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APPROACHES TO STUDY BIODIVERSITY AND SALINITY TOLERANCE MECHANISMS IN MEDITERRANEAN FRUIT TREE SPECIES

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Long Abstract

Changes in global climate are going to increase the problem of drought and soil salinity in the Mediterranean area, where increases in temperature, drought and soil and water salinity are expected. Identification of plant genotypes with positive agronomic traits, such as salt tolerance, may reduce the effects of salinity and drought on productivity. Therefore, there is considerable effort being directed toward the development of salt-tolerant genotypes through plant breeding, with the aim to the introgression of salt-tolerance traits into new cultivars. The results presented in this thesis contributed to the develop of new tools to improve knowledge on the biodiversity and salinity tolerance on two minor fruit tree crops grown in Mediterranean countries: Fig (*Ficus carica L.*) and Pistachio spp. In recent years, the interest for these two tree fruit crops is greater than before because of their nutritional values and economic importance.

The Fig tree is one of the most ancient cultivated fruit tree crops in South Italy. To explore biodiversity in the Fig, two different experiments were carried out. The main objective of the first experiment was to characterize and evaluate the genetic diversity among 181 accessions, using SSR markers. A total of 117 alleles were detected with a mean of 6.5 per locus. The average expected (He) and observed heterozygosity (Ho) were 0.56 and 0.66, respectively. The mean polymorphic information content (PIC) was 0.51, from 0.78 (LMFC30) to 0.21 (LMCF26), suggesting a high molecular diversity among the fig accessions. The UPGMA cluster analysis discriminated 174 genotypes and identified to eight groups. The accession 'Bianca d'Agosto' from Basilicata and the genotype "FCS138" from Calabria were the most diverse. These findings demonstrate the richness of the fig's genetic resources in Southern Italy, resolved cases of synonymies and homonymies, and characterized fig accessions.

In a second experiment, an analysis into the genetic variability was conducted within the "Dottato" cultivar, which has fruits that are admitted to two EU Protected Designation of Origin (PDO) "*Fico Bianco del Cilento*" (Campania) and "*Fichi di Cosenza*" (Calabria). Dottato is grown in large parts of Southern Italy and it is well regarded for its parthenocarpic fruits and their excellent organoleptic characteristics, still maintained after drying and processing. To preserve the high quality standard of the fruits admitted to PDO, it is important to know the potential genetic variation within the Dottato. The morphological and molecular diversity were studied by evaluating 24 morphological traits and by genotyping with 18 microsatellite markers. The microsatellite allelic profiles among the putative clones of 'Dottato' indicated a moderate genetic variability. The average expected and observed heterozygosity were 0.42 and 0.62, respectively. The mean polymorphic information content (PIC) was 0.4, varying from 0.08 (LMFC26) to 0.67 (FCUP 38_6), suggesting a low level of genetic diversity. The morphological clustering showed the uniformity of 19 genotypes whereas 5 were different.

Part of this PhD project was to develop new methods for studying, at cellular level, the mechanisms of plant salinity resistance by evaluating the uptake, transport and sequestration of the sodium, potassium and chloride ions in roots and in leaves. The research was conducted on "*in vitro*" plant of pistachio species (P. *atlantica*, P. *integerrima*) and their interspecific hybrid (UCB1), that are commonly used as rootstocks. The seedlings obtained from "*in vitro*" germination were cultivated on a ½ MS medium supplemented with 7gr/L of agar and 15 gr/L sucrose. The salinity treatments (100umM of NaCl) were applied on 2-month-old seedlings for 7 days. Roots and leaves were sectioned with the vibratome, and then incubated in osmolality maintaining buffer to ensure tissue viability. In roots sections, micrographs were recorded on the Confocal Fluorescence Microscopy (ZEISS LSM710/700), the CoroNa-Green AM (Invitrogen), and the Asante Potassium Green were used to detect sodium and potassium respectively. Sections of leaves of UCB-1 and P. integerrima treated and control were incubated in CoroNa-Green AM (Invitrogen) and co-stained with SNARF-1 (carboxylic acid, acetate, succinimidyl ester Molecular Probes, Inc., Eugene, OR), micrographs were recorded on the fluorescent laser scanning confocal microscopy Leica SP8/ SP8 MP Microscope was used.

