{-1}) and the stomatal conductance *gs* (mmol m^{-2} s^{-1}) for the two couples of 2x and 4x poncirus rootstocks grafted with clementine. **(B)** Maximum electron transport rate J_{max} (µmol m^{-2} s^{-1}). Symbols and vertical bars in black color represent diploid and white color represent tetraploid rootstocks. Values (mean ± se) with different letter were significantly different ($P < 0.05$, $n \geq 3$).

6 Conclusions and perspectives

6.1 Physiological and molecular determinants of salt stress in citrus

In recent years two strategies widely used have been employed for citrus rootstocks and varieties improvement for better adaptation to environmental constraints: (1) to create somatic hybrids of complementary rootstock parents that have potential for improved biotic and abiotic resistances, tree size control, and horticultural performance (Grosser and Gmitter 1990; Grosser and Gmitter 1990; Ollitrault *et al.* 1994; Ollitrault *et al.* 2000) and (2) to produce numerous hybrids of *Citrus* with related genera to broaden the germplasm base, including sexually incompatible or difficult to hybridize citrus relatives (Grosser *et al.* 2007; Ollitrault *et al.* 2000). Most genotypes used to create new varieties (triploid and hybrids) have been investigated only for fruit production and fruit quality and little interest has been given to adaptation to abiotic constraints. To withstand new challenges such as soil salinity, one may wonder if it would be possible to use parental genitors that have good fruit quality properties but also with good abiotic stress tolerance properties for example more efficient systems for detoxification of ROS and compartmentation of ions in vacuoles. By that way it would be possible to increase the tolerance to salt stress by the association of a Cl^- and Na^+ excluder rootstock and a variety better adapted to salt stress. Therefore it is important to evaluate all the genetic sources that are to our disposition, including monoembryonic genotypes, in order to be able to select specific salinity traits of tolerance.

To develop new genitor to salt tolerance a good knowledge of different mechanism involved in salt tolerance is necessary. In citrus, salt tolerant ability is usually related with exclusion of chloride ions. This ability to exclude Cl^- appears to be the combined result of several traits since chloride tolerance has often been associated with molecular, biochemical, hormonal, physiological (Gómez Cadenas *et al.* 1998; Tadeo *et al.* 2008) and morphological factors (Moya *et al.* 1999). In our experiment we observed a large diversity concerning the Cl^- accumulation in leaves. Citron species (Poncire commun, Etrog, Digite and Diamante) presented high chloride content. On the other hand, mandarin's species (Cleopatra and Sunki) accumulated lesser amount of Cl^- in leaves. The ability to restrict Cl^- at root levels of other species studied in this experiment was in between of Citron and Mandarin. Poncirus and Carrizo which are known to salt sensitive were present in the middle range (Figure 9). Citron

species have primarily evolved by self pollination and show little inter-varietals diversity (Ollitrault *et al.*, 2003). It is interesting to note that all citron under stress has the same symptoms and characteristics (large decrease in *gs* and photosynthetic capacity). This low variability in physiological parameters could be due to little inter varietals genetic diversity (Nicolosi *et al.* 2005; Ollitrault *et al.* 2003). In our experiment we observed more or less the same patterns for sodium exclusion as chloride. Under moderate salt stress, salt tolerance is attributed to Cl^- exclusion but not to Na^+. In high salt stress conditions, Na^+ is also very toxic. So it will be interesting to know what are the concentrations of Na^+ tolerable by citrus genotypes.

Froelicher *et al.* (2010) showed that the group of acidic mandarins (Cleopatra mandarin, Sunki mandarin, Shekwasha mandarin) differs from other mandarins and revealed a maternal relationship with *Citrus limonia* (Rangpur lime, Volkamer lemon) and *Citrus jambhiri* (Rough lemon). It should be noted that these genotypes belong to cultivated species used as rootstocks and that have a stronger adaptation to abiotic stresses. Also results suggest that two species classified by Swingle and Reece, *Citrus limon*, and *Citrus aurantifolia*, have multiple maternal cytoplasmic origins (Froelicher *et al.* 2010). Therefore, the existence of a link between adaptation to salt or water stress and the relatedness between genotypes can be assumed. Does salt tolerance ability of maternal plant have more impact than the parental genotypes? Also we know that cytoplasm in *Citrus* is maternally inherited (Green *et al.* 1986; Masashi Yamamoto 1993). We may ask the question for role of different cytoplasmic organelle in salt tolerance.

Mexican lime is a hybrid between *C. micrantha* and citron while Rangpur lime is hybrid have mandarin as maternal origin and citron as a paternal origin (Nicolosi *et al.* 2000). We may assume that tolerance of Rangpur lime may be due to its maternal parent. Similarly Eureka lemon which also presentsgood tolerance has a maternal origin as Sour orange and paternal origin as citron. It is known that Australian Sour orange is hybrid between pummelo and mandarin. Furthermore Star ruby grapefruit was obtained through cross between pummelo and orange. Also it was observed that most of the genotypes that have mandarin and pummelo as maternal origin were more tolerant to salt stress.

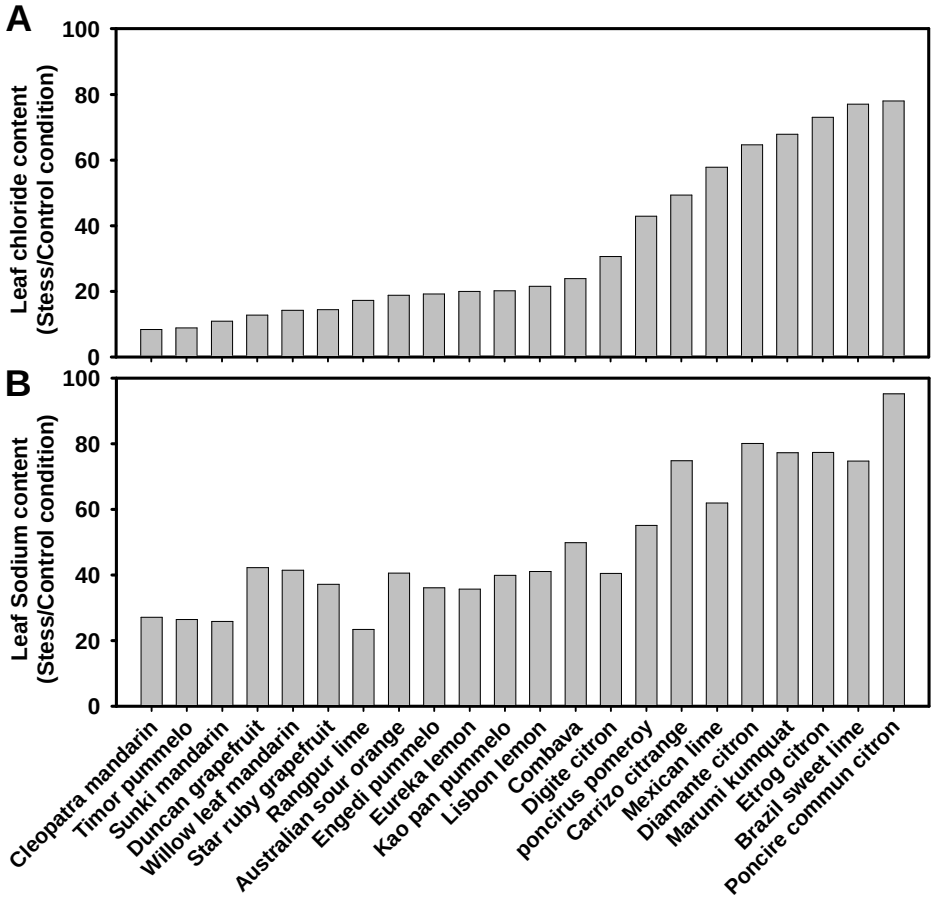


Fig. 9: A) Leaf chloride content stress/control condition and B) leaf sodium content stress/control condition after 80 days of salt stress

(In the both graph genotypes have been arranged in same order)

Mitochondria is a cytoplasmic organelle that plays an important role in salt tolerance (Jacoby *et al.* 2010; Mittler 2006). The effect of salinity on mitochondrial respiration is yet to be fully characterized; in fact, there are contradictory physiological reports showing that respiration rates increase in response to salt stress (Bloom and Epstein 1984; Livne and Levin 1967), while others show that it can decrease (Jolivet *et al.* 1990). It is likely that these responses probably differ according to species, plant organ, developmental stage, and the severity of salinity treatment (Jacoby *et al.* 2010). Furthermore link of mitochondrial proline metabolism with salinity and drought tolerance (Di Martino *et al.* 2006) and the relatively high degree of ROS-induced damage of mitochondrial proteins compared to those of other organelles in wheat cells (Bartoli *et al.* 2004) show the role of mitochondria in salinity tolerance. In citrus, it has been shown that in Cleopatra mandarin, proline content was increased in root under salt stress (Anjum 2008). Also mitochondria are equipped with an impressive array of enzymatic and nonenzymatic antioxidant defense systems posed to detoxify the ROS generated by adverse circumstances (Addabbo *et al.* 2009; Asada 2006; Di

Martino *et al.* 2006). Similarly in plants, chloroplast is the site of ROS production. This elaborates the importance of cytoplasmic genomes which should play an important role in selection of genotypes in breeding scheme. Furthermore we may hypothesized that salt tolerance is citrus is related to better detoxification of ROS species and/or high capacities to make compartmentation under salt stress.

Fortunella is a taxonomic group rarely or never studied for its behavior towards salt stress. It appears to be very sensitive to salt, because the symptoms of discoloration and defoliation observed were very pronounced and advanced. In a hierarchy of Cl^- accumulation it is just placed after Citron species. Also it is interesting to note that inter-specific genetic diversity is clearly observed in physiological processes. *Fortunella* was the most sensitive genera under salt stress followed by *Poncirus.* Within the *Citrus*, there is different physiological behavior regarding the ions accumulation, leaf symptoms and central metabolic processes (mandarins, pummelo and citron). In *Fortunella* PSII system seemed to be badly affected which is usually admitted to be a direct effect of oxidative stress while *Poncirus* and related species (e.g. Carrizo citrange) showed less damaged to PSII system as compared to *Fortunella* but presented high flow of leaf CO_2 exchange. On the other hand in *Citrus*, in spite of large diversity, salt tolerant genotypes decreased the different function of plants (growth, photosynthesis, stomatal conductance) and maintained the chlorophyll content and PSII system. This further solidifies the importance of avoiding and repairing of oxidative damage by scavenging of ROS species.

Mouhaya (2008) found that tetraploid rootstocks (autotetraploid and allotetraploid) were more tolerant to salt stress. Whether this capacity of salt tolerance was due to better detoxification mechanism? Recently it is shown that in tetraploid of *Dioscorea zingiberensis* the activity of antioxidant enzymes superoxide dismutase, peroxidase, catalase, ascorbate peroxidase and glutathione reductase were stimulated and antioxidants (ascorbic acid and glutathione) were maintained at high concentrations to tolerate the heat stress. We may suppose that better detoxification mechanism is important in salt tolerance of tetraploid citrus rootstocks. In tetraploid there is a duplication of the nuclear genome. However it is not known if it has an impact on the number of copies of cytoplasmic genomes. In citrus, if the cytoplasm plays an important role in salt tolerance properties, then we may suppose that better tolerance in allotetraploid might be obtained depending on the origin of the parental cytoplasmic organelles. It will be interesting to explore this which could ultimately affect the choice of female parent during somatic and sexual breeding.

Tetraploid genitors are widely used in varietal improvement schemse for triploid production (Ollitrault *et al.* 2008). From the results obtained in tetraploids; we may wonder if triploid plants have better detoxification abilities. If this hypothesis is right, it will be very interesting to explore and direct the scheme of triploid production toward the ability of better detoxification mechanism. A better detoxification ability in scion varieties combined with rootstock having good toxic ions exclusion ability could withstand salt and water stress. Also triploid plants have large stomata but lower stomatal density like tetraploid so we may assume that triploid plants would present a better water use efficiency like tetraploid plant (Mouhaya 2008). Hence we should select the new varieties and rootstocks for salt tolerance or for breeding purpose according to their detoxification abilities and we should use the tolerant genitor as female parent if the role of cytoplasmic genomes in stress tolerance is confirmed.

6.2 Genetic determinant of salt tolerance

To dissect the genetic determinant of salt tolerance we developed F2 population from two rootstocks with contrasting behavior under salt stress. It has been reported that salt tolerance is a complex trait involving the function of many genes (Foolad 2004; Lindsay *et al.* 2004). One of recently used ways of studying the genetic determinants of phenotypic traits is the production of genetic maps. This involves determining the relative position of loci (genes or sequences) on chromosomes. The scheduling and distance separating the loci are estimated from the frequency of recombination occurring in meiosis. A segregating population was necessary to estimate the frequency of recombination. To do this we created an F2 population by self pollination of F1 hybrid obtained from cross between (Cleopatra mandarin x Poncirus trifoliata). The choice of this hybrid has been governed by the salt stress tolerance of mandarin and sensitivity of Poncirus ensuring the segregation of the tolerance trait in the progeny. We developed the population and genetic map was constructed we also mapped different gene on this map. It will be then interesting to investigate the salt tolerance behavior of this population. It will help to identify the QTL's potentially involved in the salt tolerance. We are limited by the size of our plant population (99 hybrids) and the number of genetic markers could be increased (SSR markers 135 and 4 candidate genes). It will be more beneficial to expand the population and also search for more heterozygous markers for F1 population to increase the map density, better coverage of total genome and better identification of markers involved in QTLs.

6.3 Impact of autotetraploid and zygotic rootstocks on Clementine fruit yield and quality

Rootstocks selection depends on many factors like fruit yield, fruit quality, and tolerance to biotic and abiotic stresses. Indeed, autotetraploid citrus seedlings have shown a better tolerance to salt stress and water stress than their parental diploids (Saleh *et al*. 2008; Allario *et al*. 2010), probably because of a higher abcisic acid constitutive biosynthesis in tetraploid (Allario *et al*. 2010). Rootstock–scion relationship has extensively been discussed in literature but effect of citrus autotetraploid rootstocks on scion growth, fruit yield and quality is yet to be explored. Analysis of the number of tetraploid in a field trial showed that 2.4% of tetraploid were still present even after rouging of nursery seedlings. In a previous study, 5.4% of tetraploid were obtained among seedling of trifoliate orange Pomeroy (SRA 1074) taking account of all germinated seedlings (Saleh *et al.* 2008). Tetraploids are characterized by slower growth, typically broader, thicker and darker leaves (Barrett and Hutchison 1978; Cameron and Soost 1969). Similarly, tetraploid induction is temperature dependant and cold temperature favors the tetraploid induction Aleza et al (2011). Also these authors showed that the different genotypes have different ability to produce tetraploid seedling. A high rate of 20% of tetraploid was observed in Carrizo citrange originating from Mediterranean area. We observed the 6.6 % of plants having zygotic origin. It is important to discard the zygotic plant from rootstock trials because of uncertain effect on fruit yield and quality. However, we don't know if the rate of zygotic seedlings is still very limited among seedlings originating for a tetraploid parental tree. Therefore, further analysis will be required in order to confirm the interest of the use of tetraploid seedlings. Also we may wonder about the origin of tetraploidization. If tetraploidization is environmentally dependant phenomenon, one may wonder whether it is affected by temperature at flowering time or triggered by a hormonal effect such as ABA content or by both factors. It will be very interesting to extract the seeds of fruits from young trees grown on different temperatures at flowering stage and then to grow these seeds to observe the percentage of tetraploid seedlings.

One of the most dramatic effects of the rootstock is on tree size (Jensen *et al.* 2003). Slow growth rate of clementine grown was observed on autotetraploid rootstocks compared to diploid rootstocks. Clementine grown on diploid rootstocks was more vigorous than on tetraploid rootstocks. The mechanism by which the rootstock exerts its size control on the scion is not well understood (Basile *et al.* 2003). It could be due to reductions in nutrient and water movement (Kamboj *et al.* 1996) and changes in hormone concentration (Michalczuk

2002). It is well known that autotetraploid rootstocks exhibit low photosynthesis and stomatal conductance (Mouhaya 2008; Syvertsen *et al.* 2000) and leaf gas exchange parameters ultimately affecting the growth of plant. We observed low photosynthesis and stomatal conductance for Clementine /tetraploid rootstocks association as compared to Clementine /diploid rootstocks association. Rootstock genotype had a significant impact on scion gas exchange, water status, canopy growth and yield (Soar *et al.* 2006). We may suppose that the slower growth of Clementine and ultimately tree height is the effect of gas exchange capacity due to the use of tetraploid rootstock.

The rate of photosynthesis depends on one of these factors: 1) activity and amount of photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); 2) regeneration rate of ribulose bisphosphate (RuBP) and 3) synthesis of starch and sugar (Farquhar *et al.* 1980; Manter and Kerrigan 2004). Total leaf starch and sugar content was higher in leaves of clementine/tetraploid rootstock association. There was no difference in leaf nitrogen content when expressed in per unit mass. To author's knowledge effect of autotetraploid citrus rootstocks on scion leaf nitrogen and sugar content is not documented. From the literature, we know that production of leaf starch may decrease photosynthesis caused by feedback regulation (Iglesias *et al.* 2002; Sun *et al.* 1999). Also it is known that high sugar content in leaves can repress some genes of photosynthesis (Webber *et al.* 1994). We may then suppose that the decrease of the leaf Clementine photosynthesis grafted on tetraploid rootstock is dependant of the carbohydrate contents. To explain the large changes in sugars and starch content in leaf of Clementine grafted on tetraploid compared to Clementine grafted onto diploid rootstocks, we may suppose that the source /sink relation between roots and scion is strongly affected. Indeed, Allario *et al.* (2011) showed the root morphology is strongly changed between tetraploid and diploid plants grown in pots. Therefore, there is no doubt that the use of tetraploid rootstock may strongly affect carbohydrates balance in between root and shoot.

In contrast to photosynthesis and stomatal conductance rate, J_{max} (maximum electron flow rate under saturated light) was higher for Clementine/tetraploid rootstock association as compared to the use of Clementine/diploid rootstock association. The difference was very significant. In our knowledge we did not find any reference on effect of rootstock on scion electron flow capacity. So we may suppose that tetraploid rootstock may affect the scion maximum electron flow rate under saturated light. Poncirus autotetraploid rootstocks induced increased capacity of J_{max} in Clementine scion. Scion genes expression related to photosynthesis, transcription/translation, cell division and stress tolerance depends upon the

rootstocks used (Allario 2009; Jensen *et al.* 2003). We may also wonder if the higher J_{max} which we observed in scion is related to different gene expression? Similarly is higher J_{max} account for the better tolerance to salt stress? We previously reported that plants have developed different mechanism (ion homeostasis, osmolytes biosynthesis, compartmentation of ions, ROS scavenging etc) to cope with salt stress (Flowers and Colmer 2008; Hasegawa *et al.* 2000). All these processes places burden on plant metabolism. An alternative strategy, placing less of a metabolic burden on plant can be achieved by regulation of photosynthetic electron transport (Johnson 2005). In Thellungiella which is known to be a salt tolerant plant, gas exchanges were marginally inhibited by high salt and PSI was unaffected, but there was a large increase in electron flow involving PSII (Stepien and Johnson 2008). Salt stress tolerance may be increased by decrease in oxidative stress through better flow of electron in PSII. As tetraploid rootstocks encounter better to salt stress (Mouhaya 2008) and (Allario et al 2009 submitted). We assume that the better tolerance to salt stress in tetraploid rootstocks could be due to better electron flow rate in PSII. We were limited in our studies to the comparison of poncirus diploid and autotetraploid rootstocks. Now it would be interesting to explore other autotetraploid and allotetraploid of citrus *genera* as seedlings but also grafted and different scion varieties.

6.3.1 Yield and fruit quality

In our experiment, Clementine fruit yield on tetraploid rootstocks was very low than when using diploid rootstocks. But we also observed that plant Clementine grown on tetraploid rootstocks were not as vigorous. So we may increase the total yield by increasing the planting density in field (Wheaton *et al.* 1995). Also preliminary result obtained at IVIA research centre showed that for same volume of canopy scion variety gave more fruits on tetraploid rootstocks as compared to diploid rootstock (Raphael Morillon, personal communication). Hence we may expect to increase the fruits production when using tetraploid rootstocks by better management practices like planting distance and pruning.

Now the question is whether the fruit quality is change? It is said that tetraploid rootstocks produce more acidic fruits. The secondary metabolites (vitamin C, phenols, carotenoids, total antioxidant capacity) of scion fruit is also influenced by rootstock (Remorini *et al.* 2008). In our hands, despite of decreasing fruit yield, autotetraploid rootstocks have no or very minimal change in fruit quality parameters. Sugar content, acidity and fruit weight was same when using both diploid and tetraploid rootstocks. Also the detailed analysis of

sugar that we performed did not show any difference in fructose, sucrose and glucose content depending of the use of diploid/tetraploid rootstock. Little concern can be addressed about the different organic acid contents. Indeed, even through higher contents were measured when using one tetraploid rootstock, for the other tetraploid genotype investigated these differences were not significant (article 3).

Carotenoid contents are highly influenced by genetic factor (Fanciullino *et al.* 2006). In diploid, tetraploid and hexaploid wheat species, carotenoid content (lutein) were significantly decreased with the increase of ploidy level (Leenhardt *et al.* 2006). We did not observe any difference in carotenoid content when using diploid and tetraploid rootstocks. Recently attempts have been made to elaborate the inheritance of fruit quality traits in allotetraploid citrus hybrid. The allotetraploid hybrid (*C. reticulata* Blanco x *C. limon* (L.) Burm.) produced the same carotenoid compounds as mandarin but at very low levels. The total carotenoid content was about 10-fold lower in the allotetraploid somatic hybrid juice sacs than in mandarin, leading to global quantitative dominance of lemon at the phenotypic level (Bassene *et al.* 2009). In our hands, the use of autotetraploid rootstocks did not change fruit carotenoid contents. It would be interesting to know if autotetraploid rootstocks also behave in same way when not used as rootstock. ABA is the end product of the carotenoid biosynthetic pathway. It has been observed that tetraploid rootstocks produce more ABA then diploid rootstocks (Allario et al 2009) and it would one of the reasons that may explain why tetraploid rootstocks can withstand better in stressful environment. We may assume that tetraploid rootstocks produce less carotenoid content compared to diploid rootstocks. So it would be important to know that if autotetraploid rootstock does change the carotenoid contents of scion fruit and if there is any change in ABA content in fruit and leaves?

Interestingly we found the high hespridin content in clementine grown on tetraploid rootstocks. Hesperidin is the predominant flavonoid in most citrus fruits but its content depends upon the cultivar, environmental growing conditions and maturity stage (Mouly *et al.* 1997). Hesperidin is believed to play a role in plant defense. Also it was shown that there may the involvement of hespridine in defense mechanism of cv. Valencia Late orange (*Citrus sinensis*) plant against infection with *P. citrophthora* (Del Río *et al.* 2004). As poncirus genotype is resistant to biotic stresses we may assume that induction of high content of hesperidin in scion fruit could be a form of defense mechanism. Another role of hesperidin is as an antioxidant in cell. Salt stress preferentially enhanced the content of H_2O_2 as well as the activities of the superoxide dismutase (SOD) in plant. It was shown that hesperidin provides strong cellular antioxidant protection against the damaging effects of peroxide hydrogen

(Wilmsen and Salvador 2005). So autotetraploid rootstocks induced a better protection to adverse effect of environment. As mentioned above that autotetraploid enhanced the electron flow rate under saturated light in leaves which is a way to overcome stress. We may add that autotetraploid rootstocks induce better defense mechanism by increase in hesperidin production.

