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ORIGINAL PAPER

Genetic diversity revealed by physiological behavior of citrus genotypes subjected to salt stress

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Abstract Different physiological behavior of a wide range of varieties and species belonging to the *Citrus* genus was analyzed when subjected to salt stress with the aim to seek new sources of tolerance that might be specie-specific. Our goal was to use physiological results obtained along a salt stress in order to clarify if it would be possible to associate them with the known 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* × *P. trifoliata*). A moderate salt stress of 75 mM 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 under light. 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 citrus genus: mandarin and pummelo presented good tolerance to salt stress while citron was very sensitive. Furthermore, all secondary genotypes that presented good tolerance to salt tolerance shared mandarin or pummelo as female parent.

Keywords Citrus · Salt stress · Genetic diversity · Photosynthesis · Rootstocks

Introduction

Citrus species are considered sensitive to salt stress (Maas 1993). The adverse effects of salt stress on plant growth are related to low osmotic potential, accumulation of ions to toxic levels, and nutritional imbalance (Ashraf and Harris 2004; Byrt and Munns 2008). In citrus, effects of salt stress have been extensively reported in the literature (Anjum 2008; Atmane et al. 2003; Garcia-Sanchez and Syvertsen 2009; Hussain et al. 2012; Lopez-Climent et al. 2008; Mouhaya et al. 2010b; Saleh et al. 2008; Syvertsen et al. 2010). The observed effects of salinity are various and including symptoms of leaf injury, growth suppression and yield decline. Primary effects of salt stress in citrus are decreased stomatal conductance (Anjum 2008), which results in less CO_2 diffusion, which ultimately decline net photosynthesis (García-Sánchez and Syvertsen 2006) and ion accumulation (Hussain et al. 2012; Sudhir et al. 2005). A large diversity within citrus genus towards salt stress does exit, e.g., Rangpur lime, Sunki mandarin and Cleopatra mandarin are considered to be tolerant, while Carrizo citrange is known to salt sensitive (Maas 1993). In citrus

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damages caused by salinity are usually associated with chloride accumulation but not with sodium (Banuls et al. 1997; Hussain et al. 2012; Moya et al. 2002; Syvertsen and Garcia-Sanchez 2014). 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 Na^+ and Cl^- ions seems to be crucial in the evaluation of citrus tolerance status for salt stress (Hussain et al. 2012), 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 polyembryonnic seeds. Some citrus produce in their seeds somatic embryos in addition to the zygotic one. Those embryos are originated from nucellar cells, a maternal tissue, and therefore the regenerated plants reproduce the maternal characters. From three basic species, Citron (*C. medica*) and pummelo (*C. maxima*) are two species which are not able to produce polyembryonic seeds. This is the reason for 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, 2000, 2007, 2008). 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 (Barrett and Rhodes 1976; Scora 1975) where a large diversity of *Citrus* genus is represented by three basic species such as mandarin, citron and pummelo. They are at the origin of many secondary cultivated species such as orange, lemon, sour orange, grapefruit, clementine by several sexual crosses 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. 2001a; 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 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. 2001b). This concept gained further support from various studies using cytoplasmic DNA markers (Froelicher et al. 2010; Nicolosi et al. 2000). Therefore, in this study, different varieties were selected representing the major species on the basis of the genetic diversity of *Citrus* assuming 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, those 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 their evolution.

Materials and methods

Plant material

This study was carried out at INRA-CIRAD research station of Corsica, France. Twenty different genotypes of citrus were selected belong to major diversity of Citrus genus complemented with one intergeneric hybrid carrizo citrange (*C. sinensis* × *P. trifoliata*). A total of 8 species and one hybrid were represented (Table 1). Six plants 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 3 months after germination in 2.5-l 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

As for genotypes 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 as described by Hussain et al. (2012) using four inter-simple sequence