In roots, distinct accumulation patterns of sodium were observed at the subcellular level in UCB-1 and P. *integerrima.* The potassium signal was stronger in the endodermis of all three genotypes subjected to saline stress.

In leaves, a new methodology was developed that removed chlorophyll auto-fluorescence signal, allowing the distinction between different compartmentation ability of sodium ions between the genotypes: only in UCB1 were vacuoles stained. The higher salt tolerance of UCB1 was also confirmed by a higher survival rate and no damage in the leaves. The inability of *P. Integerrima* to accumulate sodium in vacuoles can be correlated with a higher sensitivity to salt stress, evidenced by greater damage in the leaves with chlorosis and necrosis, and a lower survival rate. The new methods developed can be transferred to other fruit crops, providing a unique opportunity to assess salt resistance.

The results presented in this thesis contributed to develop new tools to improve knowledge on the biodiversity and to have a better understanding of the salinity tolerance mechanisms.

1. General Introduction

Over the next four decades, the world's population is estimate to increase by 2 billion people to exceed 9 billion people by 2050 and 13.2 billion in 2100 (United Nations, 2017). This increasing population means that even small malfunctions in the current food production and supply systems could easily lead to severe famine and even civil unrest, especially in developing countries (Wheeler and Von Braun, 2013). According with the World development report (2008), food production will need to increase by 70 to 100% more food by 2050 (Bank, 2007). Food production is vulnerable to several environmental problems such as climate change, ozone depletion, drought, desertification, flooding, soil salinity, and soil erosion (Godfray *et al.,* 2010). Increasing temperatures, changing precipitation patterns (abundance and strength), increasing frequency of extreme weather events and natural disasters, e.g. floods and droughts, or the increase of greenhouse gases as well as a predicted rise of the sea level are going to be the most prominent climatic factors affecting agricultural production systems and, therefore, agrobiodiversity (Kotschi, 2007; Lovejoy, 2006). The Mediterranean region, as most regions in the world, is warming and becoming increasingly arid (Stocker *et al*., 2013). The Mediterranean region includes countries extending from Spain to Turkey and Cyprus and from Morocco to Syria. All are characterized by hot dry summers, mostly rainy winters and partially wet spring and autumn. In these regions, in order to avoid plant water deficits, irrigation is necessary to ensure regular crop yields and to reduce inter-annual yield variability (Collins *et al.*, 2009). Climate models developed by NASA in 2016 predict increasing temperatures and increases in the frequency and duration of drought during the twenty-first century that will have negative impact on agricultural productivity (Cook *et al.*, 2016). This study finds that the recent drought that began in 1998 in the Eastern Mediterranean Levant region is likely the worst drought of the past nine centuries (Cook *et al*., 2016). Climate changes over the past 30 years have made the Mediterranean region much less fertile, and this trend is continuing. Therefore recent changes in global climate are going to exacerbate the problem of soil salinity and aridity especially in the Mediterranean basin (Cook *et al.,* 2016). This increase of aridity comes together with associated disturbances such as floods, extreme heat and drought events and forest fires all them having strong impacts on

many ecosystems (Penuelas *et al*., 2013). Variation in important climate variables including higher temperature and less precipitation are expected to decrease water for irrigation and impose high evapotranspiration losses (Chaves *et al*., 2009; Yeo, 1998). The resulting drier conditions will further raise irrigation demands, which are often done with poor quality water which contains dissolved salts and hence increase soil salinity (Chartzoulakis and Psarras, 2005). Desertification and salinization are growing rapidly worldwide, with has resulted in more than 50% of the average productivity of major crops (Pitman and Läuchli, 2002). Loss of arable land via salinization is a major factor undermining the productivity of modern agricultural systems (Galvani, 2007).