Now what will be the effect of autotetraploid rootstocks on scion fruit quality under salt stress? In normal condition the citrus fruit quality seem to be less affected by ploidy level. We may hypothesize that under salt tolerance while giving better tolerance to salt and water stress autotetraploid genotypes will not change the fruit quality. We also hypothesize that autotetraploid rootstocks will gave better tolerance by inducing high electron transport rate and high detoxification capacity in scion leaves under salt and water stress. Similarly what would be the effect of the use of tetraploid rootstocks on triploid varieties under salt stress and water deficit? It will be very interesting to explore this. At the end, we may expect a better water use efficiency while preserving PSII system with minimal oxidative stress damage.

7 Literature cited

Abu-Abied, M.; Holland, D. The gene c-ino1 from Citrus paradisi is highly homologous to tur1 and ino1 from yeast and Spirodela encoding for myo-inositol phosphate synthase. *Plant Physiology* **1994**, 106, 1689.

Adams, K.; Cronn, R.; Percifield, R.; Wendel, J. Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. *Proc. Natl. Acad. Sci. U. S. A.* **2003**, 100, 4649.

Addabbo, F.; Montagnani, M.; Goligorsky, M. Mitochondria and reactive oxygen species. *Hypertension* **2009**, 53, 885.

Adie, B.; Perez-Perez, J.; Perez-Perez, M.; Godoy, M.; Sanchez-Serrano, J.; Schmelz, E.; Solano, R. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. *The Plant Cell Online* **2007**, 19, 1665.

Agastian, P.; Kingsley, S.; Vivekanandan, M. Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica* **2000**, 38, 287-290.

Agre, P.; Bonhivers, M.; Borgnia, M. The aquaporins, blueprints for cellular plumbing systems. *J. Biol. Chem.* **1998**, 273, 14659.

Aharon, G.; Apse, M.; Duan, S.; Hua, X.; Blumwald, E. Characterization of a family of vacuolar Na+/H+ antiporters in Arabidopsis thaliana. *Plant and soil* **2003**, 253, 245-256.

Ajay, A.; Divya, C.; Gaurav, A.; Singh, V. P. Compositional variation in beta - carotene content, carbohydrate and antioxidant enzymes in selected banana cultivars. *International Journal of Food Science & Technology* **2008**, 43, 1913-1921.

Albertini, M. V.; Carcouet, E.; Pailly, O.; Gambotti, C.; Luro, F.; Berti, L. Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. *Journal of Agricultural and Food Chemistry* **2006**, 54, 8335-8339.

Aleza, P.; Froelicher, Y.: Schwarz, S.; Agustí, M.; Hernández, M.; Juárez, J.; Morillon, R., Navarro, L.; Ollitrault, P. Tetraploidization events by chromosome doubling of nucellar cells are frequent in apomictic **citrus** and are dependent on genotype and environment. 2011.Submitted (Annals of Botany).

Alia; Saradhi, P. P. Proline accumulation under heavy-metal stress *J. Plant Physiol.* **1991**, 138, 554-558.

Alia; Hayashi, H.; Chen, T. H. H.; Murata, N. Transformation with a gene for choline oxidase enhances the cold tolerance of Arabidopsis during germination and early growth. *Plant Cell Environ.* **1998a**, 21, 232-239.

Alia; Hayashi, H.; Sakamoto, A.; Murata, N. Enhancement of the tolerance of Arabidopsis to high temperatures by genetic engineering of the synthesis of glycinebetaine. *Plant J.* **1998b**, 16, 155-161.

Alia; Mohanty, P.; Matysik, J. Effect of proline on the production of singlet oxygen. *Amino Acids* **2001**, 21, 195-200.

Allakhverdiev, S. I.; Nishiyama, Y.; Miyairi, S.; Yamamoto, H.; Inagaki, N.; Kanesaki, Y.; Murata, N. Salt stress inhibits the repair of photodamaged photosystem II by suppressing the transcription and translation of psbA genes in Synechocystis. *Plant Physiology* **2002**, 130, 1443-1453.

Allario, T. Identification de déterminants physiologiques et moléculaires de la tolérance à la contrainte saline et au déficit hydrique de porte-greffes autotétraploïdes d'agrumes. Thèse de Doctorat, Université de Corse-Pascal Paoli, Corte, **2009**, p 201.

Allario T.; Brumos J.; Colmenero-Flores J-M.; Iglesias, D.; Talon M.; Navarro L.; Ollitrault P.; Morillon R. The higher water deficit tolerance Valencia sweet orange (*Citrus sinensis*) grafted on doubled diploid Rangpur lime (*citrus limonia*) rootstock is mediated by an over expression in root of genes involved in water deficit tolerance.**2010**. (Submitted).

Allario T.; Brumos J.; Colmenero-Flores J-M.; Tadeo F.; Froelicher Y.;Talon M.; Navarro L.; Ollitrault P.; Morillon R. Large changes in anatomy and physiology between diploid Rangpur lime (*Citrus limonia*) and its autotetraploid are not associated with large changes in leaf gene expression. Journal Experimental of Botany, 2011 *In press*

Allen, D.; Ort, D. Impacts of chilling temperatures on photosynthesis in warm-climate plants. *Trends Plant Sci.* **2001**, 6, 36-42.

Amtmann, A.; Sanders, D. Mechanisms of Na+ uptake by plant cells. *Advances in Botanical Research* **1998**, 29, 75-112.

Andreyev, A. I.; Kushnareva, Y. E.; Starkov, A. A. Mitochondrial metabolism of reactive oxygen species. *Biochem.-Moscow* **2005**, 70, 200-214.

Anil, V.; Sankara Rao, K. Calcium-mediated signal transduction in plants: a perspective on the role of Ca2+ and CDPKs during early plant development. *J. Plant Physiol.* **2001**, 158, 1237-1256.

Anjum, M. A. Effect of NaCl concentrations in irrigation water on growth and polyamine metabolism in two citrus rootstocks with different levels of salinity tolerance. *Acta Physiol. Plant.* **2008**, 30, 43-52.

Apse, M.; Aharon, G.; Snedden, W.; Blumwald, E. Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. *Science* **1999**, 285, 1256.

Apse, M.; Blumwald, E. Engineering salt tolerance in plants. *Current Opinion in Biotechnology* **2002**, 13, 146-150.

Apse, M.; Sottosanto, J.; Blumwald, E. Vacuolar cation/H+ exchange, ion homeostasis, and leaf development are altered in a T-DNA insertional mutant of AtNHX1, the Arabidopsis vacuolar Na+/H+ antiporter. *The Plant Journal* **2003**, 36, 229-239.

Arbona, V.; Flors, V.; Jacas, J.; Garcia-Agustin, P.; Gomez-Cadenas, A. Enzymatic and non-enzymatic antioxidant responses of Carrizo citrange, a salt-sensitive citrus rootstock, to different levels of salinity. *Plant and Cell Physiology* **2003**, 44, 388-394.

Arbona, V.; Lopez-Climent, M. F.; Mahouachi, J.; Perez-Clemente, R. M.; Abrams, S. R.; Gomez-Cadenas, A. Use of persistent analogs of abscisic acid as palliatives against salt-stress induced damage in citrus plants. *J. Plant Growth Regul.* **2006**, 25, 1-9.

Arruda, M.; Gonçalves, E.; Schneider, M.; da Costa da Silva, A.; Morielle-Versute, E. An alternative genotyping method using dye-labeled universal primer to reduce unspecific amplifications. *Molecular biology reports* **2010**, 37, 2031-2036.

Asada, K. Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **2006**, 141, 391.

Ashari, S.; Aspinall, D.; Sedgley, M. Discrimination of zygotic and nucellar seedlings of 5 polyembryonic citrus rootstocks by isozyme analysis and seedling morphology. *J. Horticult. Sci.* **1988**, 63, 695-703.

Ashraf, M. Breeding for salinity tolerance in plants. *Crit. Rev. Plant Sci.* **1994**, 13, 17-42.

Ashraf, M.; Harris, P. J. C. Potential biochemical indicators of salinity tolerance in plants. *Plant Science* **2004**, 166, 3-16.

Atmane, R.; El-Yacoubi, H.; Abdellatif, R. Responses to NaCl stress of Citrus aurantium, Citrange troyer and Poncirus trifoliata in callus cultures: assessment of characters for evaluating salt stress responses in citrus rootstocks. *Agronomie* **2003**, 23, 643-649.

Auger, D.; Gray, A.; Ream, T.; Kato, A.; Coe Jr, E.; Birchler, J. Nonadditive gene expression in diploid and triploid hybrids of maize. *Genetics* **2005**, 169, 389.

Baker, N. A possible role for photosystem II in environmental perturbations of photosynthesis. *Physiol. Plant.* **1991**, 81, 563-570.

Banu, N. A.; Hoque, A.; Watanabe-Sugimoto, M.; Matsuoka, K.; Nakamura, Y.; Shimoishi, Y.; Murata, Y. Proline and glyclinebetaine induce antioxidant defense gene expression and suppress cell death in cultured tobacco cells under salt stress. *J. Plant Physiol.* **2009**, 166, 146-156.

Banuls, J.; Legaz, F.; Primomillo, E. Effect of salinity on uptake and distribution of chloride and sodium in some citrus scion-rootstock combinations. *J. Horticult. Sci.* **1990**, 65, 715-724.

Banuls, J.; Serna, M.; Legaz, F.; Talon, M.; Primo-Millo, E. Growth and gas exchange parameters of Citrus plants stressed with different salts. *J. Plant Physiol.* **1997**, 150, 194-199.

Bar-Joseph, M.; Lee, R. Citrus tristeza virus. *AAB descriptions of plant viruses* **1989**, 353,

Bari, R.; Jones, J. Role of plant hormones in plant defence responses. *Plant Molecular Biology* **2009**, 69, 473-488.

Barkley, N. A.; Roose, M. L.; Krueger, R. R.; Federici, C. T. Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theoretical and Applied Genetics* **2006**, 112, 1519-1531.

Barrett, H. C. Hybridization of Citrus and related genera. *Fruit varieties journal (USA)* **1985**.

Barrett, H. C.; Rhodes, A. M. A numerical taxonomic study of affinity relationships in cultivated *Citrus* and it close relatives. *Systematic Botany* **1976**, 1, 105-136.

Barrett, H. C.; Hutchison, D. J. Spontaneous tetraploidy in apomitic seedlings of Citrus. *Economic Botany* **1978**, 32, 27-45.

Barrett, H. C.; Hutchinson, D. J. Occurrence of spontaneous octoploidy in apomictic seedlings of a tetraploid *Citrus* hybrid. *Proceedings of the International Society of Citriculture* **1982**, 1, 29-30.

Bartoli, C.; Gomez, F.; Martinez, D.; Guiamet, J. Mitochondria are the main target for oxidative damage in leaves of wheat (Triticum aestivum L.). *Journal of Experimental Botany* **2004**, 55, 1663.

Basile, B.; Marsal, J.; DeJong, T. Daily shoot extension growth of peach trees growing on rootstocks that reduce scion growth is related to daily dynamics of stem water potential. *Tree Physiol.* **2003**, 23, 695.

Bassene, J. B.; Froelicher, Y.; Dhuique-Mayer, C.; Mouhaya, W.; Ferrer, R. M.; Ancillo, G.; Morillon, R.; Navarro, L.; Ollitrault, P. Non-additive phenotypic and transcriptomic inheritance in a citrus allotetraploid somatic hybrid between C. reticulata and C. limon: the case of pulp carotenoid biosynthesis pathway. *Plant Cell Reports* **2009**, 28, 1689-1697.

Bassene, J. B.; Froelicher, Y.; Dubois, C.; Ferrer, R. M.; Navarro, L.; Ollitrault, P.; Ancillo, G. Non-additive gene regulation in a citrus allotetraploid somatic hybrid between C. reticulata Blanco and C. limon (L.) Burm. *Heredity* **2010**, 105, 299-308.

Behboudian, M.; Törökfalvy, E.; Walker, R. Effects of salinity on ionic content, water relations and gas exchange parameters in some citrus scion--Rootstock combinations. *Sci. Hortic.* **1986**, 28, 105-116.

Beidler, J.; Hilliard, P.; Rill, R. Ultrasensitive staining of nucleic acids with silver. *Analytical biochemistry* **1982**, 126, 374-380.

Bellinger, Y.; Larher, F. Proline accumulation in higher plants: a redox buffer. *Plant Physiol Life Sci Adv* **1985**, 6, 23-27.

Benhayyim, G.; Kochba, J. Aspects of salt tolerance in a nacl-selected stable cell-line of citrus-sinensis

Plant Physiology **1983**, 72, 685-690.

Bienert, G.; Møller, A.; Kristiansen, K.; Schulz, A.; Møller, I.; Schjoerring, J.; Jahn, T. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J. Biol. Chem.* **2007**, 282, 1183.

Bloom, A.; Epstein, E. Varietal differences in salt-induced respiration in barley. *Plant Science Letters* **1984**, 35, 1-3.

Bohnert, H.; Jensen, R. Strategies for engineering water-stress tolerance in plants. *Trends in Biotechnology* **1996**, 14, 89-97.

Bohnert, H.; Sheveleva, E. Plant stress adaptations--making metabolism move. *Current Opinion in Plant Biology* **1998**, 1, 267-274.

Bongi, G.; Loreto, F. Gas-exchange properties of salt-stressed olive (Olea europea L.) leaves. *Plant Physiology* **1989**, 90, 1408.

Borsani, O., Cuartero, J., Fernandez, J. A., Valpuesta, V & Botella, M.A. Identification of two loci in tomato reveals distinct mechanism for salt tolerance. *Plant cell* **2001a**, 13, 873-887.

Boudsocq, M.; Willmann, M.; McCormack, M.; Lee, H.; Shan, L.; He, P.; Bush, J.; Cheng, S.; Sheen, J. Differential innate immune signalling via Ca2+ sensor protein kinases. *Nature* **2010**,

Brugnoli, E.; Björkman, O. Growth of cotton under continuous salinity stress: influence on allocation pattern, stomatal and non-stomatal components of photosynthesis and dissipation of excess light energy. *Planta* **1992**, 187, 335-347.

Brumós, J.; Colmenero-Flores, J.; Conesa, A.; Izquierdo, P.; Sánchez, G.; Iglesias, D.; López-Climent, M.; Gómez-Cadenas, A.; Talón, M. Membrane transporters and carbon metabolism implicated in chloride homeostasis differentiate salt stress responses in tolerant and sensitive Citrus rootstocks. *Functional & integrative genomics* **2009**, 9, 293-309.

Brumós, J.; Talón, M.; Bouhlal, R.; Colmenero Flores, J. Cl homeostasis in includer and excluder citrus rootstocks: transport mechanisms and identification of candidate genes. *Plant, Cell & Environment* **2010**,

Byrne, M.; Nelson, C.; Randall, D. Ploidy effects on anatomy and gas exchange of tall fescue leaves. *Plant Physiology* **1981**, 68, 891.

Byrt, C. S.; Munns, R. Living with salinity. *New Phytologist* **2008**, 179, 903-905.

Cabasson, C. M.; Luro, F.; Ollitrault, P.; Grosser, J. W. Non-random inheritance of mitochondrial genomes in Citrus hybrids produced by protoplast fusion. *Plant Cell Reports* **2001**, 20, 604-609.

Cai, Q.; Guy, C.; Moore, G. Extension of the linkage map in Citrus using random amplified polymorphic DNA (RAPD) markers and RFLP mapping of cold-acclimation-responsive loci. *TAG Theoretical and Applied Genetics* **1994**, 89, 606-614.

Camara-Zapata, J. M.; Garcia-Sanchez, F.; Martinez, V.; Nieves, M.; Cerda, A. Effect of NaCl on citrus cultivars. *Agronomie* **2004**, 24, 155-160.

Cameron, J. W.; Frost, H. B., Genetic, Breeding and nucellar embryonny. In The Citrus Industry. Reuther W BLDaWHJ (ed) University of California: Riverside, **1968**; pp 325-370.

Cameron, J. W.; Soost, R. K. Characters of new populations of *Citrus* polyploids, and the relation between tetraploidy in the pollen parent and hybrid tetraploid progeny. *Proc. 1st Int. Citrus Symp* **1969**, 1, 199-205.

Cerdá, A.; Nieves, M.; Guillen, M. Salt tolerance of lemon trees as affected by rootstock. *Irrigation Science* **1990**, 11, 245-249.

Chang, L. F.; Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **2001**, 410, 37-40.

Chapman, H. The mineral nutrition of citrus. *The citrus industry* **1968**, 2, 127-289.

Chaves, M.; Oliveira, M. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *Journal of Experimental Botany* **2004**, 55, 2365.

Chen, C. X.; Bowman, K. D.; Choi, Y. A.; Dang, P. M.; Rao, M. N.; Huang, S.; Soneji, J. R.; McCollum, T. G.; Gmitter, F. G. EST-SSR genetic maps for Citrus sinensis and Poncirus trifoliata. *Tree Genet. Genomes* **2008**, 4, 1-10.

Chen, W.; Li, P. Membrane stabilization by abscisic acid under cold aids proline in alleviating chilling injury in maize (Zea mays L.) cultured cells. *Plant, Cell & Environment* **2002**, 25, 955-962.

Chen, Z. J. Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. *Annu. Rev. Plant Biol.* **2007**, 58, 377-406.

Cheng, Y.; De Vicente, M.; Meng, H.; Guo, W.; Tao, N.; Deng, X. A set of primers for analyzing chloroplast DNA diversity in Citrus and related genera. *Tree Physiol.* **2005**, 25, 661.

Chinnusamy, V.; Jagendorf, A.; Zhu, J. K. Understanding and improving salt tolerance in plants. *Crop Science* **2005**, 45, 437-448.

Cole, P. J.; McCloud, P. I. SALINITY AND CLIMATIC EFFECTS ON THE YIELD OF CITRUS. *Australian Journal of Experimental Agriculture* **1985**, 25, 711-717.

Colmenero Flores, J.; Martínez, G.; Gamba, G.; Vázquez, N.; Iglesias, D.; Brumós, J.; Talón, M. Identification and functional characterization of cation–chloride cotransporters in plants. *The Plant Journal* **2007**, 50, 278-292.

Colombo, B.; Baccanti, M.; Theobald, J. The simultaneous determination of carbon, hydrogen, nitrogen and sulphur using the new elemental analyser EA 1108. *Labmat* **1988**, 8, 61-63.

Cooper, W. Toxicity and accumulation of salts in citrus trees on various rootstocks in Texas. In, **1961**, 95-104.

Cooper, W.; Gorton, B.; Olson, E. Ionic accumulation in citrus as influenced by rootstock and scion and concentration of salts and boron in the substrate. *Plant Physiology* **1952**, 27, 191.

Corazza-Nunes, M.; Machado, M.; Nunes, W.; Cristofani, M.; Targon, M. Assessment of genetic variability in grapefruits (Citrus paradisi Macf.) and pummelos (C. maxima (Burm.) Merr.) using RAPD and SSR markers. *Euphytica* **2002**, 126, 169-176.

Cristofani, M.; Machado, M.; Grattapaglia, D. Genetic linkage maps of Citrus sunki Hort. ex. Tan. and Poncirus trifoliata (L.) Raf. and mapping of citrus tristeza virus resistance gene. *Euphytica* **1999**, 109, 25-32.

Cuevas, J.; López-Cobollo, R.; Alcázar, R.; Zarza, X.; Koncz, C.; Altabella, T.; Salinas, J.; Tiburcio, A.; Ferrando, A. Putrescine as a signal to modulate the indispensable ABA increase under cold stress. *Plant Signaling & Behavior* **2009**, 4, 219.

Dalal, M.; Tayal, D.; Chinnusamy, V.; Bansal, K. C. Abiotic stress and ABA-inducible Group 4 LEA from Brassica napus plays a key role in salt and drought tolerance. *Journal of Biotechnology* **2009**, 139, 137-145.

Davies, P. The plant hormones: their nature, occurrence, and functions. *Plant hormones* **2010**, 1-15.

Davies, W. J.; Kudoyarova, G.; Hartung, W. Long-distance ABA signaling and its relation to other signaling pathways in the detection of soil drying and the mediation of the plant's response to drought. *J. Plant Growth Regul.* **2005**, 24, 285-295.

De Simone, M.; Russo, M.; Puleo, G.; Marsan, P.; Lorenzoni, C.; Marocco, A.; REFORGIATO, R. Construction of genetic maps for Citrus aurantium and C. latipes based on AFLP, RAPD and RFLP markers. *Fruits* **1998**, 53, 383-390.

Del Río, J.; Gómez, P.; Baidez, A.; Arcas, M.; Botia, J.; Ortuño, A. Changes in the levels of polymethoxyflavones and flavanones as part of the defense mechanism of Citrus sinensis (cv. Valencia Late) fruits against Phytophthora citrophthora. *J. Agric. Food Chem* **2004**, 52, 1913-1917.

Delauney, A.; Hu, C.; Kishor, P.; Verma, D. Cloning of ornithine delta-aminotransferase cDNA from Vigna aconitifolia by trans-complementation in Escherichia coli and regulation of proline biosynthesis. *J. Biol. Chem.* **1993**, 268, 18673.

Desikan, R.; Clarke, A.; Hancock, J. T.; Neill, S. J. H2O2 activates a MAP kinase-like enzyme in Arabidopsis thaliana suspension cultures. *Journal of Experimental Botany* **1999**, 50, 1863-1866.

Dhuique-Mayer, C.; Caris-Veyrat, C.; Ollitrault, P.; Curk, F.; Amiot, M. J. Varietal and interspecific influence on micronutrient contents in citrus from the Mediterranean area. *Journal of Agricultural and Food Chemistry* **2005**, 53, 2140-2145.

Di Martino, C.; Pizzuto, R.; Pallotta, M.; De Santis, A.; Passarella, S. Mitochondrial transport in proline catabolism in plants: the existence of two separate translocators in mitochondria isolated from durum wheat seedlings. *Planta* **2006**, 223, 1123-1133.

Diedhiou, C.; Golldack, D. Salt-dependent regulation of chloride channel transcripts in rice. *Plant Science* **2006**, 170, 793-800.

Doyle, J. J.; Doyle, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* **1987**, 19, 11-15.

Durham, R.; Liou, P.; Gmitter, F.; Moore, G. Linkage of restriction fragment length polymorphisms and isozymes in Citrus. *TAG Theoretical and Applied Genetics* **1992**, 84, 39-48.

Economides, C.; Gregoriou, C. Growth, yield, and fruit quality of nucellar frost'Marsh'grapefruit on fifteen rootstocks in Cyprus. *Journal of the American Society for Horticultural Science* **1993**, 118, 326-329.