Table 1 List of genotypes used for salt stress experiment

Common name	Tanaka system	Code	ICVN and SRA no.
Diamante citron	*C. medica* L.	CiD	SRA 540
Digite citron	*C. medica* L.	CiB	SRA 640
Etrog citron	*C. limonimedica* L.	CiE	SRA 709
Poncire commun citron	*C. medica* L.	CiP	SRA 601
Eingedi 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
Australian sour orange	*C. aurantium* L.	SoA	SRA 851
Combava	*C. hystrix* D.C.	CoK	SRA 630
Carrizo citrange	*C. sinensis* × *Poncirus trifoliata*	CaC	SRA 796

ICVN International citrus variety numbering, *SRA* Station de research agronomic

repeat (ISSR) primers: HVH$(CA)_7$T, DBDA$(CA)_7$, BDB$(CA)_7$C, HVH$(TCC)_5$ (Fang and Roose 1997). Ploidy status of plants was determined by flow cytometry using leaf samples according to Froelicher et al. (2007). 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 genotype 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.

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 experiment 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 same developmental stage were tagged randomly. These two leaves were used for the measurement of the studied parameters. For each parameter, each genotype was evaluated consecutively. Since it was not possible to measure all the parameters the same day at the same time, control and salt stress plants were split in two sets. Measurements were performed at the beginning of the experiments and after 80 days of salt stress during two consecutive sunny days for controls and stressed plants, respectively. Since the temperature and humidity were very similar during the two selected days, we expect to have limited the impact of the environment on the measurements.

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 1,000 μmol m^{-2} s^{-1}. Measurements were performed in the morning (8–11 am) to avoid high external temperature and low humidity. Leaf temperature was 28 ± 2 °C, leaf to air vapor 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 darkadapted 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 three leaves for salt treated were collected after 80 days of treatment. Mineral analysis was carried out according to Mouhaya et al. (2010a), Saleh et al. (2008). Leaves were oven dried at 60 °C for 1 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 h. The resulting ashes were dissolved in 100 mL of 0.5 N 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 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 analyzed with R statistical software to represent physiological diversity under normal growth (control) and under salt stress conditions. Data were normalized and used in principal component analysis (PCA) or to construct a dendrogram by Ward aggregation method.