A soil is considered salty when the electric conductivity (EC) of the soil solution reaches 4 dS m−1 (equivalent to 40 mM NaCl), generating an osmotic pressure of about 0.2 MPa and significantly reducing the yields of most crops (Munns and Tester, 2008). As a consequence, ion toxicity, leads to chlorosis and necrosis, mainly due to Na+ accumulation that interferes with many physiological processes in plants, with adverse effects on germination, plant growth and crop yield (Flowers and Colmer, 2015; Munns, 2002; Munns and Tester, 2008).

Most of the soil salinity is of natural origin (primary salinization), but increases in soil salinity is also the result of human activities (secondary salinization). Primary salinity results from accumulation of salts over long time, through natural processes, in the soil or groundwater. The weathering processes break down rocks which releases soluble salts like sodium chloride (the most soluble salt), calcium, magnesium and to a lesser degree sulphates and carbonates, which form deposition of oceanic salt carried in wind and rain. The secondary, or human-induced salinity, is due to a range of cultural factors. In fact, human activities alter the hydrologic balance of the soil between water applied (irrigation or rainfall) and water utilized by crops (transpiration), especially in situations with salt rich irrigation water or insufficient drainage of the soil (Ghassemi *et al*., 1995). Sodicity is a derived consequence of salinity in clay soils, where leaching, through either natural or human-induced processes, has washed soluble salts into the subsoil, leaving sodium bound to the negative charges of the clay (Pitman and Läuchli, 2002). The process of Sodicity is complex and occurs over a long period. At the beginning, salts accumulate within the soil profile, from either airborne deposition or mineral weathering, this causes the clay fraction of the soil to become saturated with sodium.

Subsequently, leaching of the profile, either by rainwater over prolonged periods, or by irrigation with fresh water, lowers the electrolyte concentration and the clay particles disperse. Further leaching washes the dispersed clay particles deeper into the profile where they block pores and hinder infiltration of water. The soil then is very slow to drain, and is readily waterlogged (Pitman and Läuchli, 2002).

The most common method of measuring salinity is to determine the level of electrical conductivity (EC) within soils and water. Increases in EC measurements are directly correlated with increases in the concentration of soluble salts or elemental ions, predominantly sodium and chloride. Electrical conductivity is expressed in units such as decisiemens per meter (dS/m) which quantify the ability of a sample to conduct electrical impulses with a resistance of 1 ohm; in the field, this can be determined using instruments such as electromagnetic (EM38) soil mapping devices (Corwin and Lesch, 2013).

Soil samples are classified as saline when EC values exceed 4 dS/m while water is considered saline at levels above 2 dS/m. Rain or distilled water has a conductivity of 0.02-0.05 dS/m whereas seawater, at the other extreme, run between 45-60 dS/m. Salinity in water is also measured by the weight of its inorganic particulates or total dissolved solids (TDS), expressed as parts per million (ppm) or milligrams per liter (mg/l): less than 1,000 ppm is considered fresh or potable, greater than 4,000 ppm is saline, and between 35,000-45,000 ppm is the standard for seawater. When comparing EC and TDS measurements, note that 1 dS/m is roughly equal to 650-700 ppm, and closer to 800 ppm at relatively higher levels of salinity (Maas and Grattan, 1999).

Salinity thresholds are generally defined as the maximum amount of salt that a plant can tolerate in its root zone without impacting growth. Other important thresholds indicate the highest level of plant salt-tolerance associated with a decline in yield or biomass (usually between 10-50%); zero yield thresholds specify levels at which a plant can no longer survive. In general, plants classified as salt-sensitive have salinity thresholds of 1-3 dS/m and zero yields at 8-16 dS/m (or less) while the 'moderately' salt-tolerant have thresholds of 5-10 dS/m and zero yields at 16-24 dS/m (Glenn *et al*., 1999; Grieve *et al*., 2012).

Date palm has been classified as "tolerant" with a threshold of 4.0 (Furr and Armstrong Jr, 1962; Furr *et al*., 1966). A study conducted by Ramoliya and Pandey (2003) has found that

certain varieties of date palm can tolerate a relatively high soil salinity level of 12.8 dS m −1 (1 dS m −1 = 640 mg l −1) with no visible effect on the seedling phenotype. Based on the classification proposed by FAO, the olive tree, fig tree and pomegranate are considered moderately salt tolerant and the date palm highly resistant (Ayers and Westcot, 1976). Information from literature are sometimes contradictory about salt tolerance in Pistachio. Pistachio is considered moderately sensitive by (Picchioni *et al*., 1990; Sepaskhah and Maftoun, 1988), but highly tolerant by (Behboudian *et al*., 1986) and (Walker *et al*., 1987). Among Pistacia species, P. *atlantica* has been described as a highly tolerant to the salinity (Ferguson *et al*., 2005).