Epitalawage, N.; Eggenberg, P.; Strasser, R. Use of fast chlorophyll a fluorescence technique in detecting drought and salinity tolerant chickpea (Cicer arietinum L) varieties. *ARCHIVES DES SCIENCES-GENEVE*- **2003**, 56, 79-93.

Epstein, E.; Norlyn, J.; Rush, D.; Kingsbury, R.; Kelley, D.; Cunningham, G.; Wrona, A. Saline culture of crops: a genetic approach. *Science* **1980**, 210, 399-404.

Erickson, L.; Reuther, W.; Batchelor, L.; Webber, H. (1968) The citrus industry: Anatomy, physiology, genetics, and reproduction. In. University of California Press

Evans, L.; Dunstone, R. Some physiological aspects of evolution in wheat. *Aust. J. Biol. Sci* **1970**, 23, 725-741.

Everard, J.; Gucci, R.; Kann, S.; Flore, J.; Loescher, W. Gas exchange and carbon partitioning in the leaves of celery (Apium graveolens L.) at various levels of root zone salinity. *Plant Physiology* **1994**, 106, 281.

FAO. *Food and Agriculture Organization*. Disponible sur: http://faostat.fao.org/.

Fallahi, E.; Rodney, D. Tree size, yield, fruit quality, and leaf mineral nutrient concentration of'Fairchild'mandarin on six rootstocks. *Journal of the American Society for Horticultural Science (USA)* **1992**,

Fanciullino, A. L.; Dhuique-Mayer, C.; Luro, F.; Casanova, J.; Morillon, R.; Ollitrault, P. Carotenoid diversity in cultivated citrus is highly influenced by genetic factors. *Journal of Agricultural and Food Chemistry* **2006**, 54, 4397-4406.

Fang, D.; Roose, M. Identification of closely related citrus cultivars with inter-simple sequence repeat markers. *TAG Theoretical and Applied Genetics* **1997**, 95, 408-417.

Fang, D.; Krueger, R.; Roose, M. Phylogenetic relationships among selected Citrus germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. *American Society for Horticultural Science (USA)* **1998**,

Farquhar, G.; Caemmerer, S.; Berry, J. A biochemical model of photosynthetic CO_2 assimilation in leaves of C 3 species. *Planta* **1980**, 149, 78-90.

Federici, C.; Fang, D.; Scora, R.; Roose, M. Phylogenetic relationships within the genus Citrus (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. *TAG Theoretical and Applied Genetics* **1998**, 96, 812-822.

Fernández-Ballester, G.; García-Sánchez, F.; Cerdá, A.; Martínez, V. Tolerance of citrus rootstock seedlings to saline stress based on their ability to regulate ion uptake and transport. *Tree Physiol.* **2003**, 23, 265.

Flagel, L.; Udall, J.; Nettleton, D.; Wendel, J. Duplicate gene expression in allopolyploid Gossypium reveals two temporally distinct phases of expression evolution. *BMC Biology* **2008**, 6, 16.

Flagel, L. E.; Wendel, J. F. Gene duplication and evolutionary novelty in plants. *New Phytologist* **2009**, 183, 557-564.

Flexas, J.; Ribas-Carbó, M.; Hanson, D.; Bota, J.; Otto, B.; Cifre, J.; McDowell, N.; Medrano, H.; Kaldenhoff, R. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO2in vivo. *The Plant Journal* **2006**, 48, 427-439.

Flowers, T. Improving crop salt tolerance. *Journal of Experimental Botany* **2004**, 55, 307.

Flowers, T.; Colmer, T. Salinity tolerance in halophytes*. *New Phytologist* **2008**, 179, 945-963.

Flowers, T. J. Improving crop salt tolerance. *Journal of Experimental Botany* **2004**, 55, 307-319.

Foolad, M. Recent advances in genetics of salt tolerance in tomato. *Plant Cell, Tissue and Organ Culture* **2004**, 76, 101-119.

Fortin, M.; Morrison, N.; Verma, D. Nodulin-26, a peribacteroid membrane nodulin is expressed independently of the development of the peribacteroid compartment. *Nucleic acids research* **1987**, 15, 813.

Fricke, W.; Akhiyarova, G.; Veselov, D.; Kudoyarova, G. Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. *Journal of Experimental Botany* **2004**, 55, 1115.

Fricke, W.; Akhiyarova, G.; Wei, W.; Alexandersson, E.; Miller, A.; Kjellbom, P.; Richardson, A.; Wojciechowski, T.; Schreiber, L.; Veselov, D. The short-term growth response to salt of the developing barley leaf. *Journal of Experimental Botany* **2006**, 57, 1079.

Froelicher, Y.; Bassene, J. B.; Jedidi-Neji, E.; Dambier, D.; Morillon, R.; Bernardini, G.; Costantino, G.; Ollitrault, P. Induced parthenogenesis in mandarin for haploid production: induction procedures and genetic analysis of plantlets. *Plant Cell Reports* **2007**, 26, 937-944.

Froelicher, Y.; Mouhaya, W.; Bassene, J.; Costantino, G.; Kamiri, M.; Luro, F.; Morillon, R.; Ollitrault, P. New universal mitochondrial PCR markers reveal new information on maternal citrus phylogeny. *Tree Genet. Genomes* **2010**, 1-13.

Fukuda, A.; Nakamura, A.; Tagiri, A.; Tanaka, H.; Miyao, A.; Hirochika, H.; Tanaka, Y. Function, intracellular localization and the importance in salt tolerance of a vacuolar Na+/H+ antiporter from rice. *Plant and Cell Physiology* **2004**, 45, 146.

Gabbay-Azaria, R.; Tel-Or, E.; Schönfeld, M. Glycinebetaine as an osmoregulant and compatible solute in the marine cyanobacterium Spirulina subsalsa. *Arch. Biochem. Biophys.* **1988**, 264, 333-339.

García-Agustín, P.; Primo-Millo, E. Selection of a NaCl-tolerant Citrus plant. *Plant Cell Reports* **1995**, 14, 314-318.

Garcia-Legaz, M.; Ortiz, J.; Garcí-Lidón, A.; Cerda, A. Effect of salinity on growth, ion content and CO2 assimilation rate in lemon varieties on different rootstocks. *Physiol. Plant.* **1993**, 89, 427-432.

García-Sánchez, F.; Syvertsen, J. Salinity tolerance of Cleopatra mandarin and Carrizo citrange citrus rootstock seedlings is affected by CO2 enrichment during growth. *J. amer. Soc. hort. Sci* **2006**, 131, 24-31.

Garcia-Sanchez, F.; Syvertsen, J. P. Substrate Type and Salinity Affect Growth Allocation, Tissue Ion Concentrations, and Physiological Responses of Carrizo Citrange Seedlings. *Hortscience* **2009**, 44, 1432-1437.

Garcia, R.; Asins, M.; Forner, J.; Carbonell, E. Genetic analysis of apomixis in Citrus and Poncirus by molecular markers. *TAG Theoretical and Applied Genetics* **1999**, 99, 511-518.

Garnsey, S. M.; Barret, H. C.; Hutchison, O. J. Identification of citrus tristeza virus resistance in citrus relatives and its potencial applications. **1987**,

Garrington, T. P.; Johnson, G. L. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* **1999**, 11, 211-218.

Ghanem, M.; Albacete, A.; Martinez-Andujar, C.; Acosta, M.; Romero-Aranda, R.; Dodd, I.; Lutts, S.; Perez-Alfocea, F. Hormonal changes during salinity-induced leaf senescence in tomato (Solanum lycopersicum L.). *Journal of Experimental Botany* **2008**, 59, 3039.

Gimeno, V.; Syvertsen, J. P.; Rubio, F.; Martinez, V.; Garcia-Sanchez, F. Growth and mineral nutrition are affected by substrate type and salt stress in seedlings of two contrasting citrus rootstocks. *J. Plant Nutr.* **2010**, 33, 1435-1447.

Giraudat, J.; Parcy, F.; Bertauche, N.; Gosti, F.; Leung, J.; Morris, P.; Bouvier-Durand, M.; Vartanian, N. Current advances in abscisic acid action and signalling. *Plant Molecular Biology* **1994**, 26, 1557-1577.

Gómez Cadenas, A.; Tadeo, F.; Primo Millo, E.; Talon, M. Involvement of abscisic acid and ethylene in the responses of citrus seedlings to salt shock. *Physiol. Plant.* **1998**, 103, 475-484.

Gomez, L.; Rubio, E.; Augé, M. A new procedure for extraction and measurement of soluble sugars in ligneous plants. *Journal of the Science of Food and Agriculture* **2002**, 82, 360-369.

Gomez-Cadenas, A.; Arbona, V.; Jacas, J.; Primo-Millo, E.; Talon, M. Abscisic acid reduces leaf abscission and increases salt tolerance in citrus plants. *J. Plant Growth Regul.* **2002**, 21, 234-240.

Gómez-Cadenas, A.; Iglesias, D.; Arbona, V.; Colmenero-Flores, J.; Primo-Millo, E.; Talón, M. in Recent research development in plant molecular biology **2003**, 1, 281-298.

Grattapaglia, D.; Sederoff, R. Genetic linkage maps of Eucalyptus grandis and Eucalyptus urophylla using a pseudo-testcross: mapping strategy and RAPD markers. *Genetics* **1994**, 137, 1121.

Green, R.; Vardi, A.; Galun, E. The plastome of Citrus. Physical map, variation among Citrus cultivars and species and comparison with related genera. *TAG Theoretical and Applied Genetics* **1986**, 72, 170-177.

Grieve, A.; Walker, R. Uptake and distribution of chloride, sodium and potassium ions in salt-treated citrus plants. *Aust. J. Agric. Res.* **1983**, 34, 133-143.

Grosser, J. W.; Gmitter, F. G., Jr. Protoplast fusion and citrus improvement. *Plant Breeding Reviews* **1990**, 8, 339-374.

Grosser, J. W.; Gmitter, F. G., Jr. Somatic hybridization of Citrus with wild relatives for germplasm enhancement and cultivar development. *Hortscience* **1990**, 25, 147-151.

Grosser, J.W.; Gmitter, F.; Sesto, F. Six new somatic citrus hybrids and their potential for cultivar improvement. *Journal of the American Society for Horticultural Science* **1992**, 117, 169-173.

Grosser, J. W.; Gmitter, F. G.; Fleming, G. H.; Chandler, J. L., Applications of biotechnology to citrus cultivar ir improvement at the Citrus Research and Education Center. In Proceedings of the First International Symposium on Citrus Biotechnology. Goren R, Goldschmidt EE (eds) International Society Horticultural Science: Leuven 1, **2000**; pp 213-220.

Grosser, J. W.; Chandler, J. L.; Duncan, L. W. Production of mandarin+pummelo somatic hybrid citrus rootstocks with potential for improved tolerance/resistance to sting nematode. *Sci. Hortic.* **2007**, 113, 33-36.

Grosser, J. W.; Gmitter, F. G., Jr. (2009) In vitro breeding provides new and unique opportunities for conventional breeding. In: Proceedings of the VI International Symposium on In Vitro Culture and Horticultural Breeding, Brisbane, Australia, 25-29 August 2008. International Society for Horticultural Science (ISHS), pp 65-72.

Grosser, J. W.; Medina-Urrutia, V.; Ananthakrishnan, G.; Serrano, P. Building a replacement sour orange rootstock: Somatic hybridization of selected mandarin plus pummelo combinations. *Journal of the American Society for Horticultural Science* **2004**, 129, 530-534.

Guei, R. G.; Wassom, C. E. Genetics of osmotic adjustment in breeding maize for drought tolerance. *Heredity* **1993**, 71, 436-441.

Gulsen, O.; Uzun, A.; Canan, I.; Seday, U.; Canihos, E. A new citrus linkage map based on SRAP, SSR, ISSR, POGP, RGA and RAPD markers. *Euphytica* **2010**, 173, 265-277.

Guo, W. W.; Prasad, D.; Serrano, P.; Gmitter, F. G.; Grosser, J. W. Citrus somatic hybridization with potential for direct tetraploid scion cultivar development. *Journal of Horticultural Science & Biotechnology* **2004**, 79, 400-405.

Guo, Y.; Qiu, Q. S.; Quintero, F. J.; Pardo, J. M.; Ohta, M.; Zhang, C. Q.; Schumaker, K. S.; Zhu, J. K. Transgenic evaluation of activated mutant alleles of SOS2

reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in Arabidopsis thaliana. *Plant cell* **2004**, 16, 435-449.

Handa, S.; Handa, A.; Hasegawa, P.; Bressan, R. Proline accumulation and the adaptation of cultured plant cells to water stress. *Plant Physiology* **1986**, 80, 938.

Hare, P. D.; Cress, W. A.; Van Staden, J. Dissecting the roles of osmolyte accumulation during stress. *Plant Cell Environ.* **1998**, 21, 535-553.

Harper, J. F.; Harmon, A. Plants, symbiosis and parasites: A calcium signalling connection. *Nat. Rev. Mol. Cell Biol.* **2005**, 6, 555-566.

Hasegawa, P. M.; Bressan, R. A.; Zhu, J. K.; Bohnert, H. J. Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **2000**, 51, 463-499.

Hattori, K.; Konomi, M.; Osumi, M.; Yamagami, M.; Schroeder, J.; Uozumi, N. Enhanced salt tolerance mediated by AtHKT1 transporter induced Na+ unloading from xylem vessels to xylem parenchyma cells. *The Plant Journal* **2005**, 44, 928-938.

Hauser, F.; Horie, T. (2009) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K+/Na+ ratio in leaves during salinity stress. In. Blackwell Publishing Ltd, pp 552-565.

Hayashi, H.; Alia; Sakamoto, A.; Nonaka, H.; Chen, T. H. H.; Murata, N. Enhanced germination under high-salt conditions of seeds of transgenic Arabidopsis with a bacterial gene (codA) for choline oxidase. *J. Plant Res.* **1998**, 111, 357-362.

Hechenberger, M.; Schwappach, B.; Fischer, W.; Frommer, W.; Jentsch, T.; Steinmeyer, K. A family of putative chloride channels from Arabidopsis and functional complementation of a yeast strain with a CLC gene disruption. *J. Biol. Chem.* **1996**, 271, 33632.

Heinen, R.; Ye, Q.; Chaumont, F. Role of aquaporins in leaf physiology. *Journal of Experimental Botany* **2009**,

Hernandez, J.; Jimenez, A.; Mullineaux, P.; Sevilia, F. Tolerance of pea (Pisum sativum L.) to long term salt stress is associated with induction of antioxidant defences. *Plant, Cell & Environment* **2000**, 23, 853-862.

Herrero, R.; Asíns, M.; Pina, J.; Carbonell, E.; Navarro, L. Genetic diversity in the orange subfamily Aurantioideae. II. Genetic relationships among genera and species. *TAG Theoretical and Applied Genetics* **1996**, 93, 1327-1334.

Holm, L. M.; Jahn, T. P.; Moller, A. L. B.; Schjoerring, J. K.; Ferri, D.; Klaerke, D. A.; Zeuthen, T. NH3 and NH4+ permeability in aquaporin-expressing Xenopus oocytes. *Pflugers Arch.* **2005**, 450, 415-428.

Horie, T.; Hauser, F.; Schroeder, J. HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. *Trends Plant Sci.* **2009**, 14, 660-668.

Howie, H.; Lloyd, J. RESPONSE OF ORCHARD WASHINGTON NAVEL ORANGE, CITRUS-SINENSIS (L) OSBECK TO SALINE IRRIGATION WATER .2. FLOWERING, FRUIT-SET AND FRUIT-GROWTH. *Aust. J. Agric. Res.* **1989**, 40, 371-380.

HU, C.; Delauney, A.; Verma, D. (1992) A bifunctional enzyme (delta'-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. In, pp 9354-9358

Hu, Y. C.; Burucs, Z.; von Tucher, S.; Schmidhalter, U. Short-term effects of drought and salinity on mineral nutrient distribution along growing leaves of maize seedlings. *Environ. Exp. Bot.* **2007**, 60, 268-275.

Huang, S.; Spielmeyer, W.; Lagudah, E.; Munns, R. Comparative mapping of HKT genes in wheat, barley, and rice, key determinants of Na+ transport, and salt tolerance. *Journal of Experimental Botany* **2008**, 59, 927.

Hulbert, S.; Ilott, T.; Legg, E.; Lincoln, S.; Lander, E.; Michelmore, R. Genetic analysis of the fungus, Bremia lactucae, using restriction fragment length polymorphisms. *Genetics* **1988**, 120, 947.

Iglesias, D. J.; Lliso, I.; Tadeo, F. R.; Talon, M. Regulation of photosynthesis through source: sink imbalance in citrus is mediated by carbohydrate content in leaves. *Physiol. Plant.* **2002**, 116, 563-572.

Iglesias, D.; Cercós, M.; Colmenero-Flores, J.; Naranjo, M.; Ríos, G.; Carrera, E.; Ruiz-Rivero, O.; Lliso, I.; Morillon, R.; Tadeo, F. Physiology of citrus fruiting. *Brazilian Journal of Plant Physiology* **2007**, 19, 333-362.

Ingram, J.; Bartels, D. The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Biol.* **1996**, 47, 377-403.

Ishikawa, F.; Suga, S.; Uemura, T.; Sato, M.; Maeshima, M. Novel type aquaporin SIPs are mainly localized to the ER membrane and show cell-specific expression in Arabidopsis thaliana. *FEBS Lett.* **2005**, 579, 5814-5820.

Izzo, R.; Navariizzo, F.; Quartacci, M. F. Growth and mineral absorption in maize seedlings as affected by increasing nacl concentrations

J. Plant Nutr. **1991**, 14, 687-699.

Jacoby, R. P.; Millar, A. H.; Taylor, N. L. Wheat Mitochondrial Proteomes Provide New Links between Antioxidant Defense and Plant Salinity Tolerance. *J. Proteome Res.* **2010**, 9, 6595-6604.

Jacquemond, C.; Rocca Serra, D. (1992) Citrus Rootstocks Selection in Corsica for 25 Years. In: Proc. Int. Soc. Citriculture, pp 246-251

Jacquemond, C.; Tison, G.; Agostini, D.; Navari, C.; Roesch, M.; Vittori, D. C32 and C35 citrange: possible new rootstocks for clementine in corsica. *proceedings of the international society of citriculture* **2004**, 1, 321-322.

Jagendorf, A.; Takabe, T. Inducers of glycinebetaine synthesis in barley. *Plant Physiology* **2001**, 127, 1827.

Jang, J.; Kim, D.; Kim, Y.; Kim, J.; Kang, H. An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in Arabidopsis thaliana. *Plant Molecular Biology* **2004**, 54, 713-725.

Jarrell, D.; Roose, M.; Traugh, S.; Kupper, R. A genetic map of citrus based on the segregation of isozymes and RFLPs in an intergeneric cross. *TAG Theoretical and Applied Genetics* **1992**, 84, 49-56.

Jenks, M. A.; Hasegawa, P. M.; Jain, S. M.; Ben-Hayyim, G.; Moore, G. A., Recent advances in breeding citrus for drought and saline stress tolerance. In Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops. Springer Netherlands, **2007**; pp 627-642.

Jensen, P. J.; Rytter, J.; Detwiler, E. A.; Travis, J. W.; McNellis, T. W. Rootstock effects on gene expression patterns in apple tree scions. *Plant Molecular Biology* **2003**, 53, 493-511.

Jentsch, T. J.; Stein, V.; Weinreich, F.; Zdebik, A. A. Molecular structure and physiological function of chloride channels. *Physiol. Rev.* **2002**, 82, 503-568.

Johanson, U.; Karlsson, M.; Johansson, I.; Gustavsson, S.; Sjovall, S.; Fraysse, L.; Weig, A.; Kjellbom, P. The complete set of genes encoding major intrinsic proteins in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. *Plant Physiology* **2001**, 126, 1358.

Johansson, I.; Karlsson, M.; Johanson, U.; Larsson, C.; Kjellbom, P. The role of aquaporins in cellular and whole plant water balance. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **2000**, 1465, 324-342.

Johnson, G. Cyclic electron transport in C3 plants: fact or artefact? *Journal of Experimental Botany* **2005**, 56, 407.

Jolivet, Y.; Larher, F.; Hamelin, J. Osmoregulation in halophytic higher-plants - the protective effect of glycine betaine against the heat destabilization of membranes. *Plant Science Letters* **1982**, 25, 193-201.

Jolivet, Y.; Hamelin, J.; Larher, F. Osmoregulation in halophytic higher-plants - the protective effects of glycine betaine and other related solutes against the oxalate destabilization of membranes in beet root-cells. *Zeitschrift Fur Pflanzenphysiologie* **1983**, 109, 171-180.

Jolivet, Y.; Pireaux, J.; Dizengremel, P. Changes in properties of barley leaf mitochondria isolated from NaCl-treated plants. *Plant Physiology* **1990**, 94, 641.

Joseph, M.; Randall, D.; Nelson, C. Photosynthesis in Polyploid Tall Fescue: II. Photosynthesis and Ribulose-1, 5-Bisphosphate Carboxylase of Polyploid Tall Fescue. *Plant Physiology* **1981**, 68, 894.

Kaldenhoff, R.; Grote, K.; Zhu, J.; Zimmermann, U. Significance of plasmalemma aquaporins for water transport in Arabidopsis thaliana. *The Plant Journal* **1998**, 14, 121-128.

Kaldenhoff, R.; Ribas-Carbo, M.; SANS, J.; Lovisolo, C.; Heckwolf, M.; Uehlein, N. Aquaporins and plant water balance. *Plant, Cell & Environment* **2008**, 31, 658-666.

Kamboj, J.; Blake, P.; Quinlan, J.; Webster, A.; Baker, D. (1996) Recent advances in studies on the dwarfing mechanism of apple rootstocks. In. ISHS, pp 75-82

Kato, T. Nitrogen metabolism and utilization in citrus. *Horticultural Reviews* **1986**, 8, 1-216.

Kawasaki, S.; Borchert, C.; Deyholos, M.; Wang, H.; Brazille, S.; Kawai, K.; Galbraith, D.; Bohnert, H. J. Gene expression profiles during the initial phase of salt stress in rice. *Plant cell* **2001**, 13, 889-905.

Khan, I.; Roose, M. Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. *Journal of American Society of Horticultural Sciences* **1988**, 113, 105-110.

Khan, I.; Kender, W. 1 Citrus Breeding: Introduction and Objectives. *Citrus genetics, breeding and biotechnology* **2007**, 1.

Kijas, J.; Thomas, M.; Fowler, J.; Roose, M. Integration of trinucleotide microsatellites into a linkage map of Citrus. *TAG Theoretical and Applied Genetics* **1997**, 94, 701-706.

Kinclova-Zimmermannova, O.; Flegelova, H.; Sychrova, H. Rice Na+/H+-antiporter Nhx1 partially complements the alkali-metal-cation sensitivity of yeast strains lacking three sodium transporters. *Folia microbiologica* **2004**, 49, 519-525.