Results and discussion

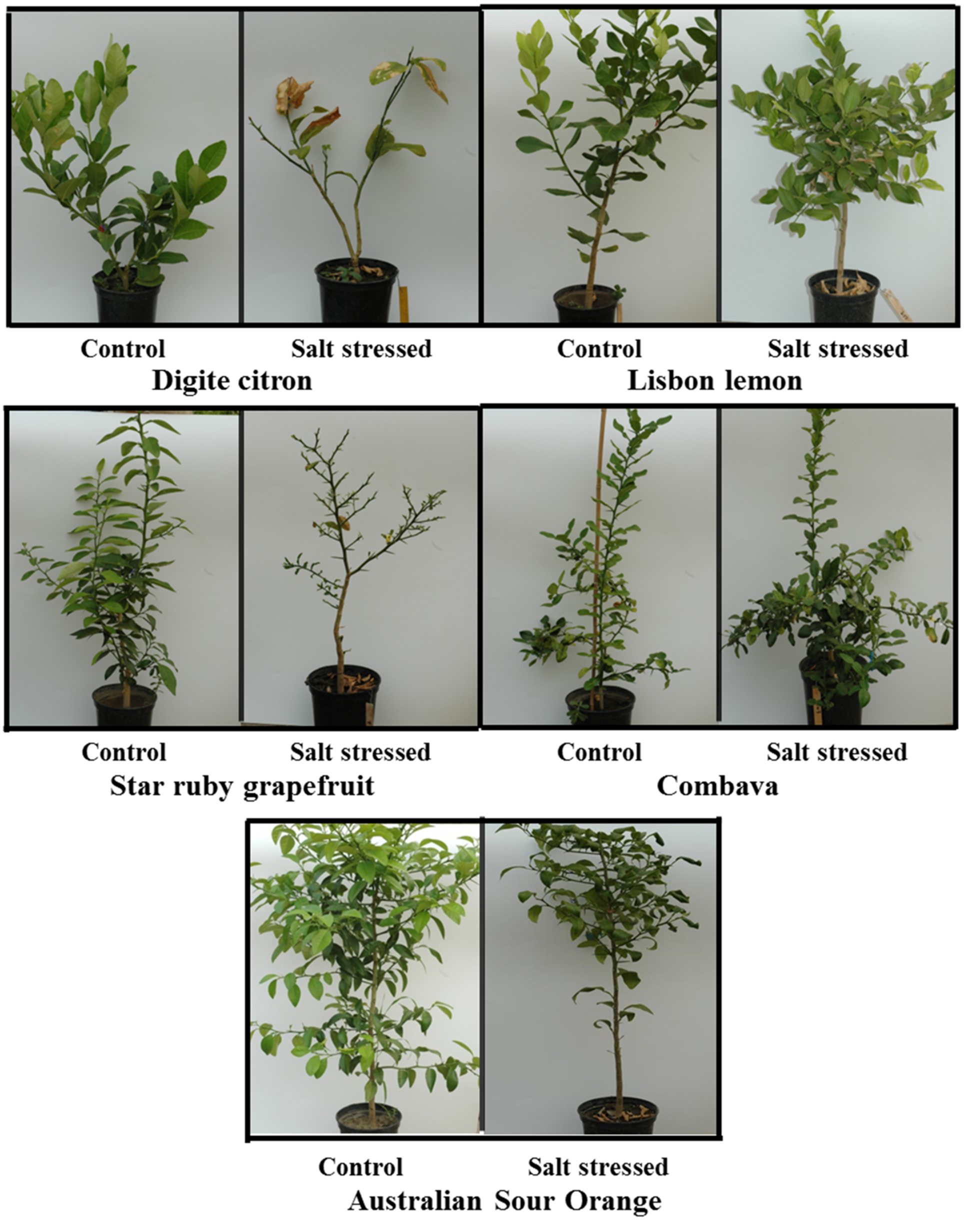
Salt stress leaf symptoms

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. Most of the trees of citron species were badly affected with only a limited number of leaves which were not affected by necrosis (Fig. 1). Theses leaves were used to measure the photosynthetic parameters. A progressive loss of leaves was observed in Mexican lime [*C. aurantifolia* (Christm.) Swing.] after 65 days onwards. Some leaves were still present on trees after 80 days of salt stress which allowed us to measure physiological parameters. After 80 days plants eventually lost all their leaves. Carrizo citrange showed leaf necrosis from the ends of leaves and first observed on lower 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 1 mm) were observed on some leaves of pummelo but we did not consider them as salt symptoms. A very unique behavior of sudden drop of all the leaves while they were still green were 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 in vigorous growing but less or not observable when the control varieties did not present a strong vegetative flush (Table 2). 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

The results obtained from different parameters when plants were grown in control condition 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 replications 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 Digite 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 comparison of behavior under stress must first be compared to control trees and then, relative values can be used to compare different varieties. The

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



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 observed ones for the control plants (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 three trees was almost identical. The PCA of diversity in behavior of genotypes relative to control under salt stress showed two clearly distinguishable varietal groups (Fig. 4). The first group (I) corresponds to varieties or species that showed lower photosynthetic activity and 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 (II) we found lemons, mandarins, pummelos, Rangpur lime, Combava, sour orange and grapefruit. This group was distinguished mainly by a lower accumulation of toxic

Table 2 Brief description of different symptoms observed during salt stress for each genotype

Common name	Symptoms
Diamante citron	Leaf necrosis were developed from the tip of the blade and then spread throughout the leaves and reached the ramifications. Furthermore, all leaves were fallen
Digite citron	
Etrog citron	
Poncire commun citron	
Eingedi pummelo	Small discoloration (less than 1 mm) on certain leaves
Kao Pan pummelo	
Timor pummelo	
Eureka lemon	No visible symptom was observed during the 80 days of salt stress
Lisbon lemon	
Brazilian sweet lime	A slight reduction in growth of plant
Mexican lime	Yellowing of leaves, progressive leaf fall
Rangpur lime	Slight reduction in plant growth
Cleopatra mandarin	No visible symptom was observed during the 80 days of salt stress
Sunki mandarin	
Willow leaf mandarin	
Duncan grapefruit	Sudden drop of all the leaves while they were still green and then emergence of new leaves
Star Ruby grapefruit	
Australian sour orange	Reduction of vegetative growth
Combava	Reduction of leaf and/or branch growth
Carrizo citrange	Leaf necrosis symptoms were first observed on lower (older) leaves, leaf necrosis start from the end of the leaves and progress towards tip, increase in vegetative growth