According to the FAO Land and Plant Nutrition Management Service, over 800 million hectares of land throughout the world are salt-affected, either by salinity (397 million ha) or the associated condition of sodicity (434 million ha). Therefore, over 6% of the world's land is salt affected by either salinity or sodicity (Table1).

Table 1- Percent of Salinity and Sodicity throughout the world (Source: FAO Land and Plant Nutrition Management Service, FAOSTAT 2014)

Regions	***Total area***	***Saline soils***	***%***	***Sodic soils***	***Percent***
Africa	1899.1	38.7	2.0	33.5	1.8
Asia, Pacific and Australia	3107.2	195.1	6.3	248.6	8.0
Europe	2010.8	6.7	0.3	72.7	3.6
Latin America	2038.6	60.5	3.0	50.9	2.5
Near East	1801.9	91.5	5.1	14.1	0.8
North America	1923.7	4.6	0.2	14.5	0.8
Total	**12781.3**	**397.1**	**3.1%**	**434.3**	**3.4%**

A significant proportion of agricultural land has become saline and more than 45 million hectares of irrigated land in the world have been damaged by salt, and 1.5 million hectares are

taken out of production each year because of high salinity levels in the soil (Munns and Tester, 2008). This constant salinization of arable land is expected to have overwhelming global effects, resulting in 30% land loss within the next 25 years, and up to 50% by the year 2050 (Wang *et al*., 2003). This progressive loss of arable land has potentially serious consequences for the expanding global population, which is progressively increasing.

In the Mediterranean countries, water shortage is becoming a problem of high concern affecting the local economy, mostly based on agriculture. Mediterranean regions are currently experiencing increasing salt stress problems resulting from seawater intrusion into aquifers and irrigation with saline water (Rana and Katerji, 2000). The decrease in good quality of water in these areas will accelerate the use of saline water for irrigation which will raise salt accumulation in soils, and increase the extent of secondary salinization (Munns and Gilliham, 2015; Yeo, 1998).

In Italy, the most salt-affected soils are spotted around semi-arid regions particularly in Sicily and in general in the Southern Italy, and amounted to 450,000 ha, including potentially salt-affected soils. Irrigation with saline waters is used in Sicily in many areas where this water represent the only source available for irrigation (only 300 million m3 of good quality water is available against the need of 1600 million m3), causing secondary salinization and sodication, (Crescimanno, 2001). Appropriate farm management practices can prevent soil and water salinization alleviating adverse effects of salinity. This approach can be carried out by implementing large engineering scheme for irrigation and drainage, planting perennials to lower water tables, but also using plant growth-promoting bacteria, which may demonstrate to provide a significant benefit to the plants to facilitate their growth in saline soils (Botella *et al*., 2005; Mayak *et al*., 2004). Even if several treatments and management practices can reduce salt levels in the soil, there are some situations where it is either impossible or too costly to realize desirably low soil salinity levels. In some cases, the only viable management option is to plant salt-tolerant crops (Godfray *et al*., 2010; Tester and Langridge, 2010).

To meet these challenges, it is required to select crops with increased salt tolerance and to improve the knowledge of their salt-tolerance capacities. For this end, it is vital to have a thorough understanding of the different adaptation mechanisms that have evolved in many plants to cope with salinity and to have a good knowledge of the regulation of these

mechanisms. It will be very important to characterize and evaluate as wide a range of germplasm as possible for avoidance, resistance or tolerance to major stresses such as drought, heat, waterlogging and soil salinity. (Frison *et al*., 2012).