Kishor, P. B. K.; Hong, Z. L.; Miao, G. H.; Hu, C. A. A.; Verma, D. P. S. Overexpression of delta-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* **1995**, 108, 1387-1394.

Kochba, J.; Benhayyim, G.; Spiegelroy, P.; Saad, S.; Neumann, H. Selection of stable salt-tolerant callus cell-lines and embryos in citrus-sinensis and citrus-aurantium

Zeitschrift Fur Pflanzenphysiologie **1982**, 106, 111-118.

Kovtun, Y.; Chiu, W. L.; Tena, G.; Sheen, J. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, 97, 2940-2945.

Krall, J. P.; Edwards, G. E.; Andreo, C. S. Protection of pyruvate, pi dikinase from maize against cold lability by compatible solutes. *Plant Physiology* **1989**, 89, 280-285.

Krug, C. Chromosome numbers in the subfamily Aurantioideae with special reference to the genus Citrus. *Botanical Gazette* **1943**, 104, 602-611.

Kruse, E.; Uehlein, N.; Kaldenhoff, R. The aquaporins. *Genome Biol.* **2006**, 7, 6.

Kudla, J.; Batistic, O.; Hashimoto, K. Calcium signals: The lead currency of plant information processing. *The Plant Cell Online* **2010**, 22, 541.

Laborem, G.; Wagner, M.; Reyes, F. Concentración de prolina como indicador de déficit hídrico en tres patrones cítricos. *Trabajo de ascenso. Universidad Rómulo Gallegos (Unerg), San Juan de Los Morros (Venezuela)* **1995**, 30,

Lan, W.; Wang, W.; Wang, S.; Li, L.; Buchanan, B.; Lin, H.; Gao, J.; Luan, S. A rice high-affinity potassium transporter (HKT) conceals a calcium-permeable cation channel. *Proceedings of the National Academy of Sciences* **2010**, 107, 7089.

Lee, I.; Kim, D.; Lim, Y.; Lee, K.; Song, H.; Lee, Y. Generation and performance evaluation of salt tolerant mutants in rice. *SABRAO Journal of Breeding and Genetics* **2003**, 35, 93-102.

Lee, L. Citrus polyploidy-origins and potential for cultivar improvement. *Aust. J. Agric. Res.* **1988**, 39, 735-747.

Leech, R.; Leese, B.; Jellings, A. Variation in cellular ribulose-1, 5-bisphosphate-carboxylase content in leaves of Triticum genotypes at three levels of ploidy. *Planta* **1985**, 166, 259-263.

Leenhardt, F.; Lyan, B.; Rock, E.; Boussard, A.; Potus, J.; Chanliaud, E.; Remesy, C. Genetic variability of carotenoid concentration, and lipoxygenase and peroxidase activities among cultivated wheat species and bread wheat varieties. *European Journal of Agronomy* **2006**, 25, 170-176.

Leidi, E. O.; Barragán, V.; Rubio, L.; El-Hamdaoui, A.; Ruiz, M. T.; Cubero, B.; Fernández, J. A.; Bressan, R. A.; Hasegawa, P. M.; Quintero, F. J.; Pardo, J. M. The AtNHX1 exchanger mediates potassium compartmentation in vacuoles of transgenic tomato. *The Plant Journal* **2010**, 61, 495-506.

Leitch, A.; Leitch, I. Genomic plasticity and the diversity of polyploid plants. *Science* **2008**, 320, 481.

Lessani, H.; Marschner, H. Relation between salt tolerance and long-distance transport of sodium and chloride in various crop species. *Aust. J. Plant Physiol.* **1978**, 5, 27-37.

Leung, J.; Giraudat, J. Abscisic acid signal transduction. *Annu Rev Plant Physiol Plant Mol Biol*. **1998**, 199-222.

Levy, Y.; Shalhevet, J.; Bielorai, H. Effect of irrigation regime and water salinity on grapefruit quality. *Journal of the American Society of Horticultural Science* **1979**, 104, 356-359.

Levy, Y.; Shalhevet, J. Ranking the salt tolerance of citrus rootstocks by juice analysis. *Sci. Hortic.* **1990**, 45, 89-98.

Lindsay, M.; Lagudah, E.; Hare, R.; Munns, R. A locus for sodium exclusion (Nax1), a trait for salt tolerance, mapped in durum wheat. *Funct Plant Biol* **2004**, 31, 1105-1114.

Ling, P.; Yu, C.; Deng, Z.; Chen, C.; Huang, S.; Wendell, M.; Gmitter Jr, F. Citrus genome mapping with AFLP markers. In: Plant & Animal Genomes XIII San Diego, CA, USA.**1999**.

Liou, P. A molecular study of the Citrus genome through analysis of restriction fragment length polymorphism and isozyme mapping. PhD, Gainseville, USA, **1990**, p

Liu, D. C.; He, L. G.; Wang, H. L.; Xu, M.; Sun, Z. H. Molecular cloning, characterization and expression analysis of PtrHOS1, a novel gene of cold responses from trifoliate orange [Poncirus trifoliata (L.) Raf.]. *Acta Physiol. Plant.* **2010**, 32, 271-279.

Liu, H. U. A.; Wang, Q.; Yu, M.; Zhang, Y.; Wu, Y.; Zhang, H. Transgenic salt-tolerant sugar beet (Beta vulgaris L.) constitutively expressing an Arabidopsis thaliana vacuolar Na+/H+ antiporter gene, AtNHX3, accumulates more soluble sugar but less salt in storage roots. In. Blackwell Publishing Ltd, **2008**, 1325-1334

Liu, J.; Ishitani, M.; Halfter, U.; Kim, C.; Zhu, J. The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, 97, 3730.

Liu, J. P.; Zhu, J. K. Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of arabidopsis. *Plant Physiology* **1997**, 114, 591-596.

Liu, J.; Zhu, J. A calcium sensor homolog required for plant salt tolerance. *Science* **1998**, 280, 1943.

Livne, A.; Levin, N. Tissue respiration and mitochondrial oxidative phosphorylation of NaCl-treated pea seedlings. *Plant Physiology* **1967**, 42, 407.

Lloyd, J.; Kriedemann, P.; Aspinall, D. Comparative sensitivity of 'Prior Lisbon'lemon and 'Valencia'orange trees to foliar sodium and chloride concentrations. *Plant, Cell & Environment* **1989**, 12, 529-540.

Lloyd, J.; Kriedemann, P.; Aspinall, D. Contrasts between Citrus species in response to salinisation: An analysis of photosynthesis and water relations for different rootstock scion combinations. *Physiol. Plant.* **1990**, 78, 236-246.

Logan, B. Reactive oxygen species and photosynthesis. *Antioxidants and Reactive Oxygen Species in Plants* **2005**, 250–267.

Longley, A. E. Triploid citrus. *Journal of Washington Academy of Science* **1926**, 16, 543-545.

Lopez-Climent, M. F.; Arbona, V.; Perez-Clemente, R. M.; Gomez-Cadenas, A. Relationship between salt tolerance and photosynthetic machinery performance in citrus. *Environ. Exp. Bot.* **2008**, 62, 176-184.

Louzada, E. S.; del Rio, H. S.; Setamou, M.; Watson, J. W.; Swietlik, D. M. Evaluation of citrus rootstocks for the high pH, calcareous soils of South Texas. *Euphytica* **2008**, 164, 13-18.

Luan, S. The CBL-CIPK network in plant calcium signaling. *Trends Plant Sci.* **2009**, 14, 37-42.

Lurin, C.; Geelen, D.; Barbier-Brygoo, H.; Guern, J.; Maurel, C. Cloning and functional expression of a plant voltage-dependent chloride channel. *The Plant Cell Online* **1996**, 8, 701.

Luro, F.; Laigret, F.; Lorieux, M.; Ollitrault, P. Citrus genome mapping with molecular markers: two maps obtained by segregation analysis of progeny of one intergeneric cross. In, **1996**, 862-866.

Luro, F.; Rist, D.; Ollitrault, P. Evaluation of genetic relationships in Citrus genus by means of sequence tagged microsatellites. *Acta Horticulturae* **2001**, 237-242.

Maas, E. V. Salinity and citriculture. *Tree Physiol.* **1993**, 12, 195-216.

Maathuis, F.; Amtmann, A. K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. *Annals of Botany* **1999**, 84, 123.

Maathuis, F.; Filatov, V.; Herzyk, P.; Krijger, G.; Axelsen, K.; Chen, S.; Green, B.; Li, Y.; Madagan, K.; Sánchez-Fernández, R. Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. *The Plant Journal* **2003**, 35, 675-692.

Mademba-Sy, F. Contribution à la recherche d'indicateurs physiologiques et biochimiques de la tolérance des agrumes à la salinité. *Thèse de doctorat, Université de Rennes 1* **2004**, 164.

Mademba-Sy, F.; Bouchereau, A.; Larher, F. R. Proline accumulation in cultivated citrus and its relationship with salt tolerance. *Journal of Horticultural Science & Biotechnology* **2003**, 78, 617-623.

Mahajan, S.; Pandey, G. K.; Tuteja, N. Calcium- and salt-stress signaling in plants: Shedding light on SOS pathway. *Arch. Biochem. Biophys.* **2008**, 471, 146-158.

Mäkelä, P.; Munns, R.; Colmer, T.; Peltonen-Sainio, P. Growth of tomato and an ABA-deficient mutant (sitiens) under saline conditions. *Physiol. Plant.* **2003**, 117, 58-63.

Manter, D.; Kerrigan, J. A/Ci curve analysis across a range of woody plant species: influence of regression analysis parameters and mesophyll conductance. *Journal of Experimental Botany* **2004**, 55, 2581.

Mäntylä, E.; Lång, V.; Palva, E. Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LTI78 and RAB18 proteins in Arabidopsis thaliana. *Plant Physiology* **1995**, 107, 141-148.

Marmagne, A.; Vinauger-Douard, M.; Monachello, D.; De Longevialle, A.; Charon, C.; Allot, M.; Rappaport, F.; Wollman, F.; Barbier-Brygoo, H.; Ephritikhine, G. Two members of the Arabidopsis CLC (chloride channel) family, AtCLCe and AtCLCf, are associated with thylakoid and Golgi membranes, respectively. *Journal of Experimental Botany* **2007**,

Masashi Yamamoto, S. Phylogenic Relationships of Citrus Revealed by RFLP Analysis of Mitochondrial and Chloroplast DNA. *Japanese Journal of Breeding* **1993**, 43, 355-365.

Mäser, P.; Eckelman, B.; Vaidyanathan, R.; Horie, T.; Fairbairn, D.; Kubo, M.; Yamagami, M.; Yamaguchi, K.; Nishimura, M.; Uozumi, N. Altered shoot/root Na+ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na+ transporter AtHKT1. *FEBS Lett.* **2002**, 531, 157-161.

Masojidek, J.; Hall, D. Salinity and drought stresses are amplified by high irradiance in sorghum. *Photosynthetica* **1992**, 27, 159-171.

Masterson, J. Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. *Science* **1994**, 264, 421.

Matsumoto, K.; Yamaguchi, H. Increased variation of NaCl-tolerance in adventitious embryoids of trifoliate orange using an in vitro technique. *Revista Brasileira de Genética* **1984**, 7.

Maurel, C.; Verdoucq, L.; Luu, D.; Santoni, V. Plant aquaporins: membrane channels with multiple integrated functions. *Plant Biology* **2008**, 59, 595.

Maxwell, K.; Johnson, G. Chlorophyll fluorescence--a practical guide. *Journal of Experimental Botany* **2000**, 51, 659.

McAinsh, M.; Schroeder, J. 4 Crosstalk in Ca2+ Signaling Pathways. *Signal Crosstalk in Plant Stress Responses* **2009**, 59.

McCormack, E.; Tsai, Y. C.; Braam, J. Handling calcium signaling: Arabidopsis CaMs and CMLs. *Trends Plant Sci.* **2005**, 10, 383-389.

Melgar, J.; Syvertsen, J.; Martínez, V.; García-Sánchez, F. Leaf gas exchange, water relations, nutrient content and growth in citrus and olive seedlings under salinity. *Biol. Plant.* **2008**, 52, 385-390.

Melgar, J. C.; Dunlop, J. M.; Syvertsen, J. P. Growth and physiological responses of the citrus rootstock Swingle citrumelo seedlings to partial rootzone drying and deficit irrigation. *J. Agric. Sci.* **2010**, 148, 593-602.

Mestre, P. F.; Asins, M. J.; Carbonell, E. A.; Navarro, L. New gene(s) involved in the resistance of Poncirus trifoliata (L.) Raf. To citrus tristeza virus. *Theoretical and Applied Genetics* **1997**, 95, 691-695.

Michalczuk, L. Indole-3-acetic acid level in wood, bark and cambial sap of apple rootstocks differing in growth vigour. *Acta Physiol. Plant.* **2002**, 24, 131-136.

Mishra, S.; Subrahmanyam, D.; Singhal, G. Interrelationship between salt and light stress on primary processes of photosynthesis. *Journal of plant physiology (Germany)* **1991**,

Mittler, R. Abiotic stress, the field environment and stress combination. *Trends Plant Sci.* **2006**, 11, 15-19.

Molin, W.; Meyers, S.; Baer, G.; Schrader, L. Ploidy Effects in Isogenic Populations of Alfalfa: II. Photosynthesis, Chloroplast Number, Ribulose-1, 5-Bisphosphate Carboxylase, Chlorophyll, and DNA in Protoplasts. *Plant Physiology* **1982**, 70, 1710.

Molinari, H.; Marur, C. Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (Citrus sinensis Osb. x Poncirus trifoliata L. Raf.) overproducing proline. *Plant Science* **2004**, 167, 1375-1381.

Moore, G. A.; Castle, W. S. Morphological and ispzymic analysis of open-pollinated citrus rootstocks populations. *J. Hered.* **1988**, 79, 59-63.

Morales, F.; Abadía, A.; Gómez-Aparisi, J.; Abadía, J. Effects of combined NaCl and CaCl2 salinity on photosynthetic parameters of barley grown in nutrient solution. *Physiol. Plant.* **1992**, 86, 419-426.

Motosugi, H.; Yamamoto, Y.; Naruo, T.; Yamaguchi, D. Growth and fruit quality of 'Kyoho' grapevines grafted on autotetraploid rootstocks. *Journal of the Japanese Society for Horticultural Science* **2007**, 76, 271-278.

Mouhaya, W. Caractérisation physiologique et moléculaire de la tolérance au stress salin chez les porte-greffe tetraploïdes d'agrumes. Thèse de Doctorat, Université de Corse-Pascal Paoli, Corte, **2008**, p 202.

Mouhaya, W.; Allario, T.; Brumos, J.; Andres, F.; Froelicher, Y.; Luro, F.; Talon, M.; Ollitrault, P.; Morillon, R. Sensitivity to high salinity in tetraploid citrus seedlings increases with water availability and correlates with expression of candidate genes. *Functional Plant Biology* **2010**, 37, 674-685.

Mouly, P. P.; Gaydou, E. M.; Faure, R.; Estienne, J. M. Blood orange juice authentication using cinnamic acid derivatives. Variety differentiations associated with flavanone glycoside content. *Journal of Agricultural and Food Chemistry* **1997**, 45, 373-377.

Mourão Filho, F.; Pio, R.; Mendes, B.; de Azevedo, F.; Schinor, E.; Entelmann, F.; Alves, A.; Cantuarias-Avilés, T. Evaluation of citrus somatic hybrids for tolerance to Phytophthora nicotianae and citrus tristeza virus. *Sci. Hortic.* **2008**, 115, 301-308.

Moya, J. L.; Primo-Millo, E.; Talon, M. Morphological factors determining salt tolerance in citrus seedlings: the shoot to root ratio modulates passive root uptake of chloride ions and their accumulation in leaves. *Plant Cell Environ.* **1999**, 22, 1425-1433.

Moya, J. L.; Tadeo, F. R.; Gomez-Cadenas, A.; Primo-Millo, E.; Talon, M. Transmissible salt tolerance traits identified through reciprocal grafts between sensitive Carrizo and tolerant Cleopatra citrus genotypes. *J. Plant Physiol.* **2002**, 159, 991-998.

Moya, J.; Gomez-Cadenas, A.; Primo-Millo, E.; Talon, M. Chloride absorption in salt-sensitive Carrizo citrange and salt-tolerant Cleopatra mandarin citrus rootstocks is linked to water use. *Journal of Experimental Botany* **2003**, 54, 825.

Munns, R. Comparative physiology of salt and water stress. *Plant Cell Environ.* **2002**, 25, 239-250.

Munns, R. Genes and salt tolerance: bringing them together. *New Phytologist* **2005**, 167, 645-663.

Munns, R.; Termaat, A. Whole-plant responses to salinity. *Aust. J. Plant Physiol* **1986**, 13, 143-160.

Munns, R.; Tester, M. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* **2008**, 59, 651-681.

Murata, N.; Mohanty, P. S.; Hayashi, H.; Papageorgiou, G. C. Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen-evolving complex. *FEBS Lett.* **1992**, 296, 187-189.

Murata, Y.; Pei, Z.; Mori, I.; Schroeder, J. Abscisic acid activation of plasma membrane Ca2+ channels in guard cells requires cytosolic NAD (P) H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. *The Plant Cell Online* **2001**, 13, 2513.

Naot, D.; Ben-Hayyim, G.; Eshdat, Y.; Holland, D. Drought, heat and salt stress induce the expression of a citrus homologue of an atypical late-embryogenesis Lea5 gene. *Plant Molecular Biology* **1995**, 27, 619-622.

Neumann, D.; zurNieden, U.; Schwieger, W.; Leopold, I.; Lichtenberger, O. Heavy metal tolerance of Minuartia verna. *J. Plant Physiol.* **1997**, 151, 101-108.

Neves-Piestun, B. G.; Bernstein, N. Salinity-induced changes in the nutritional status of expanding cells may impact leaf growth inhibition in maize. *Functional Plant Biology* **2005**, 32, 141-152.

Ni, Z.; Kim, E.-D.; Ha, M.; Lackey, E.; Liu, J.; Zhang, Y.; Sun, Q.; Chen, Z. J. Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. *Nature* **2009**, 457, 327-331.

Nicolosi, E. Origin and taxonomy. *Citrus Genetics, Breeding and Biotechnology. CAB International, Wallingford, UK* **2007**, 19–43.

Nicolosi, E.; Deng, Z. N.; Gentile, A.; La Malfa, S.; Continella, G.; Tribulato, E. Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theoretical and Applied Genetics* **2000**, 100, 1155-1166.

Nicolosi, E.; La Malfa, S.; El-Otmani, M.; Negbi, M.; Goldschmidt, E. The search for the authentic citron (Citrus medica L.): Historic and genetic analysis. *Hortscience* **2005**, 40, 1963-1968.

Nienhuis, J.; Slocum, T.; Ruggero, M.; Schaefer, B. Restriction Fragment Length Polymorphism Analysis of Loci Associated with Insect Resistance in Tomato1. *Crop Science* 27, 797.

Nieves, M.; Cerda, A.; Botella, M. Salt tolerance of two lemon scions measured by leaf chloride and sodium accumulation. *J. Plant Nutr.* **1991**, 14, 623-636.

Niu, X.; Bressan, R.; Hasegawa, P.; Pardo, J. Ion homeostasis in NaCl stress environments. *Plant Physiology* **1995**, 109, 735.

Noble, C.; Rogers, M. Arguments for the use of physiological criteria for improving the salt tolerance in crops. *Plant and soil* **1992**, 146, 99-107.

Nolte, K. D.; Hanson, A. D.; Gage, D. A. Proline accumulation and methylation to proline betaine in Citrus: Implications for genetic engineering of stress resistance. *Journal of the American Society for Horticultural Science* **1997**, 122, 8-13.

Nuccio, M. L.; Russell, B. L.; Nolte, K. D.; Rathinasabapathi, B.; Gage, D. A.; Hanson, A. D. The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. *Plant J.* **1998**, 16, 487-496.

Oh, D. H.; Lee, S. Y.; Bressan, R. A.; Yun, D. J.; Bohnert, H. J. Intracellular consequences of SOS1 deficiency during salt stress. *Journal of Experimental Botany* **2010**, 61, 1205-1213.

Ohta, M.; Hayashi, Y.; Nakashima, A.; Hamada, A.; Tanaka, A.; Nakamura, T.; Hayakawa, T. Introduction of a Na+/H+ antiporter gene from Atriplex gmelini confers salt tolerance to rice. *FEBS Lett.* **2002**, 532, 279-282.

Ollitrault, P.; Dambier, D.; Cabasson, C.; Teisson, C.; Luro, F. Protoplast fusion in Citrus. *Fruits (Paris)* **1994**, 49, 401-403, 481-482.

Ollitrault, P.; Dambier, D.; Froelicher, Y.; Bakry, F.; Aubert, B. Rootstock breeding strategies for the Mediterranean citrus industry; the somatic hybridization potential. *Fruits (Paris)* **1998**, 53, 335-344.

Ollitrault, P.; Dambier, D.; Froelicher, Y.; Carreel, F.; d'Hont, A.; Luro, F.; Bruyere, S.; Cabasson, C.; Lotfy, S.; Joumaa, A.; Vanel, F.; Maddi, F.; Treanton, K.; Grisoni, M. Somatic hybridisation potential for Citrus germplasm utilization. *Cahiers Agricultures* **2000**, 9, 223-236.

Ollitrault, P.; Froelicher, Y.; Dambier, D.; Seker, M. Rootstock breeding by somatic hybridisation for the Mediterranean citrus industry. *Acta Horticulturae* **2000**, 157-162.

Ollitrault, P.; Guo, W.; Grosser, J. W., Somatic hybridization. In Citrus genetics, breeding and biotechnology. **2007**; pp 235-260.

Ollitrault, P.; Dambier, D.; Luro, F.; Froelicher, Y. Ploidy manipulation for breeding seedless triploid citrus. *Plant Breeding Reviews* **2008**, 30, 323.

Ollitrault, P.; Jacquemond, C. Facultative apomixis, spontaneous polyploidization and inbreeding in Citrus volkameriana seedlings= Apomixie facultative, polyploïdisation spontanée et dépression de consanguinité dans les semis de Citrus volkameriana. *Fruits* **1994**, 49, 479-480.

Ollitrault, P.; Jacquemond, C.; Dubois, C.; Luro, F., Citrus. In Genetic diversity of cultivated tropical plants. Perla H, Marc S, Xavier P, Glaszmann JC (eds) CIRAD: Montpellier, **2003**; pp 193-217.

Ollitrault, P.; Jacquemond, C.; Dubois, C.; Luro, F. (eds) Citrus. Science Publishers and Cirad, Paris, 2003.

Ollitrault, P.; Luro, F. Amélioration des agrumes et biotechnologie= Citrus breeding and biotechnology. *Fruits* **1995**, 50, 267-279.

Omura, M.; Ueda, T.; Kita, M.; Komatsu, A.; Takanokura, Y.; Shimada, T.; Endo-Inagaki, T.; Nesumi, H.; Yoshida, T. EST mapping of Citrus. In: Proceedings of the International Society of Citriculture IX Congress, Orlando, FL, USA, 2000, 71–74.