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 species 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 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 as mother plant 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 ability 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 then be required to analyze the genetics of tolerance to salt stress and extend the experiment not only 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 inheritance of salt stress tolerance. Undoubtedly the analysis of nuclear inheritance provides insights into the

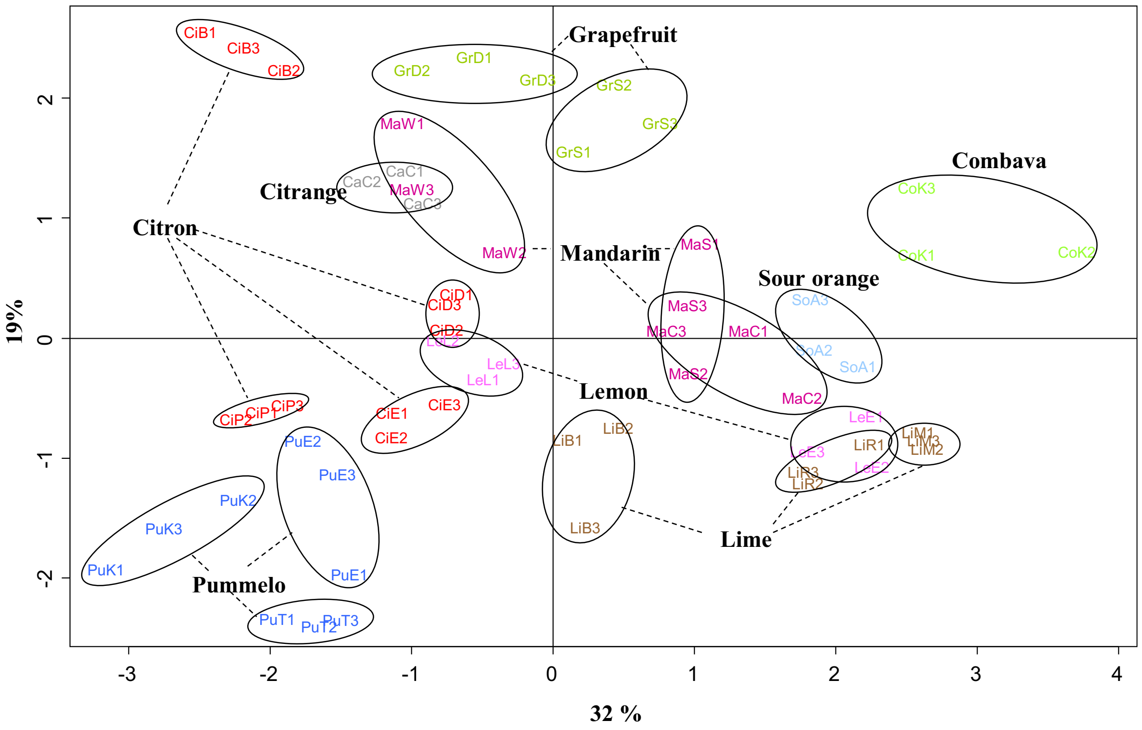


Fig. 2 PCA of citrus varieties and trees repartition on the two first axes representing 51 % of the population diversity calculated from physiological parameters estimated on the unstressed plants. *Circles* group the 3 repetitions of each genotype