1.2 Effects of salinity on plants

Plants are regularly exposed to several environmental stresses such as drought, high salinity, high temperature, cold and heavy metals that can disturb growth and productivity (Anjum *et al*., 2011). Salinity is one of the most important abiotic stresses, limiting crop production in arid and semi-arid regions, where soil salt content is naturally high and precipitation can be insufficient for leaching (Meehl *et al*., 2007).

Most fruit trees are sensitive to salinity including *Malus domestica, Prunus armeniaca, Prunus domestica, Prunus persica*, and *Citrus spp*.; Pistachio (*Pistacia spp*), *Olea europaea* and *Ficus spp*. are moderately tolerant but *Phoenix dactylifera* is very tolerant (Gucci and Tattini, 1997, Kozlowski, 1997). Olive (*Olea europaea L*.) and pistachio (*Pistacia vera L*.) are classified as well–adapted crops to drought and salt stress. Both species can develop well in arid climate and salinity conditions (Behboudian, Walker, *et al*., 1986, Chartzoulakis and Klapaki, 2000). Fig (*Ficus carica L*.) has ability of to tolerate water deficit and moderate salinity stress making it a suitable species for cultivation in semi-arid environments such as the Mediterranean and middle-East where it is an important crop plant (Golombek and Lüdders, 1993; Metwali *et al*., 2014). According to the reactions of plants in growing in saline environments, they can be classified in Halophytes and Glycophytes (Levitt, 1980; Shannon, 1997). Their response to salt stress differs in terms of toxic ion uptake, ion compartmentation and/or exclusion, osmotic regulation, CO2 assimilation, photosynthetic electron transport, chlorophyll content and fluorescence, reactive oxygen species (ROS) generation, and antioxidant defenses (Grieve *et al*., 2012; Munns, 2005; Negrão *et al*., 2016; Tang *et al*., 2015).

Glycophytes, which includes most fruit crop plants, cannot grow in the presence of high salt levels; their growth is inhibited or even completely prevented by NaCl concentrations of 100–200 mM, resulting in plant death (Munns and Termaat, 1986). The halophytes are able to grow and multiply in soils containing high NaCl concentrations (300–500 mM), due to specific

mechanisms of salt tolerance developed during their phylogenetic adaptation (Flowers and Colmer, 2008; Parida and Das, 2005). Some of them have developed adaptive specific morphological structures, such as *Mesembryanthemum crystallinum* (ice plant) that accumulates salt in salt glands located on the surface of leaves and release it as crystals (Adams *et al*., 1998).

Salinity tolerance is a complex phenomenon that involves several physiological processes that are under a multigene control (Flowers, 2004; Lovelock and Ball, 2002). Salt tolerance varies widely among species and genotypes. Plants have evolved several mechanisms that allow them to adapt, grow and reproduce under high salinity conditions. Munns, Tester (2008), Tester, Davenport (2003), and Kumari *et al.,* (2014), have reported these three mechanisms:

1) **Osmotic stress tolerance**, which is controlled by long distance signals that reduce shoot growth and is initiated before shoot Na+ accumulation.

2) **Na+ or Cl− exclusion**, that tend to prevent Na+ and Cl− uptake and transport processes in roots in order to reduce the accumulation of these ions to a toxic concentrations within leaves.

3) **Tissue tolerance**, that try to compartmentalize Na+ or Cl− in the leaves vacuoles to prevent salt injury to the sensitive thylakoid membrane of the chloroplasts.

Munns *et al*., (2012) and Roy *et al*., (2014) have suggested that these three mechanisms of salt tolerance are not mutually exclusive. Therefore, the manifestation of one does not prevent the other. However, it might be possible that each of these tolerance mechanisms is more effective in a particular circumstance and/or genotype and growth stage dependent. (Roy *et al*., 2014).

Salinity tolerance of pistachio and fig, as many woody species, have been associated to:

- Growth reduction (Okubo and Utsunomiya, 1996; Picchioni *et al*., 1990; Sepaskhah and Maftoun, 1988).
- Decrease in photosynthetic activity (including high chlorophyll index, chlorophyll fluorescence, and chlorophyll content) and transpiration rate (Caruso *et al*., 2017; Golombek and Lüdders, 1993; González-Rodríguez and Peters, 2010; Karimi and Kuhbanani, 2015; Walker *et al*., 1988). These mechanisms are based on stomatal

closure and a reduced leaf area, in order to minimize water loss via transpiration (Behboudian *et al*., 1986; Karimi and Tavallali, 2017).