Ouerghi, Z.; Rémy, R.; Ouelhazi, L.; Ayadi, A.; Brulfert, J. Two-dimensional electrophoresis of soluble leaf proteins, isolated from two wheat species (Triticum durum and Triticum aestivum) differing in sensitivity towards NaCl. *Electrophoresis* **2000**, 21, 2487-2491.

Papp, J.; Ball, M.; Terry, N. A comparative study of the effects of NaCl salinity on respiration, photosynthesis, and leaf extension growth in Beta vulgaris L.(sugar beet). *Plant, Cell & Environment* **1983**, 6, 675-677.

Pardo, J. M.; Cubero, B.; Leidi, E. O.; Quintero, F. J. Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *Journal of Experimental Botany* **2006**, 57, 1181-1199.

Parida, A.; Das, A.; Mittra, B. Effects of NaCl stress on the structure, pigment complex composition, and photosynthetic activity of mangrove Bruguiera parviflora chloroplasts. *Photosynthetica* **2003**, 41, 191-200.

Perez-Perez, J. G.; Syvertsen, J. P.; Botia, P.; Garcia-Sanchez, F. Leaf water relations and net gas exchange responses of salinized Carrizo citrange seedlings during drought stress and recovery. *Annals of Botany* **2007**, 100, 335-345.

Peynado, A.; Young, R. (1969) Relation of salt tolerance to cold hardiness of ‘Redblush’grapefruit and ‘Valencia’orange trees on various rootstocks. In, pp 1793-1802

Piao, H.; Lim, J.; Kim, S.; Cheong, G.; Hwang, I. Constitutive over-expression of AtGSK1 induces NaCl stress responses in the absence of NaCl stress and results in enhanced NaCl tolerance in Arabidopsis. *The Plant Journal* **2001**, 27, 305-314.

Piqueras, A.; Hernandez, J. A.; Olmos, E.; Hellin, E.; Sevilla, F. Changes in antioxidant enzymes and organic solutes associated with adaptation of citrus cells to salt stress. *Plant Cell Tissue Organ Cult.* **1996**, 45, 53-60.

Popova, L.; Stoinova, Z.; Maslenkova, L. Involvement of abscisic acid in photosynthetic process in Hordeum vulgare L. during salinity stress. *J. Plant Growth Regul.* **1995**, 14, 211-218.

Postaire, O.; Tournaire-Roux, C.; Grondin, A.; Boursiac, Y.; Morillon, R.; Schaffner, A. R.; Maurel, C. A PIP1 Aquaporin Contributes to Hydrostatic Pressure-Induced Water Transport in Both the Root and Rosette of Arabidopsis. *Plant Physiology* **2010**, 152, 1418-1430.

Prasad, G.; Coury, L.; Finn, F.; Zeidel, M. Reconstituted aquaporin 1 water channels transport CO2 across membranes. *J. Biol. Chem.* **1998**, 273, 33123.

Purvis, A. C.; Yelenosky, G. Translocation of carbohydrates and proline in young grapefruit trees at low-temperatures

Plant Physiology **1983**, 73, 877-880.

Qi, Z.; Verma, R.; Gehring, C.; Yamaguchi, Y.; Zhao, Y.; Ryan, C.; Berkowitz, G. Ca2+ signaling by plant Arabidopsis thaliana Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca2+ channels. *Proceedings of the National Academy of Sciences* **2010**, 107, 21193.

Qiu, Q. S.; Guo, Y.; Quintero, F. J.; Pardo, J. M.; Schumaker, K. S.; Zhu, J. K. Regulation of vacuolar Na+/H+ exchange in Arabidopsis thaliana by the salt-overly-sensitive (SOS) pathway. *J. Biol. Chem.* **2004**, 279, 207-215.

Rao, M. N.; Soneji, J. R.; Chen, C. X.; Huang, S.; Gmitter, F. G. Characterization of zygotic and nucellar seedlings from sour orange-like citrus rootstock candidates using RAPD and EST-SSR markers. *Tree Genet. Genomes* **2008**, 4, 113-124.

Rapp, R.; Udall, J.; Wendel, J. Genomic expression dominance in allopolyploids. *BMC biology* **2009**, 7, 18.

Ream, C.; Furr, J. Salt tolerance of some Citrus species, relatives and hybrids tested as rootstocks. *J. Am. Soc. Hortic. Sci* **1976**, 101, 265–267.

Reddy, M.; Sanish, S.; Iyengar, E. Photosynthetic studies and compartmentation of ions in different tissues of Salicornia brachiata Roxb. under saline conditions. *Photosynthetica* **1992**, 26, 173-179.

Remorini, D.; Tavarini, S.; Degl'Innocenti, E.; Loreti, F.; Massai, R.; Guidi, L. Effect of rootstocks and harvesting time on the nutritional quality of peel and flesh of peach fruits. *Food Chemistry* **2008**, 110, 361-367.

Richards, R. Defining selection criteria to improve yield under drought. *Plant Growth Regulation* **1996**, 20, 157-166.

Riddle, N. C.; Jiang, H. M.; An, L. L.; Doerge, R. W.; Birchler, J. A. Gene expression analysis at the intersection of ploidy and hybridity in maize. *Theoretical and Applied Genetics* **2010**, 120, 341-353.

Robinson, S.; Downton, W.; Millhouse, J. Photosynthesis and ion content of leaves and isolated chloroplasts of salt-stressed spinach. *Plant Physiology* **1983**, 73, 238.

Rodrigo, M.; Alquezar, B.; Zacarias, L. Cloning and characterization of two 9-cis-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (Citrus sinensis L. Osbeck). *Journal of Experimental Botany* **2006**, 57, 633.

Rodríguez Rosales, M.; Jiang, X.; Gálvez, F.; Aranda, M.; Cubero, B.; Venema, K. Overexpression of the tomato K+/H+ antiporter LeNHX2 confers salt tolerance by improving potassium compartmentalization. *New Phytologist* **2008**, 179, 366-377.

Rodriguez, S.; Petersen, M.; Mundy, J. Mitogen-Activated Protein Kinase Signaling in Plants. *Annu. Rev. Plant Biol.* **2010**, 61, 621-649.

Romero-Aranda, R.; Bondada, B.; Syvertsen, J.; Grosser, J. Leaf characteristics and net gas exchange of diploid and autotetraploid citrus. *Annals of Botany* **1997**, 79, 153.

Romero-Aranda, R.; Moya, J. L.; Tadeo, F. R.; Legaz, F.; Primo-Millo, E.; Talon, M. Physiological and anatomical disturbances induced by chloride salts in sensitive and tolerant citrus: beneficial and detrimental effects of cations. *Plant Cell Environ.* **1998**, 21, 1243-1253.

Roose, M. Mapping and Marker-assisted Selection. *Citrus genetics, breeding and biotechnology* **2007.**

Roose, M.; Feng, D.; Cheng, F.; Tayyar, R.; Federici, C.; Kupper, R. Mapping the Citrus genome. In. *ISHS*, **1998,** 25-32.

Roose, M. L.; Kupper, R. S. Causes and Consequences of variability in rootstocks. *Proceedings of the first international seminar on citriculture in Pakistan* **1992**, 122-130.

Roose, M. L.; Traugh, S. N. Identification and performance of citrus trees on nucellar and zygotic rootstocks. *Journal of the American Society for Horticultural Science* **1988**, 113, 100-105.

Ruiz, C.; Asins, M. Comparison between Poncirus and Citrus genetic linkage maps. *TAG Theoretical and Applied Genetics* **2003**, 106, 826-836.

Rus, A.; Yokoi, S.; Sharkhuu, A.; Reddy, M.; Lee, B.; Matsumoto, T.; Koiwa, H.; Zhu, J.; Bressan, R.; Hasegawa, P. AtHKT1 is a salt tolerance determinant that controls Na+ entry into plant roots. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, 98, 14150.

Rus, A.; Lee, B.; Munoz-Mayor, A.; Sharkhuu, A.; Miura, K.; Zhu, J.; Bressan, R.; Hasegawa, P. AtHKT1 facilitates Na+ homeostasis and K+ nutrition in planta. *Plant Physiology* **2004**, 136, 2500.

Sakamoto, A.; Murata, N. The role of glycine betaine in the protection of plants from stress: clues from transgenic plants. *Plant, Cell & Environment* **2002**, 25, 163-171.

Saleh, B.; Allario, T.; Dambier, D.; Ollitrault, P.; Morillon, R. Tetraploid citrus rootstocks are more tolerant to salt stress than diploid. *C. R. Biol.* **2008**, 331, 703-710.

Sanders, D.; Pelloux, J.; Brownlee, C.; Harper, J. Calcium at the crossroads of signaling. *The Plant Cell Online* **2002**, 14, 401.

Saneoka, H.; Nagasaka, C.; Hahn, D. T.; Yang, W. J.; Premachandra, G. S.; Joly, R. J.; Rhodes, D. Salt tolerance of glycinebetaine-deficient and glycinebetaine-containing maize lines. *Plant Physiology* **1995**, 107, 631-638.

Sankar, A.; Moore, G. Evaluation of inter-simple sequence repeat analysis for mapping in Citrus and extension of the genetic linkage map. *TAG Theoretical and Applied Genetics* **2001**, 102, 206-214.

Santner, A.; Estelle, M. Recent advances and emerging trends in plant hormone signalling. *Nature* **2009**, 459, 1071-1078.

Schäffner, A. Aquaporin function, structure, and expression: are there more surprises to surface in water relations? *Planta* **1998**, 204, 131-139.

Schroeder, J.; Allen, G.; Hugouvieux, V.; Kwak, J.; Waner, D. Guard cell signal transduction. *Annu. Rev. Plant Biol.* **2001**, 52, 627-658.

Schwarz, S. Autotetraploides espontáneos en patrones de cítricos: Incidencia, características y comportamiento en vivero y campo. Ph.D thesis, Universidad Politécnica de Valencia, Valencia, **2001**, p

Seker, M.; Tuzcu, O.; Ollitrault, P. Comparison of nuclear DNA content of citrus rootstock populations by flow cytometry analysis. *Plant Breeding* **2003**, 122, 169-172.

Seki, M.; Narusaka, M.; Ishida, J.; Nanjo, T.; Fujita, M.; Oono, Y.; Kamiya, A.; Nakajima, M.; Enju, A.; Sakurai, T. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *The Plant Journal* **2002**, 31, 279-292.

Serrano, R.; Mulet, J.; Rios, G.; Marquez, J.; De Larrinoa, I.; Leube, M.; Mendizabal, I.; Pascual-Ahuir, A.; Proft, M.; Ros, R. A glimpse of the mechanisms of ion homeostasis during salt stress. *Journal of Experimental Botany* **1999**, 50, 1023.

Shabala, S. Regulation of potassium transport in leaves: from molecular to tissue level. *Annals of Botany* **2003**, 92, 627.

Shi, H. Z.; Ishitani, M.; Kim, C. S.; Zhu, J. K. The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, 97, 6896-6901.

Shi, H. Z.; Lee, B. H.; Wu, S. J.; Zhu, J. K. Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. *Nature biotechnology* **2003**, 21, 81-85.

Shinozaki, K.; Yamaguchi-Shinozaki, K. Molecular responses to drought and cold stress. *Current Opinion in Biotechnology* **1996**, 7, 161-167.

Sibole, J. V.; Montero, E.; Cabot, C.; Poschenrieder, C.; Barcelo, J. Relationship between carbon partitioning and Na+, Cl- and ABA allocation in fruits of salt-stressed bean. *J. Plant Physiol.* **2000**, 157, 637-642.

Sibole, J. V.; Cabot, C.; Poschenrieder, C.; Barcelo, J. Efficient leaf ion partitioning, an overriding condition for abscisic acid-controlled stomatal and leaf growth responses to NaCl salinization in two legumes. *Journal of Experimental Botany* **2003**, 54, 2111-2119.

Siefritz, F.; Tyree, M.; Lovisolo, C.; Schubert, A.; Kaldenhoff, R. PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. *The Plant Cell Online* **2002**, 14, 869.

Silva-Ortega, C. O.; Ochoa-Alfaro, A. E.; Reyes-Agüero, J. A.; Aguado-Santacruz, G. A.; Jiménez-Bremont, J. F. Salt stress increases the expression of p5cs gene and induces proline accumulation in cactus pear. *Plant Physiology and Biochemistry* **2008**, 46, 82-92.

Singh, A.; Saini, M. L.; Behl, R. K. Seed germination and seedling growth of citrus (Citrus species) rootstocks under different salinity regimes. *Indian J. Agric. Sci.* **2004**, 74, 246-248.

Smirnoff, N.; Cumbes, Q. J. Hydroxyl radical scavenging activity of compatible solutes *Phytochemistry* **1989**, 28, 1057-1060.

Soar, C. J.; Dry, P. R.; Loveys, B. R. Scion photosynthesis and leaf gas exchange in Vitis vinifera L. cv. Shiraz: Mediation of rootstock effects via xylem sap ABA. In. Blackwell Publishing Ltd, **2006**,82-96.

Soost, R.; Cameron, J.; Janick, J.; Moore, J. Advances in fruit breeding. In. Purdue University Press,**1975**.

Spiegel-Roy, P.; Ben-Hayyim, G. Selection and breeding for salinity tolerance in vitro. *Plant and Soil* **1985**, 89, 243-252.

Spiegel-Roy, P.; Goldschmidt, E., Biology of citrus. Cambridge Univ Pr**1996**; p

Spollen, W.; Sharp, R.; Saab, I.; Wu, Y. Regulation of cell expansion in roots and shoots at low water potentials. *Water Deficits: Plant Responses from Cell to Community* **1993**, 37–52.

Stace, H.; Armstrong, J.; James, S. Cytoevolutionary patterns inRutaceae. *Plant Systematics and Evolution* **1993**, 187, 1-28.

Stam, P.; Van Ooijen, J. JoinMap (tm) version 2.0: Software for the calculation of genetic linkage maps. *CPRO-DLO, Wageningen* **1995**,

Steffens, D.; Sutter, S.; Roemer, S. An alternate universal forward primer for improved automated DNA sequencing of M13. *BioTechniques* **1993**, 15, 580.

Stepien, P.; Johnson, G. Contrasting responses of photosynthesis to salt stress in the glycophyte Arabidopsis thaliana and the halophyte Thellungiella halophila. Role of the plastid terminal oxidase as an alternative electron sink. *Plant Physiology* **2008**,

Storey, R.; Walker, R. R. Citrus and salinity. *Sci. Hortic.* **1998**, 78, 39-81.

Stupar, R. M.; Bhaskar, P. B.; Yandell, B. S.; Rensink, W. A.; Hart, A. L.; Ouyang, S.; Veilleux, R. E.; Busse, J. S.; Erhardt, R. J.; Buell, C. R.; Jiang, J. M. Phenotypic and transcriptomic changes associated with potato autopolyploidization. *Genetics* **2007**, 176, 2055-2067.

Sudhir, P. R.; Pogoryelov, D.; Kovacs, L.; Garab, G.; Murthy, S. D. S. The effects of salt stress on photosynthetic electron transport and thylakoid membrane proteins in the cyanobacterium Spirulina platensis. *J. Biochem. Mol. Biol.* **2005**, 38, 481-485.

Sun, J.; Okita, T.; Edwards, G. Modification of carbon partitioning, photosynthetic capacity, and O2 sensitivity in Arabidopsis plants with low ADP-glucose pyrophosphorylase activity. *Plant Physiology* **1999**, 119, 267.

Swingle, W.; Reece, P. The botany of Citrus and its relatives. *The citrus industry. Berkeley: University of California* **1967**, 1, 190-430.

Sykes, S. R. Effects of seedling age and size on chloride accumulation by juvenile citrus seedlings treated with sodium-chloride under glasshouse conditions. *Australian Journal of Experimental Agriculture* **1985**, 25, 943-953.

Sykes, S. The inheritance of salt exclusion in woody perennial fruit species. *Plant and soil* **1992**, 146, 123-129.

Syvertsen, J. P.; Lee, L. S.; Grosser, J. W. Limitations on growth and net gas exchange of diploid and tetraploid Citrus rootstock cultivars grown at elevated CO2. *Journal of the American Society for Horticultural Science* **2000**, 125, 228-234.

Székely, G.; Ábrahám, E.; Csépl , Á.; Rigó, G.; Zsigmond, L.; Csiszár, J.; Ayaydin, F.; Strizhov, N.; Jásik, J.; Schmelzer, E. Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. *The Plant Journal* **2008**, 53, 11-28.

Tadeo, F.; Cercós, M.; Colmenero-Flores, J.; Iglesias, D.; Naranjo, M.; R os, G.; Carrera, E.; Ruiz-Rivero, O.; Lliso, I.; Morillon, R. Molecular physiology of development and quality of citrus. *Advances in Botanical Research* **2008**, 47, 147-223.

Tadeo, F. R.; Cercós, M.; ColmeneroFlores, J. M.; Iglesias, D. J.; Naranjo, M. A.; Ríos, G.; Carrera, E.; RuizRivero, O.; Lliso, I.; Morillon, R.; Ollitrault, P.; Talon, M.; Jean-Claude, K.; Michel, D., Molecular Physiology of Development and Quality of Citrus. In Advances in Botanical Research. Academic Press, **2008**; pp 147-223.

Tanaka, T. Citrologia, Semi-centennial Commemoration Papers on Citrus Studies. *Osaka: Citrologia Supporting Foundation* **1961**, 114,

Taungbodhitham, A.; Jones, G.; Wahlqvist, M.; Briggs, D. Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables. *Food Chemistry* **1998**, 63, 577-584.

Teakle, N. L.; Tyerman, S. D. Mechanisms of Cl- transport contributing to salt tolerance. *Plant Cell Environ.* **2010**, 33, 566-589.

Teige, M.; Scheikl, E.; Eulgem, T.; Dóczi, R.; Ichimura, K.; Shinozaki, K.; Dangl, J.; Hirt, H. The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. *Molecular Cell* **2004**, 15, 141-152.

Tester, M.; Davenport, R. Na+ Tolerance and Na+ Transport in Higher Plants. *Annals of Botany* **2003**, 91, 503-527.

Thivend, P.; Mercier, C.; Guilbot, A. Determination of starch with glucoamylase. *Methods in carbohydrate chemistry* **1972**, 6, 100–105.

Thompson, A.; Jackson, A.; Symonds, R.; Mulholland, B.; Dadswell, A.; Blake, P.; Burbidge, A.; Taylor, I. Ectopic expression of a tomato 9-cis-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. *The Plant Journal* **2000**, 23, 363-374.

Torres, A.; Mau-Lastovicka, T.; Williams, T.; Soost, R. Segregation distortion and linkage of Citrus and Poncirus isozyme genes. *J. Hered.* **1985**, 76, 289.

Tozlu, I.; Guy, C. L.; Moore, G. A. QTL analysis of Na+ and Cl- accumulation related traits in an intergeneric BC1 progeny of Citrus and Poncirus under saline and nonsaline environments. *Genome* **1999**, 42, 692-705.

Tozlu, I.; Moore, G. A.; Guy, C. L. Regulation of growth and differential tissue dry mass accumulation by Citrus grandis, Poncirus trifoliata, and their F-1 under salinized and non-salinized environments. *Aust. J. Plant Physiol.* **2000**, 27, 27-33.

Tozlu, I.; Guy, C.; Moore, G. A., Breeding Citrus for salt tolerance. In Proceedings of the International Symposium on Techniques to Control Salination for Horticultural Productivity. Aksoy U, Anac D, Anac S, Beltrao J, BenAsher J, Cuartero J, Flowers TJ, Hepaksoy S (eds) International Society Horticultural Science: Leuven 1, **2002**; pp 255-264.

Tracy, F.; Gilliham, M.; Dodd, A.; Webb, A.; Tester, M. NaCl induced changes in cytosolic free Ca2+ in Arabidopsis thaliana are heterogeneous and modified by external ionic composition. *Plant, Cell & Environment* **2008**, 31, 1063-1073.

Turner, N. C., Further progress in crop water relations. In Advances in Agronomy, Vol 58. Academic Press Inc: San Diego, **1997**; pp 293-338.

Turner, N. C.; Wright, G. C.; Siddique, K. H. H., Adaptation of grain legumes (pulses) to water-limited environments. In Advances in Agronomy, Vol 71. Academic Press Inc: San Diego, **2001**; pp 193-231.

Tutberidze, B. D.; Kalandarishvili, T. L. The results of studies on the qualities of Poncirus trifoliata "Tetraploid No. 1" as a rootstock for citrus crops. *Subtropicheskie Kul'tury* **1975**, 74-79.

Ueda, A.; Kathiresan, A.; Inada, M.; Narita, Y.; Nakamura, T.; Shi, W. M.; Takabe, T.; Bennett, J. Osmotic stress in barley regulates expression of a different set of genes than salt stress does. *Journal of Experimental Botany* **2004**, 55, 2213-2218.

Uehlein, N.; Lovisolo, C.; Siefritz, F.; Kaldenhoff, R. The tobacco aquaporin NtAQP1 is a membrane CO2 pore with physiological functions. *Nature* **2003**, 425, 734-737.

Ulm, R.; Ichimura, K.; Mizoguchi, T.; Peck, S. C.; Zhu, T.; Wang, X.; Shinozaki, K.; Paszkowski, J. Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. *Embo J.* **2002**, 21, 6483-6493.

Urban, L.; Lu, P.; Thibaud, R. Inhibitory effect of flowering and early fruit growth on leaf photosynthesis in mango. *Tree Physiol.* **2004**, 24, 387.

Vartanian, N.; Marcotte, L.; Giraudat, J. Drought Rhizogenesis in Arabidopsis thaliana (differential responses of hormonal mutants). *Plant Physiology* **1994**, 104, 761.

Voisin, A.; Reidy, B.; Parent, B.; Rolland, G.; Redondo, E.; Gerentes, D.; Tardieu, F.; Muller, B. Are ABA, ethylene or their interaction involved in the response of leaf growth to soil water deficit? An analysis using naturally occurring variation or genetic transformation of ABA production in maize. *Plant, Cell & Environment* **2006**, 29, 1829-1840.

Walker, R. R. Sodium exclusion and potassium-sodium selectivity in salt-treated trifoliate orange (Poncirus trifoliata) and Cleopatra mandarin (Citrus reticulata) plants. *Aust. J. Plant Physiol* **1986**, 13, 293–303.

Walker, R. R.; Douglas, T. J. Effect of salinity level on uptake and distribution of chloride, sodium and potassium-ions in citrus plants. *Aust. J. Agric. Res.* **1983**, 34, 145-153.

Wan, S.; Deng, Z.; Deng, X.; Ye, X. (1991) Advances made in vitro mutation breeding in Citrus. In. PLANT MUTATION BREEDING FOR CROP IMPROVEMENT, Vienna, pp 263-269

Wang, J.; Tian, L.; Lee, H.; Wei, N.; Jiang, H.; Watson, B.; Madlung, A.; Osborn, T.; Doerge, R.; Comai, L. Genome-wide non-additive gene regulation in Arabidopsis allotetraploids. *Genetics* **2005**,

Ward, R.; Durrett, R. Subfunctionalization: How often does it occur? How long does it take? *Theoretical Population Biology* **2004**, 66, 93-100.