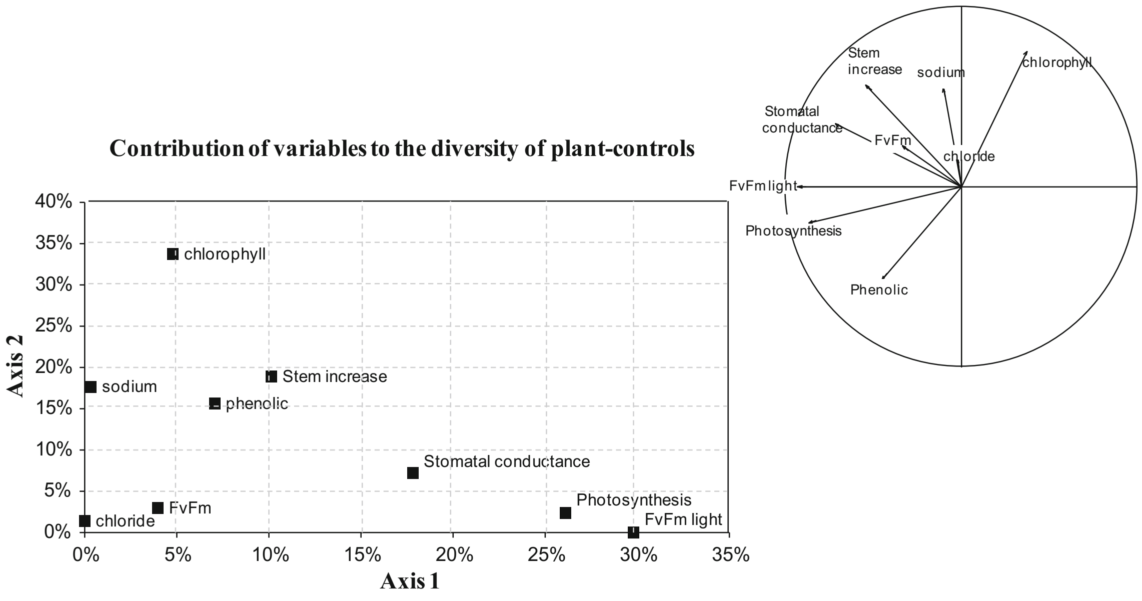


Fig. 3 Contribution of each physiological factor to the two first axes of PCA explains the diversity under control condition

mechanisms of adaptation to salt stress which is also under the control of nuclear genes. To study nucleo-cytoplasmic interaction and nuclear and cytoplasmic inheritance of salt tolerance, two paths could be explored. The first strategy could be to produce cybrid with different combinations of nucleus and cytoplasm inherited from tolerant and sensitive varieties. Unfortunately cybrid production is quite difficult and time consuming in perennial crops. The second option would be to perform Mendelian heredity studies based on segregating progenies resulting from parents with