- Smaller amounts of salt ions transported to the leaves (Ferguson *et al*., 2002; Jafari *et al*., 2018; Picchioni *et al*., 1990; Walker *et al*., 1987).
- Enzymatic activities (Chelli-Chaabouni *et al*., 2010; Gholami *et al*., 2012; Hajiboland *et al*., 2014). Some authors have correlated salt tolerance with higher constitutive levels of certain antioxidant enzymes. In contrast, salt-sensitive species show an unaffected response or even a decrease in antioxidant defenses, and they have a lower constitutive antioxidant enzyme levels than salt-tolerant species (Abdoli Nejad and Shekafandeh, 2014; Ashraf, 2009; Tavallali *et al*., 2010).
- Osmotic adjustments, mainly involving the intake of inorganic solutes from the soil under salt stress situations to facilitate the maintenance of leaf turgor (Esmaeilpour *et al*., 2016; Hajiboland *et al*., 2014; Ju *et al*., 1999).

Very little is known about these physiological components, so understanding the effects of salinity on these processes needs further investigations, because numerous factors can influence plants' responses to salinity due to the complex nature of salinity tolerance (Negrão *et al*., 2016).

The salt sensibility of some crops varies largely and shows different behavior. For fruit trees, the choice of the more suitable rootstocks for saline condition is particularly important because the salt resistance in most glycophytes is associated with the restriction of Na+ and/or Cl transport from the root to the shoot (Gucci and Tattini, 1997). The use of salt tolerant rootstock was demonstrated to be a valid strategy in increasing the salt tolerance of pistachio (Ferguson *et al*., 2002).

1.3 Managing salinity in agricultural production

Appropriate farm management practices can prevent soil and water salinization alleviating adverse effects of salinity. This approach can be carried out by implementing large

engineering scheme for irrigation and drainage, planting perennials to lower water tables, but also using plant growth-promoting bacteria which may demonstrate to provide a significant benefit to the plants to facilitate their growth in saline soils (Botella *et al*., 2005; Mayak *et al*., 2004). Even if several treatments and management practices can reduce salt levels in the soil, there are some situations where it is impossible or too expensive to realize desirably low soil salinity levels. In some cases, the only viable management option is to plant salt-tolerant crops. The tolerant plants can be obtained with several approaches such as classic breeding methods, traditional mutagenesis, molecular marker-assisted selection techniques and biotechnology (Botella *et al*., 2005; Hasegawa *et al*., 2000). Identification of plant genotypes with tolerance to salt, and incorporation of desirable traits into economically useful crop plants, may reduce the effects of salinity on productivity. Developing crop plants tolerant to salinity has the potential of making an important contribution to food production in many countries. This will permit the use of low quality water and thereby reduce some of the demand for higher quality water. Great effort is, therefore, being directed toward the development of salt-tolerant crop genotypes through the use of plant-breeding strategies involving the introgression of the genetic background from salt-tolerant wild species into cultivated plants (Pitman and Läuchli, 2002), also improving research on underutilized crop (Botella *et al*., 2005; Hasegawa *et al*., 2000).

1.4 Importance of "Neglected or Underutilized" Species

In recent centuries, agricultural systems have promoted the cultivation of a very limited number of crop species and many crops have been relegated to the status of neglected or underutilized crop species, and largely ignored (Chivenge *et al*., 2015). Numerous terms have been employed to characterize them, including "minor crops," "underutilized" species, "neglected species" or "orphan crops," "underexploited" and "underdeveloped" species. These species persist because they are still useful to local people, occupying special niches in marginal areas (Padulosi *et al*., 2002; Williams, 2002). Most underused crop are adapted to a range of ecological niches, low input agriculture and may have tolerance to abiotic and biotic stresses. Neglected and underutilized crop species are often described as "drought tolerant" (Collins *et al*., 2009) and could therefore prove vital in fighting hunger. These traditional