Warner, D.; Edwards, G. Effects of polyploidy on photosynthetic rates, photosynthetic enzymes, contents of DNA, chlorophyll, and sizes and numbers of photosynthetic cells in the C4 dicot Atriplex confertifolia. *Plant Physiology* **1989**, 91, 1143.

Wasilewska, A.; Vlad, F.; Sirichandra, C.; Redko, Y.; Jammes, F.; Valon, C.; Frey, N.; Leung, J. An Update on Abscisic Acid Signaling in Plants and More. *Molecular Plant* **2008**, 1, 198.

Webber, A. N.; Nie, G. Y.; Long, S. P. Acclimation of photosynthetic proteins to rinsing atmospheric CO2. *Photosynthesis Research* **1994**, 39, 413-425.

Weig, A.; Deswarte, C.; Chrispeels, M. The major intrinsic protein family of Arabidopsis has 23 members that form three distinct groups with functional aquaporins in each group. *Plant Physiology* **1997**, 114, 1347.

Weinl, S.; Kudla, J. The CBL-CIPK Ca2+-decoding signaling network: function and perspectives. *New Phytologist* **2009**, 184, 517-528.

Wheaton, T.; Castle, W.; Whitney, J.; Tucker, D. Performance of citrus scion cultivars and rootstocks in a high-density planting. *Hortscience* **1991**, 26, 837-840.

Wheaton, T.; Whitney, J.; Castle, W.; Muraro, R.; Browning, H.; Tucker, D. Citrus scion and rootstock, topping height, and tree spacing affect tree size, yield, fruit quality, and economic return. *JOURNAL-AMERICAN SOCIETY FOR HORTICULTURAL SCIENCE* **1995**, 120, 861-861.

Willekens, H.; Chamnongpol, S.; Davey, M.; Schraudner, M.; Langebartels, C.; Van Montagu, M.; Inzé, D.; Van Camp, W. Catalase is a sink for H2O2 and is indispensable for stress defence in C3 plants. *The EMBO Journal* **1997**, 16, 4806-4816.

Wilmsen, P.; Salvador, M. Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. *J. Agric. Food Chem* **2005**, 53, 4757-4761.

Wood, T.; Takebayashi, N.; Barker, M.; Mayrose, I.; Greenspoon, P.; Rieseberg, L. The frequency of polyploid speciation in vascular plants. *Proceedings of the National Academy of Sciences* **2009**, 106, 13875.

Wu, C.; Gao, X.; Kong, X.; Zhao, Y.; Zhang, H. Molecular cloning and functional analysis of a Na+/H+ antiporter gene ThNHX1 from a halophytic plant Thellungiella halophila. *Plant Molecular Biology Reporter* **2009**, 27, 1-12.

Wu, S.; Ding, L.; Zhu, J. SOS1, a genetic locus essential for salt tolerance and potassium acquisition. *The Plant Cell Online* **1996**, 8, 617.

Wu, Y.; Chen, Q.; Chen, M.; Chen, J.; Wang, X. Salt-tolerant transgenic perennial ryegrass (Lolium perenne L.) obtained by Agrobacterium tumefaciens-mediated transformation of the vacuolar Na+/H+ antiporter gene. *Plant Science* **2005**, 169, 65-73.

Xiang, C.; Roose, M. L. Frequency and characteristics of nucellar and zygotic seedlings in 12 citrus rootstocks. *Sci. Hortic.* **1988**, 37, 47-59.

Xin, Z.; Li, P. Relationship between proline and abscisic acid in the induction of chilling tolerance in maize suspension-cultured cells. *Plant Physiology* **1993**, 103, 607.

Xiong, L.; Schumaker, K.; Zhu, J. Cell signaling during cold, drought, and salt stress. *The Plant Cell Online* **2002**, 14, 165.

Xiong, L. M.; Zhu, J. K. Abiotic stress signal transduction in plants: Molecular and genetic perspectives. *Physiol. Plant.* **2001**, 112, 152-166.

Xiong, L.; Zhu, J. Regulation of abscisic acid biosynthesis. *Plant Physiology* **2003**, 133, 29.

Xu, D.; Duan, X.; Wang, B.; Hong, B.; Ho, T.; Wu, R. Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiology* **1996**, 110, 249.

Xue, Z.; Zhi, D.; Xue, G.; Zhang, H.; Zhao, Y.; Xia, G. Enhanced salt tolerance of transgenic wheat (Tritivum aestivum L.) expressing a vacuolar Na+/H+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na+. *Plant Science* **2004**, 167, 849-859.

Yamaguchi, T.; Blumwald, E. Developing salt-tolerant crop plants: challenges and opportunities. *Trends Plant Sci.* **2005**, 10, 615-620.

Yamamoto, M.; Tominaga, S. Chromosome identification in haploid clementine (Citrus clementina hort. ex Tanaka) by fluorescent staining. *Sci. Hortic.* **2004**, 101, 201-206.

Yancey, P.; Clark, M.; Hand, S.; Bowlus, R.; Somero, G. Living with water stress: evolution of osmolyte systems. *Science* **1982**, 217, 1214.

Yang, Q.; Chen, Z. Z.; Zhou, X. F.; Yin, H. B.; Li, X.; Xin, X. F.; Hong, X. H.; Zhu, J. K.; Gong, Z. Z. Overexpression of SOS (Salt Overly Sensitive) Genes Increases Salt Tolerance in Transgenic Arabidopsis. *Molecular Plant* **2009**, 2, 22-31.

Yasar, F.; Kusvuran, S.; Ellialtioglu, S. Determination of anti-oxidant activities in some melon (Cucumis melo L.) varieties and cultivars under salt stress. *Journal of Horticultural Science & Biotechnology* **2006**, 81, 627-630.

Yasuda, M.; Ishikawa, A.; Jikumaru, Y.; Seki, M.; Umezawa, T.; Asami, T.; Maruyama-Nakashita, A.; Kudo, T.; Shinozaki, K.; Yoshida, S. Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in Arabidopsis. *The Plant Cell Online* **2008**, 20, 1678.

Yokoi, S.; Quintero, F. J.; Cubero, B.; Ruiz, M. T.; Bressan, R. A.; Hasegawa, P. M.; Pardo, J. M. Differential expression and function of Arabidopsis thaliana NHX Na+/H+ antiporters in the salt stress response. *Plant J.* **2002**, 30, 529-539.

Yoshiba, Y.; Kiyosue, T.; Katagiri, T.; Ueda, H.; Mizoguchi, T.; Yamaguchi-Shinozaki, K.; Wada, K.; Harada, Y.; Shinozaki, K. Correlation between the induction of a gene for 1-pyrroline-5-carboxylate synthetase and the accumulation of proline in Arabidopsis thaliana under osmotic stress. *The Plant Journal* **1995**, 7, 751-760.

Yoshiba, Y.; Kiyosue, T.; Nakashima, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Regulation of levels of proline as an osmolyte in plants under water stress. *Plant and Cell Physiology* **1997**, 38, 1095.

Zeevaart, J.; Creelman, R. Metabolism and physiology of abscisic acid. *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **1988**, 39, 439-473.

Zekri, M. Effects of NaCl on growth and physiology of sour orange and Cleopatra mandarin seedlings. *Sci. Hortic.* **1991**, 47, 305-315.

Zekri, M. (1996) Leaf mineral concentration, growth, yield, fruit quality, and economics of 'Ambersweet'orange on two rootstocks. In, pp 92-96

Zekri, M.; Al-Jaleel, A. Evaluation of rootstocks for Valencia and Navel orange trees in Saudi Arabia. *Fruits* **2004**, 59, 91-100.

Zekri, M.; Parsons, L. Salinity tolerance of citrus rootstocks: Effects of salt on root and leaf mineral concentrations. *Plant and soil* **1992**, 147, 171-181.

Zhanao, D.; Wencai, Z.; Shuyuan, W. In Vitro Induction and Protoplast Plant Regeneration from NaCl-Tolerant Lines in Citrus [J]. *Acta Horticulturae Sinica* **1993**, 2,

Zhang, H.; Blumwald, E. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature biotechnology* **2001**, 19, 765-768.

Zhang, H.; Hodson, J.; Williams, J.; Blumwald, E. Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, 98, 12832.

Zhang, J.; Flowers, T.; Wang, S. Mechanisms of sodium uptake by roots of higher plants. *Plant and soil* **2010**, 326, 45-60.

Zhang, W.; Hao, H.; Ma, L.; Zhao, C.; Yu, X. Tetraploid muskmelon alters morphological characteristics and improves fruit quality. *Sci. Hortic.* **2010**, 125, 396-400.

Zhang, X.; Hu, C.; Yao, J. Tetraploidization of diploid Dioscorea results in activation of the antioxidant defense system and increased heat tolerance. *J. Plant Physiol.* **2010**, 167, 88-94.

Zhou, C.; Cai, Z.; Guo, Y.; Gan, S. An Arabidopsis mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. *Plant Physiology* **2009**, 150, 167.

Zhou, S. F.; Chen, X. Y.; Zhang, X. G.; Li, Y. X. Improved salt tolerance in tobacco plants by co-transformation of a betaine synthesis gene BADH and a vacuolar Na+/H+ antiporter gene SeNHX1. *Biotechnol. Lett.* **2008**, 30, 369-376.

Zhu, B.; Su, J.; Chang, M.; Verma, D.; Fan, Y.; Wu, R. Overexpression of a [delta] 1-pyrroline-5-carboxylate synthetase gene and analysis of tolerance to water-and salt-stress in transgenic rice. *Plant Science* **1998**, 139, 41-48.

Zhu, J. Genetic analysis of plant salt tolerance using Arabidopsis. *Plant Physiology* **2000**, 124, 941.

Zhu, J. K. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* **2002**, 53, 247-273.

Zhu, J. Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology* **2003**, 6, 441-445.

Zhu, J. K.; Liu, J. P.; Xiong, L. M. Genetic analysis of salt tolerance in Arabidopsis: Evidence for a critical role of potassium nutrition. *Plant cell* **1998**, 10, 1181-1191.

Zou, J.; Liu, A.; Chen, X.; Zhou, X.; Gao, G.; Wang, W.; Zhang, X. Expression analysis of nine rice heat shock protein genes under abiotic stresses and ABA treatment. *J. Plant Physiol.* **2009**, 166, 851-861.

Zou, J. J.; Wei, F. J.; Wang, C.; Wu, J. J.; Ratnasekera, D.; Liu, W. X.; Wu, W. H. Arabidopsis Calcium-Dependent Protein Kinase CPK10 Functions in Abscisic Acid- and Ca2+-Mediated Stomatal Regulation in Response to Drought Stress. *Plant Physiology* **2010**, 154, 1232-1243.

8 Substantial summary in French

8.1 Introduction

Originaires du Sud-Est asiatique, les agrumes sont cultivés en Chine depuis plusieurs millénaires et se seraient ensuite répandus dans le monde grâce aux échanges commerciaux et aux invasions. Aujourd'hui, le bassin méditerranéen est considéré comme le second berceau d'implantation et de diffusion des agrumes. Ceux-ci constituent la première production fruitière mondiale avec plus de 100 millions de tonnes/an dont près de 60% sont des oranges. L'agrumiculture, en constante expansion, reste localisée surtout aux Amériques, avec le Brésil (20%) comme principal pays producteur, puis les Etats Unis (16%) et la Chine (10%), mais aussi sur le pourtour méditerranéen qui représente environ 20% de la production mondiale, dont l'Espagne et le Maroc sont les principaux producteurs. La culture pratiquée sous irrigation permet de s'affranchir des problèmes de contraintes hydriques en raison de sa dissémination, la culture des agrumes est confrontée à de nombreuses contraintes biotiques. En effet, dès le XIX^{e} siècle les vergers d'agrumes ont été décimés par l'apparition de nouvelles maladies (par exemple la Tristeza due à un virus *Citrus Tristeza Virus* (CTV), la gommose (due à un champignon), *Phytophthora* sp., le Mal Secco lié à *Deuterophoma tracheiphila*…). A partir de 1840, des porte-greffes d'agrumes résistant au *Phytophthora* sp ont été utilisés. Dès lors, de nombreuses études ont été réalisées, montrant l'influence de l'association porte-greffe / greffon en ce qui concerne leur comportement physiologique vis-à-vis des conditions environnementales. Selon les espèces de porte-greffes utilisées en combinaison avec des variétés d'intérêt, les effets seront multiples et variés, et pourront affecter positivement ou négativement le rendement, la chute, la qualité et le calibre des fruits, etc.

Il faut rappeler que l'agrumicuture peut-être soumise à des déficits hydriques très importants si l'irrigation n'est pas suffisante. De même, les vergers peuvent être soumis à des stress salins importants en raison de la salinité prononcée des eaux d'irrigation. La création de porte-greffes plus tolérants aux contraintes abiotiques (sécheresse, salinité, sols calcaires…), mais également biotiques (bio-agresseurs…), tout en maintenant le rendement et la qualité de production, constitue un enjeu majeur pour l'agrumiculture.

8.1.1 Utilisation de porte-greffes tétraploïdes

Les porte-greffes d'agrumes ont la particularité de produire spontanément des génotypes tétraploïdes au sein des semis diploïdes. Ils proviennent de doublements chromosomiques lors de la méiose des embryons somatiques. Leur fréquence est très élevée pour certains porte-greffes. Ainsi, dans les vergers d'agrumes espagnols greffés sur citrange carrizo, 5 à 10% des porte-greffes seraient tétraploïdes. Bien que leur existence soit connue depuis longtemps, peu de travaux se sont intéressés à leur potentialité agronomique sous contrainte abiotique.

8.1.1.1 Différenciation physiologique, morphologique et anatomique de génotypes tétraploïdes

Les génotypes tétraploïdes présentent une physiologie, une morphologie et une anatomie très différente de celle des diploïdes parentaux. Leur croissance est moindre et les résultats de l'équipe agrumes du CIRAD suggèrent qu'une régulation plus importante des échanges respiratoires au travers des stomates des feuilles en serait à l'origine. Leurs graines sont plus grandes, les feuilles plus épaisses et vert foncé, les entre-nœuds des tiges plus courts et les racines secondaires plus trapues. Au niveau anatomique, nous avons observé que la taille de leurs cellules était toujours plus grande. De très grandes différences, physiologiques et anatomiques existent donc entre génotypes diploïdes et tétraploïdes de porte-greffes d'agrumes. Pourtant, dans le cadre d'études de l'expression des génomes diploïdes et tétraploïdes au moyen des puces à ADN et par PCR en temps réel au niveau des feuilles, il n'a observé que très peut de différences suggérant qu'il existe des mécanismes de régulation de l'expression des génomes qui seraient spécifiques chez les génotypes tétraploïdes (Allario *et al*. 2011)

8.1.1.2 Différenciation dans la tolérance au stress hydrique et salin

En situation de sécheresse, les plants tétraploïdes présentent une tolérance beaucoup plus forte que les diploïdes. De même, nous avons observé que les porte-greffe tétraploïdes greffés avec une variété diploïde comme l'oranger Valencia étaient également plus tolérants à la sécheresse. Toutefois, dans les deux cas, il semble que la perception du stress ait lieu au même moment, conduisant à une fermeture concomitante des stomates.

En situation de contrainte saline, la régulation des échanges respiratoires observée chez les génotypes tétraploïdes serait également à l'origine d'une limitation de l'absorption des ions chlorure et sodium au niveau racinaire. Le transfert jusqu'aux feuilles de ces ions

toxiques dans le flux de transpiration serait plus limité et rendrait ainsi les génotypes tétraploïdes plus tolérants au stress salin. Dans une première série d'expérimentations, nous avons en effet démontré que des porte-greffes autotétraploïdes d'agrumes sont plus tolérants que les porte-greffes diploïdes respectifs (Saleh *et al* 2008). De même, cette tolérance semble transmise au greffon lorsque les plants autotétraploïdes sont utilisés comme porte-greffe. Une autre série d'expérimentations a mis en évidence une faible différence de tolérance entre diploïdes et autotétraploïdes lorsque l'on s'affranchit de la composante hydrique du stress salin (Mouhaya *et al* 2010). Il semble donc probable que la composante hydrique du stress salin soit la composante prépondérante pouvant expliquer la tolérance accrue des porte-greffes autotétraploïdes.

8.1.1.3 Hypothèse ABA et son implication

En situation de contrainte hydrique, la régulation de l'ouverture des stomates est principalement contrôlée par la synthèse d'acide abscissique (ABA) au niveau racinaire. Cette hormone est alors véhiculée dans le flux de sève jusqu'aux stomates dont elle induit la fermeture. En l'absence de stress, nous avons montré que les porte-greffes tétraploïdes synthétisent constitutivement au niveau racinaire davantage d'ABA que les diploïdes. Les études d'expression des génomes diploïdes et tétraploïdes au niveau des racines au moyen des puces à ADN et par PCR en temps réel confirment ces résultats. La production accrue d'ABA serait à l'origine de la diminution des échanges gazeux (eau transpirée et CO_2 absorbé) et pourrait ainsi expliquer la plus grande tolérance des plants tétraploïdes vis-à-vis d'un stress hydrique. Cette capacité constitutive du porte-greffe tétraploïde apporterait, à l'association porte-greffe / greffon, une meilleure résistance au manque d'eau. D'autre part, elle serait à l'origine de la moindre croissance de ces plantes et de leur plus grande tolérance au stress salin.

8.1.1.4 Perspectives pour l'agrumiculture

En Afrique du Nord, mais également dans de nombreux autres pays du Sud, un pourcentage important des ressources en eau est utilisé pour l'agriculture. L'utilisation de porte-greffes tétraploïdes pourrait permettre de limiter l'irrigation et ainsi préserver les ressources en eau. De manière à orienter efficacement les schémas de création variétale il est nécessaire de pouvoir sélectionner des porte-greffes plus tolérants aux contraintes environnementales. Pour cela, il est impératif d'identifier les déterminants physiologiques et moléculaires pouvant être à l'origine de la tolérance accrue à telle ou telle contrainte. La

première partie de cette thèse est donc consacrée à la caractérisation physiologique de la tolérance au stress salin de porte-greffe utilisés classiquement mais également de génotypes représentatifs de la diversité des agrumes.

Afin d'optimiser les résultats déjà obtenus sur les porte-greffes tétraploïdes, le CIRAD cherche à développer de nouveaux hybrides tétraploïdes associant tolérance aux stress abiotiques et résistances aux maladies. Toutefois, dans la mesure où les porte-greffes autotétraploïdes d'agrumes seraient à l'origine d'un accroissement de tolérances aux contraintes environnementales, il était nécessaire d'évaluer l'impact de l'utilisation de tels génotypes sur la production, mais également la présence de génotypes zygotiques dans un verger d'agrumes sur les rendements et la qualité des fruits. La seconde partie de cette thèse est donc consacrée à une réanalyse d'un ancien essai porte-greffe évalué en sélection en tenant compte de la présence de porte-greffes zygotiques et autotétraploïdes.

8.2 Objectifs

Dans le cadre des travaux déjà initiés sur la tolérance au stress salin chez les agrumes à San Giuliano et à l'IVIA par l'INRA et le CIRAD, il a été proposé au cours de cette thèse de 1) caractériser au niveau physiologique le comportement de tolérances au stress salin de génotypes représentatifs du complexe d'espèces des agrumes. 2) caractériser quel pouvait être l'impact de l'utilisation de porte-greffes tétraploides et zygotiques sur les rendements et la qualité de clémentines.

Le premier objectif de ce travail a eu pour but de mettre en évidence des mécanismes physiologiques pouvant être spécifiques chez des génotypes d'agrumes représentatifs de la diversité génétique du genre *Citrus* et pouvant expliquer des comportments de sensibilité/tolérance au stress salin. Ainsi, 22 génotypes différents d'agrumes appartenant à une large diversité chez les genres *Citrus, Poncirus et Fortunela* ont été étudiés. Dans un premier article nous avons cherché à caractériser la réponse physiologique au travers de différents paramètres physiologiques à partir d'un nombre limité i.e. 12 de génotypes aux comportements contrastés exposés à un stress salin modéré (chapitre V, article n ° 1). Dans un second temps, nous avons cherché à réexplorer la diversité des agrumes en fonction de la réponse physiologique vis-à-vis du stress salin de l'ensemble des génotypes étudiés afin de développer de nouvelles pistes de recherches concernant les sources de tolérance (chapitre V,article n° 2).

Afin de décortiquer les mécanismes génétiques à l'origine de la tolérance au stress salin chez les agumes, nous avons finalisé la réalisation d'une descendance F2 issue d'une

autofécondation d'un parent F1 issu du croisement entre le mandarinier Cléopatre (*Citrus reshni Hort. ex Tan*) et le Poncirus (*Poncirus trifoliata*. (L.) Raf.). Les Poncirus sont des génotypes très sensibles au stress salin et aux sols calcaires mais résistants au virus de la tristeza et aux nématodes alors que le mandarinier Cléopâtre est tolérant au sel mais sensible au Phytophtora. A partir de cette population F2 en ségrégation, une carte génétique du génome de l'hybride F1 a été réalisée (chapitre V).Il sera donc possible dans le futur d'analyser lors d'un stress salin les propriétés de tolérance/sensibilité de chacun des génotypes de cette population F2 et donc de réaliser des anlayses QTLs permettant l'identification de gènes impliqués dans la tolérance au stress salin.

Les travaux que nous avons réalisés montrent que des porte-greffes tétraploides permettent d'augmenter les propriétés de tolérance aux stress salin et hydrique chez les agrumes. Toutefois, l'impact de l'utilisation de tels porte-greffes sur la productivité et la qualité des fruits n'a, pour l'instant, pas encore été documenté dans la littérature. Le deuxième objectif de cette thèse a donc été d'étudier quels sont les effets de l'utilisation de génotypes de *Poncirus trifoliata* tétraploïdes comme porte-greffes sur les rendements et sur la qualité des clémentines. Pour répondre à cette question nous avons réanalysé un essai porte-greffe planté il y a près de 40 ans. La production et la qualité des fruits de 36 *Poncirus trifoliata* ont été réanalysées. Par cytométrie en flux et en utilisant des marqueurs moléculaires, nous avons identifié les génotypes de porte-greffes non génétiquement conformes (génotypes zygotiques ou autotétraploïdes). Nous avons caractérisé l'impact de la présence de génotypes zygotiques et de génotypes autotétraploides spontanés sur la production et la qualité des clémentines (chapitre V, article n°3). De même, nous avons comparé les rendements de fruits mais également la qualité (composition en sucres, acides, caroténoïdes, composés phénoliques etc…) de deux génotypes porte-greffes autotétraploides de Poncirus et leurs génotypes diploïdes respectifs. Nous avons analysé la physiologie du scion (clémentinier) greffé sur ces deux types de porte-greffes (photosynthèse, conductence stomatique, composition en sucres solubles et contenus en azote des feuilles) de manière à vérifier s'il était possible d'expliquer les rendements en fruits, mais également les critères de qualité des fruits obtenus avec le fonctionnement de l'arbre (chapitre V, article n°4).