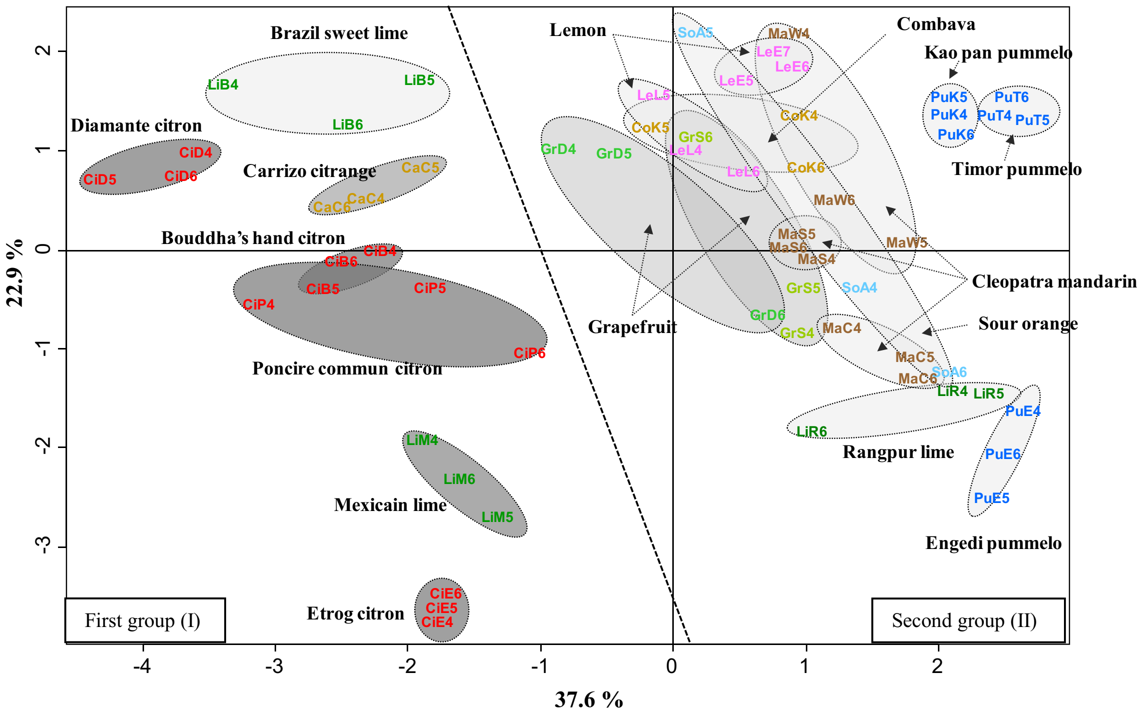


Fig. 4 PCA of citrus varieties and trees repartition on the two first axes representing 61 % of the population diversity calculated from physiological parameters estimated on the stressed plants. *Circles* group indicates 3 repetitions of each genotype

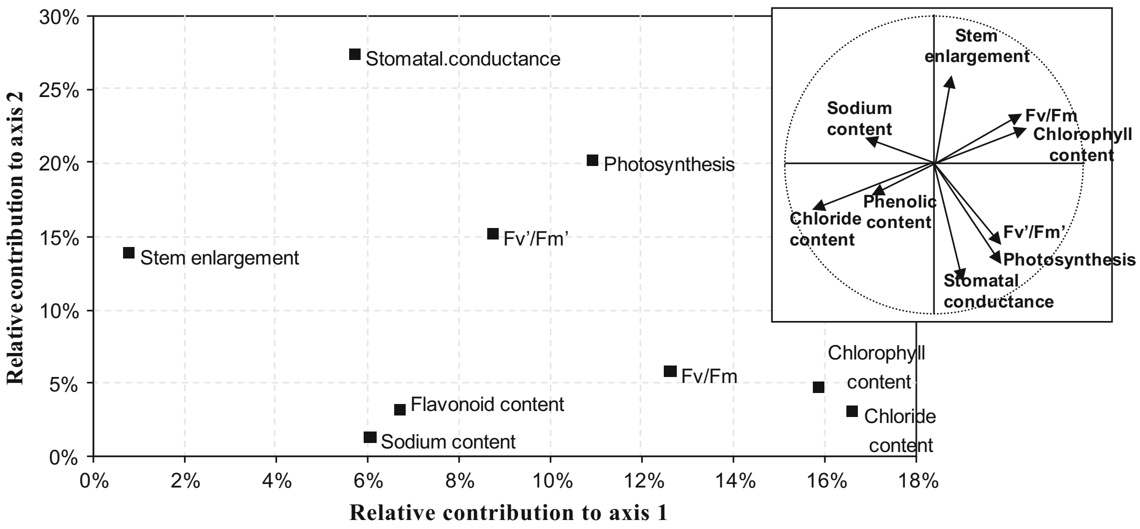


Fig. 5 Contribution of each physiological factor to first two axes of PCA explaining the diversity of stressed plant compared relatively to control

contrasted properties of tolerance when subjected to salt stress. Because of the maternal heredity of cytoplasmic organelles, all sexual crosses should then be done in both senses (male and female). At the end the salt tolerance cytoplasmic inheritance should be also investigated. However, 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 slat stress 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 pummelo groups since those genotypes seem to be at the origin of new traits of salt stress tolerance.

Author contribution statement Sajjad Hussain is a student and main author. Raphaël Morillon was involved in the write-up, planning and discussion of results. Muhammad Akbar Anjum was involved in the write-up and discussion of the manuscript. Patrick Ollitrault did the planning of experiment, helped in paper discussion and was the leader of the Citrus team. Gilles Costantino did data analysis and helped in research trial. François Luro helped in research trial and data analysis and discussion of result.

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