Le manuscrit de thèse est composé de 7 chapitres. Les deux premiers chapitres sont consacrés à une synthèse bibliographique. Le troisième est conscré aux objectifs et le quatrième chapitre rend compte brièvement des matériels et méthodes utilisés. Le cinquième chapitre est dédié aux resultats qui sont présentés sous forme d'articles en anglais. Le sixième

chapitre est consacré aux conclusions et aux perspectives. Enfin, le dernier chapitre est dédié aux réferances bibliographiques.

8.3 Travaux réalisés

8.3.1 Chapitres V : Caractérisation de la diversité de comportement physiologiques d'un panel de génotypes d'agrumes.

8.3.1.1 Introduction

L'analyse du comportement physiologique d'un panel de variétés et d'espèces du genre *Citrus* soumis à un stress salin a pour objectif de rechercher de nouvelles sources de tolérance mais également de détailler des mécanismes de tolérance qui pourraient être spécifiques d'une espèce. On connait de part la littérature le comportement de plusieurs variétés et plus particulièrement celles utilisées comme porte-greffe dans la mesure où la principale contrainte due à un stress salin réside dans la remontée des sels dans les sols. Une des réponses principales des plantes pour pallier cette contrainte se caractériserait par la capacité des plantes à exclure les ions chlorure et sodium au niveau des racines. Le mandarinier Cléopâtre (*C. reshni* Hort. Ex Tan.) ou la lime Rangpur (*C. limonia* Osb.) sont connus pour être tolérants au stress salin tandis que le Poncirus trifoliata est connu pour y être très sensible. L'obtention d'hybrides par croisements entre génotypes sensibles et résistants est la voie la plus utilisée pour améliorer les porte-greffes en combinant différents caractères de résistances biotiques et abiotiques. Pour accroître les possibilités d'amélioration par croisement, il est nécessaire d'étudier un large éventail de variétés et d'espèces pour diversifier les mécanismes de tolérance et éventuellement accroître les gains génétiques dans les schémas d'amélioration. Pour cela il n'est pas nécessaire de se cantonner aux seuls agrumes susceptibles d'être utilisés comme porte-greffe, c'est-à-dire des génotypes produisant des graines polyembryonnées. Les espèces monoembryonnées sont également importantes à étudier puisque l'hybridation est la voie d'amélioration choisie et que l'on espère récupérer par cette voie là le caractère de multiplication apomictique.

8.3.1.2 Matériels et Méthodes

L'échantillonnage variétal retenu permet d'avoir une représentation des grandes espèces à la base de la diversité génétique des agrumes en supposant que les mécanismes de tolérance acquis au cours de l'évolution sont partagés par plusieurs variétés d'une même espèce et que les spécificités devraient être observées chez les grands taxons ou dans leurs descendances.

Les trois taxons de base, portant la majeure partie de la diversité génétique du genre *Citrus*, pamplemoussier, mandarinier et cédratier, ont été sur-représentés par rapport aux espèces secondaires. Au total 8 espèces étaient représentées.

Notre étude de diversité génétique s'est déroulée dans des conditions contrôlées sur des arbres en pots ayant acquis un développement suffisant (de 12 à 24 mois) et cultivés sous serre. Le principe de l'étude réside dans l'application d'un stress modéré (NaCl, 75mM), stress comparable aux situations de culture dans de nombreux pays du pourtour méditerranéen, sur des arbres de chaque variété et de comparer leur comportement physiologique à celui d'arbres non soumis au stress (témoins). Pour valider les observations, un nombre d'arbres suffisant par variété a été nécessaire pour les traitements statistiques. Les observations réalisées sont de deux natures : l'une concernant les effets visuels et l'apparition de symptômes foliaires (croissance, brûlures, nécroses, chutes foliaires) et l'autre concernant des paramètres affectant la photosynthèse la conductance stomatique et l'accumulation de métabolites secondaires (polyphénols).

8.3.1.3 Principaux résultats obtenus

Les paramètres observés des arbres témoins (non soumis au stress salin) montrent au sein des espèces l'existence d'une grande disparité de comportements physiologiques. La vitesse de croissance ou la photosynthèse des arbres pourtant dans des conditions supposées homogènes, ne sont pas identiques pour chaque variété. Par contre au sein d'une même variété tous les arbres ont le même comportement ce qui prouve que les différences sont bien d'ordre génétique et non provoquées par une possible variation de conditions de culture. On peut donc pour chaque paramètre attribuer une valeur moyenne à chaque variété. Les pamplemoussiers ont la meilleure efficience photosynthétique et le plus fort taux de polyphénols alors que le cédratier Main de Bouddha, les pomélos, le mandarinier Willow Leaf et le citrange Carrizo ont la plus forte croissance et des taux de sodium foliaires les plus élevés. Ce résultat conforte l'idée que la comparaison des comportements sous stress doit avant tout se faire par rapport aux arbres témoins et qu'ensuite ces valeurs relatives pourront être utilisées pour comparer les variétés entre elles. Concernant les plants soumis au stress salin les réponses des arbres de chaque variété peuvent être plus hétérogènes sans toutefois remettre en cause un comportement variétal spécifique. Dans l'analyse de la diversité de comportement relatif aux témoins par une approche d'analyse en composante principale (ACP) deux entités distinctes apparaissent distinctement: celle correspondant à des variétés ou espèces ayant accumulé des ions chlorure et sodium à fortes doses (au niveau des feuilles) de

même que des composés phénoliques ; dans ce groupe on trouve tous les cédratiers, le citrange Carrizo et les limettiers Mexicain et Brazil Sweet. On peut aussi noter que le cédratier Main de Bouddha et le limettier Mexicain ont une conductance stomatique élevée et une croissance réduite. Dans le deuxième groupe on trouve les citronniers, les mandariniers, les pamplemoussiers, le combava, la lime Rangpur, le bigaradier et les pomelos. Ce groupe se singularise essentiellement par une moindre accumulation des ions mais aussi par une activité photosynthétique plus importante que les variétés du premier groupe. La réduction de croissance mesurée par l'augmentation du diamètre de la tige au niveau du collet par rapport aux plants témoins ne semble pas être un bon indicateur de la différenciation entre les deux groupes. Sur le plan génétique on peut aussi conclure que la bipartition reflète le degré d'apparentement de certaines espèces secondaires. Par exemple le bigaradier a un comportement assez proche de ses deux parents supposés (mandariniers et pamplemoussiers). Le citronnier probablement issu d'un croisement à l'origine entre un bigaradier et un cédratier aurait hérité du comportement du bigaradier tandis que les limettiers Mexicain et Brazil Sweet auraient hérité du cédratier reconnu comme leur parent paternel. Le limettier Rangpur, contrairement aux autres limettiers, présente un apparentement avec les mandariniers, par conséquent son comportement peut être assimilé à celui des variétés du second groupe.

Si l'on croise cette dispersion variétale avec les symptômes observés on s'aperçoit que le premier groupe, à l'exception du limettier Brazil Sweet, présente des nécroses foliaires, des chutes de feuilles et pour les cédratiers un desséchement des rameaux conduisant à la mortalité. Le deuxième groupe variétal se singularise par une absence de symptômes, observés chez les arbres du premier groupe. Cependant on note chez les pomelos un comportement singulier qui s'est manifesté par une chute totale des feuilles au bout de deux mois de stress, suivie par la formation de nouvelles feuilles. Il est possible que ce comportement soit un mécanisme de défense que l'on peut qualifier d'résilience et qui consiste à se débarrasser des feuilles ayant accumulé des ions toxiques pour les arbres et à fabriquer de nouvelles feuilles pour maintenir la photosynthèse et la croissance des plants.

En conclusion, nous pouvons proposer que la recherche de nouvelles sources de tolérance soit orientée sur la prospection des groupes mandariniers et pamplemoussiers qui renferment la plus forte diversité génétique. Par ailleurs, le comportement des pomelos probablement hérité des pamplemoussiers (bien que nous n'ayons pas observé de telles manifestations chez ces derniers) est une nouvelle forme de tolérance originale pour la première fois observée chez les agrumes.

8.3.2 Chapitre V : Réalisation d'une carte génétique d'un hybride inter-générique (F1) (mandarinier Cléopâtre x *Poncirus trifoliata*).

8.3.2.1 Introduction

Une des voies d'étude des déterminismes génétiques des caractères phénotypiques est la réalisation de cartes génétiques. Cela consiste à déterminer les positions relatives des loci (gènes ou séquences d'ADN) sur un chromosome. L'ordonnancement et la distance séparant les *loci* sont estimés à partir des fréquences de recombinaison survenant à la méiose. Pour cela il faut disposer d'une population en ségrégation, c'est à dire une descendance issue du croisement entre deux géniteurs. Le balisage par des marqueurs génétiques des chromosomes appelés en cartographie génétique « groupes de liaison » permet ensuite de positionner des gènes supposés intervenir dans l'expression du caractère étudié. De même, par l'étude de la ségrégation du caractère phénotypique dans cette même descendance il est possible de repérer les zones chromosomiques contenant les gènes impliqués dans l'expression de ce caractère. Si le caractère est une valeur quantitative les zones chromosomiques sont désignées par le vocable « QTL » pour Quantitative Trait Loci. Nous pouvons ainsi dénombrer, positionner mais aussi mesurer l'effet de ces QTL (positif ou négatif) sur le caractère exprimé. On peut ensuite décider à partir de la matrice de balisage (carte génétique) d'essayer d'identifier les gènes sous jaccents contenus dans les QTL par une série d'approche techniques mettant en jeu du clonage de grands fragments d'ADN du séquençage et de la vérification par transformation. Une autre possibilité d'exploitation des cartes génétiques et l'utilisation des marqueurs les plus proches des QTL pour prédire leur présence chez les hybrides de généalogies établies dans un schéma de sélection récurrente. Cette approche permet de mieux contrôler la sélection et aussi d'accélérer l'obention d'hybrides renfermant les caractères souhaités. Dans le cadre de l'étude génétique de la tolérance au stress salin nous avons entrepris l'étude des déterminants génétiques par la réalisation d'une carte génétique. Pour cela nous avons réalisé une autofécondation d'un hybride F1 (mandarinier Cléopatre x Poncirus trifoliata) afin de générer une population F2 nécessaire pour les études de ségrégation. Le choix de cet hybride a été gouverné par la tolérance au stress salin du mandarinier et la sensibilité du Poncirus garantissant la ségrégation du caractère de tolérance dans la descendance. Par ailleurs, les équipes CIRAD et INRA de la station de recherche de San Giuliano ont œuvré pour l'obtention de plusieurs centaines de marqueurs génétiques de séquences microsatellites (SSR) utiles pour le balisage des chromosomes.

8.3.2.2 Matériels et Méthodes

Au moyen de quatre marqueurs SSR, il a été verifié que les individus de la population F2 obtenus à partir de l'hybride F1 mandarinier Cléopâtre x *Poncirus trifoliata*, étaient bien zygotiques. En effet cet hybride produit des graines polyembryonnées suceptibles de produire après semis des plants nucellaires identiques au parent. Au total près de 1500 plants ont été analysés sur gel d'acrylamide. Quatre-vingt-dix neuf génotypes ont été retenus et 135 marqueurs SSRs ont été utilisés pour réaliser la carte génétique de l'hybride F1. Enfin quatre gènes candidats, connus pour être impliqués dans la tolérance au stress salin et présentant des « indels » permettant de différencier les allèles respectivement hérités des parents Poncirus et mandarinier cléopâtre, ont été cartographiés.

8.3.2.3 Principaux résultats obtenus

Seuls les hybrides zygotiques nous sont utiles. Parmi les 110 hybrides initialement sélectionnés seulement 60 se sont révélés effectivement zygotiques. La moitié des 60 hybrides présentait un défaut d'homozygotie c'est-à-dire qu'ils ne portaient pas un pourcentage équilibré de loci hétérozygotes et homozygotes comme suggéré par la théorie de ségrégation mendélienne. Dans les cas extrêmes, ce défaut se matérialisait par un pourcentage de locus homozygote minimum (1 seul pour 20 loci hétérozygotes). Les autres hybrides présentaient une proportion de loci homozygotes et hétérozygote qui suivait une distribution normale. Ce résultat aurait pu suggèrer des dysfonctionnements de la méiose chez l'hybride inter-générique ou des problèmes de viabilité pour certaines combinaisons allèliques chez les hybrides F2.

Une vérification par une nouvelle série d'empreintes génétiques a été faite sur les individus présentant une anomalie de constitution allèlique (défaut de locus homozygotes) en renouvelant toutes les amplifications et les analyses par électrophorèse pour les marqueurs incriminés dans la divergence. Les résultats premiers ont été infirmés pour tous les individus et sur tous les locus étudiés. Ces individus s'avèreraient être des clones du parent F1 (origine nucellaire) car ne présentaient aucun locus homozygote. Ce résultat a eu deux conséquences fondamentales sur la poursuite expérimentale : la première, est que les conditions de PCR par « Tailing M13 » peuvent sur certains marqueurs conduire à des amplifications aberrantes et notamment un défaut d'amplification d'un allèle. Par conséquent, il est nécessaire à l'avenir de doubler toutes les amplifications sur les génotypes parentaux en changeant la méthode d'amplification des amplicons (par électrophorèse classique sur gel de poly-acrylamide et sur capillaire de séquenceur automatique) afin de vérifier la stabilité des empreintes. La deuxième

conséquence concerne la taille de la population F2 qui, de fait, se trouve réduite à une trentaine d'individus (totalement inappropriée à l'établissement d'une carte génétique). Par conséquent, une deuxième série d'autofécondations de l'hybride F1 a été réalisée afin d'augmenter l'effectif de la population. Après récolte des fruits, les graines isolées ont été mises à germer dans des caissettes quadrillées pour mieux repérer les plantules. Environ 700 plantules ont germé et une analyse génétique a été réalisée à l'aide de 4 marqueurs microsatellites afin de repérer les individus zygotiques et éliminer les individus issus du développement des embryons nucellaires. Soixante neuf plantules hybrides supplémentaires ont été sélectionnées et ont ensuite été génotypées à l'aide de 135 marqueurs de séquences microsatellites choisis pour leur statut d'hétérozygotie chez l'hybride F1. Par ailleurs 4 marqueurs de gènes supposés intervenir dans les réactions cellulaires et physiologiques en situation de stress salin ont également été utilisés pour être cartographiés. L'analyse de ségrégation nous a conduit à l'obtention de 15 groupes de liaisons. Cinquante-quatre marqueurs présentent une ségrégation mendélienne normale et 85 ne suivent pas la loi de distribution normale (distortion de ségrégation). Ces distortions se manifestent par un excès de fréquence d'un allèle par rapport à l'autre ou d'un déséquilibre de la fréquence homozygote / hétérozygote. Le pourcentage élevé de marqueurs à distor de ségrégation (57%) pourrait survenir d'un défaut de complémentarité entre chromosomes hérités du mandarinier et du poncirus et conduisant à d'éventuels dysfonctionnement d'appariement lors de la méiose. De telles situations sont fréquemment rencontrées dans la littérature où les parents des généalogies sont génétiquement éloignés. Par ailleurs nous avons comparé la carte génétique de l'hybride F1 que nous avons obtenue avec la carte génétique de référence internationale du clémentinier. Trente cinq marqueurs sont communs aux deux cartes génétiques et pour la plupart d'entre eux la colinéarité est conservée. Néanmoins, quelques inversions d'ordre sont aussi observées dont l'explication peut soit découler des effets de distortion de ségrégation sur les traitements statistiques, soit de modifications chromosomiques au sein du génome de l'hybride intergénérique F1.

En conclusion, cette carte génétique est un premier élément de travail qui servira à l'analyse des QTL de la tolérance au stress salin chez les agrumes. Hormis l'addition de nouveaux marqueurs pour densifier le balisage, elle devra dans l'avenir être aussi complétée par une expérimentation sur la population F2 pour évaluer la réponse physiologique des arbres à un traitement stress salin.

8.3.3 Chapitre V : Etude d'un essai agronomique. Impact de la présence de porte-greffes zygotiques ou autotétraploïdes spontanés sur le rendement et la qualité des fruits

8.3.3.1 Introduction

Tous les porte-greffes d'agrumes commerciaux sont polyembryonnés et sont multipliés au moyen de semences polyembryonnées permettant, la plupart du temps, la propagation conforme du matériel végétal. Toutefois, compte tenu du caractère partiel de la reproduction apomictique (présence de l'embryon zygotique) et de la faculté des embryons somatiques à doubler leur stock chromosomique, des plants zygotiques ou polyploïdes peuvent, comme on l'a vu dans le chapitre précédant, survenir dans les semis de porte-greffes. L'objectif de notre étude était de comprendre comment la présence des porte-greffes zygotiques ou tétraploïdes peuvent affecter un essai de sélection de porte-greffes. Pour cela, un essai porte-greffe de génotypes de Poncirus greffés avec une variété de clémentinier plantés en 1974 a été ré-analysé en tenant compte de la présence des porte-greffes non conformes.

8.3.3.2 Matériels et Méthodes

Des plants appartenant à 32 porte-greffe de Poncirus (*Poncirus trifoliata* (L.) Raf.) ont été sélectionnés au sein de semis et ont été ensuite greffés avec de la clémentine commune (*Citrus reticulata* Blanco x *Citrus sinensis* (L.) Osb.). Les arbres ont été plantés dans un essai randomisé (neuf blocs, neuf répétitions) à la station de recherche INRA-CIRAD en Corse, en France en 1974. Les fruits ont été récoltés et les rendements ont étés analysés de 1979 à 1990. La qualité des fruits de la clémentine a été étudiée pendant 5 années consécutives de 1981 à 1985 (pourcentage et densité des jus, sucrosité, acidité titrable, index de maturité).

Dans le cadre de cette thèse, les porte-greffes tétraploïdes et zygotiques de l'essai ont été identifiés par cytométrie en flux et en utilisant des marqueurs de séquences microsatellites (SSR) en utilisant des prélèvements d'écorce ou des feuilles de rejets de porte-greffes pour en extraire l'ADN ou les cellules. L'ensemble des données obtenues dans les années passées a été analysé de nouveau à la lumière de la présence de ces génotypes non conformes au sein de l'essai porte-greffe.

8.3.3.3 Principaux résultats obtenus

Au moyen de la cytométrie en flux, 2,4% des porte-greffes parmi les 288 arbres se sont avérés être tétraploïdes. De même, à l'aide de marqueurs SSR, 6,6% des porte-greffes de

l’essai ont pu être identifiés comme étant zygotiques. Les données sur les rendements ont montré que les porte-greffes tétraploïdes spontanés réduisaient considérablement les productions (environ 45%) de clémentine. Les porte-greffes zygotiques n'ont pas affecté la production moyenne de fruits dans la mesure où leur présence a pu induire des augmentations mais également des diminutions de production. Les résultats concernant la sélection des porte-greffes, en supprimant les génotypes non conformes (zygotiques et autotétraploïdes), ne sont pas conformes à ceux obtenus précédemment par les sélectionneurs. En fait le meilleur porte-greffe est un clone précédemment qualifié de moyennement productif parmi les 32 porte-greffes évalués. L'analyse de la qualité des fruits réalisée pendant les premières années, indépendamment de la présence des porte-greffes zygotiques n'a pas révélé de changements significatifs. Nos résultats suggèrent donc que la présence de porte-greffes tétraploïdes spontanés a un impact très fort sur la production de fruits dans les vergers et l’élimination préalable de ces plants avant toute plantation d’un essai agronomique apparaît comme étant absolument nécessaire.

8.3.4 Chapitre V : Etude d’un essai agronomique : analyse de l’effet de la polyploïdisation des porte-greffes sur le rendement et la qualité des clémentines

8.3.4.1 Introduction

Dans le cadre des travaux réalisés par le CIRAD, il a été montré que les porte-greffes tétraploïdes ont une physiologie très différentes de leur diploïdes respectifs. En effet, ces porte-greffes polyploïdes permettraient d’accroître de façon très significative la tolérance des associations porte-greffe/greffon à la sécheresse. Pour confirmer l’intérêt de tels génotypes il était nécessaire de vérifier dans quelle mesure la qualité et les rendements des productions fruitières pouvaient être affectés. Dans le cadre de l’essai agronomique présenté dans le chapitre VI, l’impact de l’utilisation de deux autres génotypes porte-greffes tétraploides mais également leurs diploïdes respectifs a été étudié.

8.3.4.2 Matériels et Méthodes

Il a été vérifié au moyen des outils de cytométrie en flux au moyen de 5 marqueurs moléculaires, la conformité génétique des deux couples diploïdes et tétraploïdes de porte-greffe de Poncirus (*Poncirus trifoliata* (L.) Raf.). Les rendements obtenus pour les deux couples de porte-greffes ont été analysés de 1979 à 1990. La qualité des clémentines pour

chacun des génotypes de porte-greffe a été étudiée pendant 5 années consécutives de 1981 à 1985 (pourcentage et densité des jus, solides solubles, acidité titrable, index de maturité). Une analyse fine des contenus en sucres et acides organiques, caroténoïdes et hespéridines dans les clémentines a été réanalysée sur la récolte 2008/2009. En parallèle, pour compléter cette étude, une analyse physiologique des arbres au champ (contenus en chlorophylle et flavonoïdes et azote des feuilles, mais également taux de croissance des arbres, conductance stomatique et photosynthèse) a été initiée.

8.3.4.3 Principaux résultats obtenus

Il a pu être vérifié que les neuf arbres pour les deux génotypes autotétraploïdes étaient bien autotétraploïdes. Parmi les 34 génotypes diploïdes de l'essai, seulement deux génotypes ont présenté des profils de migration éléctrophorétique identiques à l'un ou l'autre des génotypes tétraploïdes. Il a été également vérifié qu'aucun tétraploïde spontané ou zygotique n'était présent parmi les génotypes putativement diploïdes.

L'analyse des rendements a montré que les porte-greffes tétraploïdes réduisaient considérablement les productions de clémentines par rapport à l'utilisation des porte-greffes diploïdes respectifs. Une analyse fine de la qualité des fruits n'a pas permis de mettre en évidence de différence significative entre fruits ayant poussé sur les associations porte-greffe diploïde/clémentinier et porte-greffe tétraploïde/clémentinier.

Des mesures de croissance des arbres ont permis de vérifier que l'utilisation de génotypes tétraploïdes induisait une diminution de croissance des arbres. De même, des mesures de flux de transpiration ont montré que le clémentinier greffé sur porte-greffe autotétraploïde présentait des conductances stomatiques plus faibles mais également une photosynthèse moindre dans le cas des clémentiniers greffés sur porte-greffes tétraploïdes par rapport à l'utilisation de porte-greffes diploïdes. Toutefois, le flux de transport d'électrons en condition de lumière saturante s'est révélé être supérieur dans les feuilles de clémentinier greffé sur porte-greffe tétraploïde suggérant que les porte-greffes tétraploïdes conféreraient une meilleure capacité adaptative de la photosynthèse en fonction des conditions environnementales.

Annexe

Date used for Diversity analysis with the help of PCA (principal component analysis) (Control condition)

Control	Chloro-phyll	Phenolic content	Fv/Fm	Gs	A (net)	Fv'/Fm'	Cl^-	Na^+	Diameter T0	Diameter T 80
CiE1	54.05	0.95	0.85	82.50	10.12	0.25	1.46	0.24	9.79	14.40
CiE2	54.90	1.04	0.85	78.04	9.97	0.25	1.46	0.24	9.79	14.40
CiE3	53.20	0.85	0.85	72.19	9.94	0.25	1.46	0.24	9.79	14.40
mean	54.05	0.95	0.85	77.58	10.01	0.25	1.46	0.24	9.79	14.40
SoA1	69.10	0.85	0.85	44.45	6.63	0.17	2.05	0.08	12.11	15.50
SoA2	65.95	0.84	0.85	44.90	6.76	0.17	2.05	0.08	9.78	14.61
SoA3	72.00	0.84	0.85	44.61	6.68	0.18	2.05	0.08	8.64	13.30
mean	69.02	0.84	0.85	44.65	6.69	0.17	2.05	0.08	10.18	14.47
MaW1	73.15	0.86	0.84	73.56	11.79	0.25	1.47	0.65	6.14	10.40
MaW2	64.85	0.79	0.83	71.45	11.64	0.24	1.47	0.65	6.58	8.20
MaW3	69.10	0.89	0.84	75.78	11.63	0.25	1.47	0.65	7.48	11.21
mean	69.03	0.84	0.84	73.60	11.69	0.25	1.47	0.65	6.73	9.94
CoK1	67.70	0.52	0.82	59.19	7.66	0.17	2.14	0.14	10.04	12.07
CoK2	69.30	0.34	0.79	53.09	6.09	0.15	2.14	0.14	6.68	8.86
CoK3	70.55	0.40	0.84	53.20	7.78	0.16	2.14	0.14	7.11	11.04
mean	69.18	0.42	0.81	55.16	7.18	0.16	2.14	0.14	7.94	10.66
MaC1	65.05	0.57	0.81	56.48	10.43	0.18	1.44	0.22	5.13	7.85
MaC2	54.60	0.50	0.81	57.54	9.52	0.17	1.44	0.22	5.35	7.07
MaC3	54.90	0.62	0.84	54.17	10.31	0.18	1.44	0.22	4.43	7.61
mean	58.18	0.56	0.82	56.06	10.09	0.18	1.44	0.22	4.97	7.51
LeE1	61.95	0.70	0.82	38.52	7.92	0.18	1.28	0.20	12.19	16.00
LeE2	59.60	0.86	0.82	35.58	6.12	0.19	1.28	0.20	11.40	14.92
LeE3	60.78	0.78	0.82	41.14	8.87	0.18	1.28	0.20	11.80	15.46
mean	60.78	0.78	0.82	38.41	7.64	0.19	1.28	0.20	11.80	15.46
CaC1	73.60	0.69	0.81	80.97	13.17	0.25	1.91	0.27	4.35	8.55
CaC2	71.30	0.70	0.84	81.42	13.38	0.25	1.91	0.27	4.94	8.90
CaC3	71.75	0.67	0.80	80.81	13.22	0.25	1.91	0.27	4.35	8.48
mean	72.22	0.69	0.82	81.07	13.26	0.25	1.91	0.27	4.55	8.64
CiB1	61.10	0.60	0.84	119.71	11.46	0.24	1.83	0.16	6.88	18.89
CiB2	65.85	0.56	0.85	121.40	11.51	0.24	1.83	0.16	6.30	13.75
CiB3	63.48	0.58	0.85	118.01	11.41	0.24	1.83	0.16	6.59	16.32
mean	63.48	0.58	0.85	119.71	11.46	0.24	1.83	0.16	6.59	16.32
CiD1	65.15	0.57	0.84	98.18	12.28	0.23	1.17	0.08	11.50	17.42
CiD2	71.70	0.78	0.83	100.94	12.45	0.23	1.17	0.08	9.23	13.25
CiD3	68.43	0.67	0.84	99.56	12.36	0.23	1.17	0.08	10.37	15.34
mean	68.43	0.67	0.84	99.56	12.36	0.23	1.17	0.08	10.37	15.34
CiP1	55.55	0.79	0.80	128.98	14.32	0.25	1.40	0.18	8.43	12.42
CiP2	52.90	0.76	0.80	133.42	14.71	0.25	1.40	0.18	8.43	12.42
CiP3	58.20	0.82	0.80	124.55	13.94	0.25	1.40	0.18	8.43	12.42
mean	55.55	0.79	0.80	128.98	14.32	0.25	1.40	0.18	8.43	12.42
GrS1	68.35	0.90	0.83	78.00	9.51	0.18	1.40	0.60	7.61	13.88
GrS2	74.20	0.80	0.84	79.48	9.35	0.18	1.40	0.60	9.88	17.00
GrS3	67.85	0.71	0.83	77.42	8.81	0.18	1.40	0.60	8.84	14.81
mean	70.13	0.80	0.83	78.30	9.23	0.18	1.40	0.60	8.78	15.23
GrD1	73.25	0.61	0.84	89.78	9.76	0.23	0.94	0.35	7.77	16.48
GrD2	69.70	0.70	0.84	89.78	9.76	0.23	0.94	0.35	6.33	14.77
GrD3	79.65	0.64	0.84	89.78	9.76	0.23	0.94	0.35	9.29	16.52
mean	74.20	0.65	0.84	89.78	9.76	0.23	0.94	0.35	7.80	15.92
LeL1	61.25	0.87	0.83	60.27	11.56	0.23	1.18	0.25	9.53	16.07
LeL2	59.10	0.82	0.84	60.93	11.63	0.23	1.18	0.25	7.54	13.93
LeL3	60.18	0.84	0.83	59.57	11.30	0.22	1.18	0.25	8.54	15.00
mean	60.18	0.84	0.83	60.25	11.50	0.23	1.18	0.25	8.54	15.00
MaS1	63.20	0.57	0.84	51.14	9.41	0.18	1.78	0.14	4.65	8.76
MaS2	58.65	0.67	0.84	50.52	9.87	0.19	1.78	0.14	8.44	12.68
MaS3	60.93	0.62	0.84	51.91	9.48	0.19	1.78	0.14	6.55	10.72
mean	60.93	0.62	0.84	51.19	9.58	0.19	1.78	0.14	6.55	10.72
LiB1	67.05	0.92	0.84	68.73	10.67	0.21	0.98	0.04	10.30	14.73
LiB2	71.65	0.90	0.83	67.87	10.61	0.21	0.98	0.04	11.51	15.62
LiB3	63.00	1.00	0.82	66.50	10.66	0.21	0.98	0.04	11.04	15.20
mean	67.23	0.94	0.83	67.70	10.65	0.21	0.98	0.04	10.95	15.18
LiR1	62.55	0.75	0.80	54.16	9.48	0.14	1.26	0.17	12.17	16.52
LiR2	57.90	0.87	0.80	53.65	9.31	0.14	1.26	0.17	9.34	14.43
LiR3	57.20	0.87	0.82	52.50	9.04	0.14	1.26	0.17	10.73	15.14
mean	59.22	0.83	0.81	53.44	9.28	0.14	1.26	0.17	10.75	15.36
LiM1	57.25	0.59	0.81	54.79	9.05	0.12	1.12	0.13	9.87	13.78
LiM2	56.20	0.57	0.80	52.37	8.93	0.13	1.12	0.13	9.87	13.78
LiM3	58.30	0.60	0.81	50.53	8.68	0.13	1.12	0.13	9.87	13.78
mean	57.25	0.59	0.81	52.56	8.89	0.13	1.12	0.13	9.87	13.78
PuT1	36.10	0.87	0.85	69.69	13.56	0.25	1.37	0.04	6.64	10.22
PuT2	46.00	1.01	0.83	69.69	13.56	0.25	1.37	0.04	6.93	10.22
PuT3	36.20	0.77	0.83	69.69	13.56	0.25	1.37	0.04	6.79	10.22
mean	39.43	0.88	0.84	69.69	13.56	0.25	1.37	0.04	6.79	10.22
PuE1	53.60	1.02	0.82	71.56	13.30	0.26	1.13	0.13	7.27	10.22
PuE2	49.20	0.80	0.84	71.56	13.30	0.26	1.13	0.13	5.35	10.22

PuE3	50.40	0.70	0.83	71.56	13.30	0.26	1.13	0.13	6.31	10.22
mean	51.07	0.84	0.83	71.56	13.30	0.26	1.13	0.13	6.31	10.22
PuK1	38.70	1.30	0.85	71.79	13.37	0.26	2.19	0.20	5.36	10.22
PuK2	37.80	0.88	0.82	71.58	13.62	0.26	2.19	0.20	5.36	10.22
PuK3	38.25	1.09	0.84	71.69	13.49	0.26	2.19	0.20	5.36	10.22
mean	38.25	1.09	0.84	71.69	13.49	0.26	2.19	0.20	5.36	10.22

Date used for Diversity analysis with the help of PCA (principal component analysis)(Stressed condition)

CiE4	28.60	1.15	0.54	32.15	4.63	0.15	106.41	16.63	11.26	11.26
CiE5	28.60	1.15	0.54	32.15	4.63	0.15	106.41	16.63	13.32	13.92
CiE6	28.60	1.15	0.54	32.15	4.63	0.15	106.41	16.63	8.65	9.45
SoA4	54.25	0.98	0.79	13.24	2.41	0.13	53.84	10.84	11.39	12.89
SoA5	53.05	1.05	0.82	1.80	0.33	0.10	34.60	7.79	9.79	13.25
SoA6	57.60	1.06	0.81	16.99	3.11	0.14	27.36	7.57	10.68	13.49
MaW5	63.40	0.73	0.82	17.33	4.60	0.12	12.88	6.82	7.86	8.88
MaW6	60.75	0.95	0.77	9.22	2.24	0.15	28.75	10.76	10.21	11.33
MaW7	71.70	0.79	0.82	2.28	1.21	0.12	20.87	9.15	7.69	9.46
CoK4	64.45	0.60	0.75	5.62	2.01	0.09	12.76	12.30	9.33	12.30
CoK5	61.75	0.68	0.72	3.37	0.81	0.09	33.22	9.90	9.17	10.72
CoK7	64.60	0.56	0.78	5.55	2.11	0.09	12.76	12.30	9.18	10.70
MaC7	59.55	1.07	0.83	17.72	4.81	0.12	8.02	5.66	5.09	6.30
MaC8	53.45	1.07	0.81	13.42	3.73	0.12	20.22	7.84	6.56	7.63
MaC9	55.50	1.09	0.82	17.40	4.24	0.13	8.02	3.97	5.72	6.50
LeE5	51.05	1.14	0.82	3.81	1.81	0.07	20.87	7.41	4.50	7.19
LeE6	50.10	0.94	0.79	2.76	1.80	0.10	15.43	4.94	5.95	9.64
LeE7	54.05	1.03	0.81	4.14	1.89	0.08	25.34	10.67	5.22	7.88
CaC6	40.60	1.39	0.63	3.56	2.00	0.08	86.46	20.28	5.05	7.92
CaC7	31.40	1.09	0.57	3.56	2.00	0.08	48.75	13.05	4.22	6.47
CaC8	42.55	0.99	0.61	3.56	2.00	0.08	60.26	14.91	4.30	6.69
CiB4	34.90	1.10	0.79	7.23	2.34	0.07	62.48	10.27	8.22	10.85
CiB5	32.30	1.05	0.68	7.23	2.34	0.07	60.26	8.11	6.87	8.57
CiB6	27.90	1.08	0.72	4.62	2.31	0.07	44.74	7.71	8.86	11.80
CiD4	32.40	1.25	0.67	3.83	0.88	0.07	80.08	11.97	10.79	15.21
CiD5	37.65	1.12	0.68	2.66	1.24	0.06	135.76	22.17	9.87	12.97
CiD9	34.63	1.15	0.71	3.36	1.02	0.07	97.66	17.52	10.16	12.66
Cip4	17.50	1.20	0.76	13.78	2.51	0.11	143.96	26.27	11.60	13.05
Cip5	40.20	1.10	0.75	18.37	1.28	0.11	100.34	19.07	8.88	9.98
Cip6	17.85	1.15	0.82	30.57	4.10	0.17	83.02	16.06	9.82	12.41
GrS4	55.65	1.23	0.81	21.17	3.26	0.11	22.54	9.29	11.75	14.55
GrS5	53.30	1.11	0.81	21.22	3.22	0.11	22.54	4.57	10.44	14.53
GrS6	50.50	1.02	0.82	2.03	0.96	0.11	15.22	10.10	10.45	13.71
GrD6	46.70	1.04	0.77	3.54	1.47	0.09	23.68	11.89	11.72	15.94
GrD7	48.55	0.82	0.85	17.47	4.43	0.15	7.20	7.74	12.44	15.80
GrD8	46.25	0.91	0.79	3.53	1.58	0.09	5.15	7.61	13.38	17.19
LeL5	58.30	0.90	0.74	4.56	1.28	0.08	34.91	11.47	10.31	13.46
LeL6	58.60	1.10	0.77	7.81	2.61	0.10	20.04	5.88	10.91	14.02
LeL7	57.70	0.94	0.75	4.67	2.14	0.09	26.31	8.77	12.31	13.98
MaS4	50.05	0.80	0.81	8.12	2.66	0.12	13.47	4.96	7.09	7.58
MaS5	51.90	0.86	0.81	6.45	2.52	0.12	22.23	4.57	9.57	11.09
MaS6	50.65	0.90	0.78	8.19	2.97	0.11	22.54	7.14	8.19	10.21
LiB5	44.65	1.16	0.73	1.74	0.79	0.04	40.88	9.13	11.67	12.64
LiB6	44.20	1.07	0.73	8.11	1.37	0.05	83.77	12.88	11.38	12.36
LiB7	40.40	1.13	0.73	7.12	1.41	0.06	102.63	26.17	10.30	12.45
LiR4	51.35	1.24	0.79	15.82	4.17	0.13	21.74	4.11	9.33	11.54
LiR5	43.90	1.19	0.82	16.75	4.35	0.14	13.47	3.04	8.83	11.13
LiR6	45.45	1.27	0.73	12.63	3.78	0.12	29.95	7.97	11.95	12.36
LiM4	40.50	1.20	0.64	8.99	2.06	0.10	80.80	14.72	11.71	12.56
LiM5	32.50	1.17	0.67	13.38	2.80	0.12	73.17	16.21	12.57	13.43
LiM6	36.50	1.19	0.65	11.18	2.43	0.11	76.99	15.47	12.14	13.00
PuT4	57.65	0.93	0.84	13.63	4.84	0.16	13.78	5.62	7.27	10.46
PuT5	64.80	0.90	0.81	15.00	5.32	0.16	17.82	5.74	5.35	7.93
PuT6	61.23	0.91	0.83	14.32	5.08	0.16	15.80	5.68	6.31	9.20
PuE4	57.20	0.87	0.83	35.35	7.11	0.17	42.96	7.59	6.64	8.55
PuE5	42.00	0.89	0.80	36.32	8.50	0.17	26.04	7.92	6.93	8.07
PuE6	49.60	0.88	0.81	35.83	7.81	0.17	34.50	7.76	6.79	8.31
PuK4	53.50	0.84	0.84	10.52	4.08	0.12	28.11	6.84	5.36	7.22
PuK5	54.20	0.92	0.84	9.74	4.21	0.12	28.11	6.84	5.36	7.22
PuK6	53.50	0.84	0.84	9.12	4.38	0.12	28.11	6.84	5.36	7.22

RESUME EN FRANÇAIS

Titre : Caractérisation physiologique de génotypes d'agrume : études de tolérance au stress salin et impacts de la présence de porte-greffes zygotiques et autotétraploïdes»

Les agrumes sont classés parmi les arbres fruitiers les plus sensibles au stress salin. Cependant, une forte diversité existe pour ce caractère au sein des agrumes: *Poncirus trifoliata* (L.) Raf. est connu pour être très sensible alors que *Citrus reshni Hort.* ex Tan. (Mandarinier Cléopâtre) est l'un des génotypes les plus tolérants. La stratégie habituelle pour améliorer la résistance des porte-greffes est basée sur l'hybridation entre des parents ayant des caractères intéressants complémentaires. Une autre façon d'acquérir une tolérance au stress salin de porte-greffe est liée à la tétraploïdie par le doublement du nombre de chromosomes. L'analyse génétique et physiologique de la tolérance au stress salin de tout nouveau génotype est donc requise pour les programmes de sélection de variétés plus adaptées. Des études combinant des approches génétiques (cartographie du génome) ainsi que des approches physiologiques liées à la diversité du groupe des agrumes ont été réalisées afin d'être en mesure de corréler dans le futur les phénotypes de tolérance au stress salin observés avec l'expression des génomes respectifs.

Une population F2 résultant de la pollinisation d'un hybride F1 (*C. reshni* x *P. trifoliata*) a été réalisée. L'étude de la ségrégation de 135 marqueurs microsatellites et de 4 gènes candidats a permis l'établissant de 15 groupes de liaison. La majorité des marqueurs (57%) montrent une ségrégation non mendélienne sans doute due à un dysfonctionnement lors de l'appariement des chromosomes intergénériques lors de la méiose chez le parent hybride. La comparaison de la carte génétique obtenue avec la carte génétique de référence de clémentinier montre que la colinéarité des marqueurs a été respectée. De même, la tolérance au stress salin de vingt-deux génotypes représentant la diversité des agrumes a été étudiée. Les différents génotypes ont ensuite été soumis à un stress salin. Plusieurs paramètres physiologiques tels que le taux de croissance, la teneur en chlorophylle, la teneur totale en composés phénoliques, le rendement du transport d'électrons du PSII, la conductance stomatique ainsi que le taux de photosynthèse ont été mesurés. Différents comportements physiologiques de tolérance au stress salin en fonction des espèces d'agrumes étudiées ont été observés suggérant l'existence de différents mécanismes à l'origine de la tolérance au stress salin. Les cédratiers se sont révélés être les plus sensibles alors que tous les mandariniers et pamplemoussiers étaient tolérants. De nombreux génotypes ont présenté des symptômes de chlorose, des accumulations d'ions chlorure et sodium dans les feuilles et des changements des paramètres physiologiques. Les profils spécifiques de tolérance étaient quant à eux associés à un maintien de la photosynthèse même si de plus faibles valeurs de conductance stomatique ont pu être observées. Dans le même temps, la croissance des plantes était maintenue avec de faibles accumulations en ions chlorure et sodium. Certaines espèces comme les pomelos ont montré à la fin de l'essai une chute des feuilles suivie par une nouvelle pousse que nous avons interprétée comme une réponse d'adaptation.

Des travaux réalisés par le CIRAD montrent que des porte-greffes tétraploïdes permettent d'augmenter les propriétés de tolérance aux stress salin et hydrique chez les agrumes. Il était donc utile de caractériser l'impact de la présence de porte-greffes zygotiques ou tétraploïdes sur les rendements et la qualité des fruits du scion, en relation avec la physiologie des arbres. Les résultats que nous avons obtenus suggèrent que les porte-greffes zygotiques n'affectent pas les rendements moyens pour un génotype donné. Au contraire, les porte-greffes tétraploïdes diminuent de façon très importante les productions sans toutefois changer la qualité des fruits.

La physiologie du clémentinier greffé sur deux porte-greffes diploïdes et de leurs tétraploïdes respectifs a également été analysée et suggère que la moindre croissance des associations porte-greffe tétraploïde/clémentinier est due à une photosynthèse plus limitée. Toutefois, le flux de transport d'électrons en condition de lumière saturante s'est révélé être supérieur dans les feuilles de clémentinier greffé sur porte-greffes tétraploïdes suggérant que ces mêmes porte-greffes confèreraient une meilleure capacité adaptative de la photosynthèse aux conditions environnementales.

Mots-clés: Agrume, Diversité, Qualité des fruits, Stress salin, Tétraploïde.

ABSTRACT

Title: Physiological characterization of citrus genotypes: salt stress tolerance studies and impact of the presence of zygotic and autotetraploid rootstocks

Citrus are classified among the most sensitive tree crops to salt stress. However, strong diversity exists for this trait in the citrus gene pool e.g. *Poncirus trifoliata* (L.) Raf. which is highly susceptible while *Citrus reshni* Hort. ex Tan. (Cleopatra mandarin) is one of the most tolerant genotypes. Usual strategy to improve the resistance of rootstocks is based on hybridization between parent sharing complementary interesting characters. One other way to gain a salt stress tolerance for rootstocks is related to tetraploidy by chromosome doubling. Therefore genetic and physiological analysis for salt stress tolerance of any genotype is required for breeding programs and selection of more adapted varieties. We initiated studies combining genetic approaches (genome mapping) as well as physiological approaches related to the diversity of the citrus group in order to be able to correlate in the future the specific phenotypical traits of tolerance for different citrus genotypes with their genome expression. A F2 population resulting from self pollination of hybrid F1 (*C. reshni* x *P. trifoliata*) was created, and the segregation of 135 SSR markers plus 4 candidate genes was studied allowing establishing of 15 linkage groups. A majority of the markers (57%) showed a skewed segregation probably due to the intergeneric chromosome pairing during meiosis of the hybrid parent. Furthermore, markers colinearity was respected by comparing this map to the reference clementine genetic map.

In the meantime, we tested salt stress tolerance of twenty two citrus genotypes representing the citrus diversity among the more usual scions and rootstocks. The different genotypes were then subjected to salt stress. Physiological parameters such as growth rate, chlorophyll content, total phenolic compounds content, quantum yield of PSII electron transport, stomatal conductance as well as photosynthesis rate were monitored along the stress. Different physiological behaviors for salt stress tolerance depending of the citrus species were observed suggesting existence of different mechanisms for salt stress tolerance. Citrons were the most sensitive while all mandarins and pummelo were tolerant. All genotypes affected by salt stress were characterized by chlorosis symptom induction, chloride and sodium accumulation in leaves and by the change of physiological parameters. Specific profile of tolerance was associated with photosynthesis maintaining even though lower values of stomatal conductance were observed. In the meantime, plant growth was maintained with chloride and sodium accumulations. Some species such as grapefruits showed at the end of the assay an extended leaf drop completed by a growth of new leaf that we interpreted as a response of adaptation.

Work conducted by CIRAD showed that the use of tetraploid rootstocks lead to increased salt stress and water deficit tolerance properties in citrus. Therefore, it was interesting to characterize the impact of the presence of zygotic or tetraploid rootstocks on yields and fruit quality parameters related to the tree physiology. Results we obtained suggest that the presence of zygotic rootstocks did not affect the average yields of fruit for any given genotype. On the contrary, tetraploid rootstocks decrease dramatically fruit production without changing the quality of fruit when compared to the use of diploid rootstocks. The physiology of the tree of two rootstocks at the diploid and tetraploid levels was also analyzed. Results suggest that the reduced growth of tetraploid rootstock / clementine associations was due to a more limited photosynthesis. However, the maximum electron flow rate under saturated light was found to be higher in leaves of clementine grafted on tetraploid rootstock suggesting that tetraploid rootstocks confer a greater adaptive capacity of photosynthesis to environmental conditions.

Key words: Citrus, diversity, Fruit quality, Salt stress, Tetraploid.