plants, crops and crop varieties and their use have often been the victims of progress. They are unattractive in comparison to modern, exportable crops produced in much simpler production systems. Often, such valuable genetic resources can be lost before they can be fully characterized and effectively used (Mal, 2007). Another plant genetic resource that needs to be protected is crop wild relatives. These are wild plant species which are closely related to cultivated crops, including their wild ancestors – the wild 'cousins' of our cultivated plants. For plant breeders, crop wild relatives are source of rare material for crop improvement. They may serve as source of useful genes for new traits pest and disease resistance, or tolerance to heat, drought and other stresses (Hunter, 2012). In addition, the conservation and use of crop wild relatives are essential elements for increasing food security, eliminating poverty, and maintaining the environment (Hunter 2012). Nowadays there is a great effort by several national, regional, and international organizations and institutions, such as Biodiversity International, the Food and Agriculture Organization of the United Nations (FAO), and Crops for the Future (CFF) in the research and development of underutilized plant species (Food, 2010). Crop diversification, can be not only a strategy to protect global food supplies, but also a tool to combat hunger, malnutrition, and over nutrition (Hawkesworth *et al*., 2010). It is also a way to improve adaptability to extreme climatic conditions, provide resilience to biotic and abiotic stresses and produce harvestable yields where major crops may fail (Hawkesworth *et al.*, 2010; Padulosi and Hoeschle-Zeledon, 2004). Some genetic studies have been done on fig and pistachio diversity in the world (Baraket *et al*., 2011; Caruso *et al*., 1997; Essid *et al*., 2015; Giraldo *et al*., 2008; Ikegami *et al*., 2009; Kafkas and Perl-Treves, 2001; Parfitt and Badenes, 1997).

Special efforts are needed also to improve the cultivation, management, harvesting and post-harvesting of underutilized species. Minor crops are less competitive with the major crop that are supported by seed supply systems, production and post-harvest technologies and extension services. In addition, their markets are well-established and consumers are familiar to using them. In order to bring underutilized species back into cultivation, is necessary to promote scientific research including agronomy, breeding, post-harvest handling and value addition, and linking farmers to markets (Chivenge *et al*., 2015; Padulosi and Hoeschle-Zeledon, 2004).

In the Mediterranean countries, the most important fruits, in terms of the volume of harvested products, are apples (9.6 million tons), oranges (13 million tons) and peaches (5.5 million tons) (Table 2). Other Mediterranean crops, such as figs, loquats, pomegranates, pistachios, have received little attention but are now being re-emphasized in areas with Mediterranean climates for diversification and revitalization of local agriculture (Tous and Ferguson, 1996). The variety of fruit crops grown in a given region is influenced from climate, accessibility, trade and ability of plant breeders. Many other fruits such as pistachios and figs are often produced on a limited scale and they are consumed and traded locally, despite their good quality, tolerance to grow in arid areas and dry, and salinity lands.

Table 2 - Fruits crop production in Mediterranean countries (Source: FAO, Statistics Division FAOSTAT 2014).

Item	***Value***
Grapes	28440558
Oranges	13353083
Apples	9667298
Tangerines, mandarins, clementines, satsumas	6909108
Peaches and nectarines	5544972
Lemons and limes	2805949
Pears	2391007
Plums and sloes	1247082
Cherries	856005
Figs	848588
Almonds, with shell	612927
Hazelnuts, with shell	552257
Carobs	143406
Pistachios	94270

1.5 Underutilized fruit crops and salt tolerance

Climate change, degradation of land and water resources shortage have led to growing interest with crops and species that are adapted to challenging environments. Climate changes over the past 30 years have made the region much less fertile. This will accentuate

water deficit in this region, reduce length of growing seasons and force large areas of marginal agriculture out of production (Cook *et al*., 2016). Most of these underutilized crops have many remarkable characteristic such as the ability to withstand drought, flooding, temperature extremes, and pests and diseases largely than current major staples. Their potential is given first, by providing genetic traits for adaptation and second by strengthening the resilience of agroecosystems through crop diversification (Mayes *et al*., 2011; Padulosi *et al*., 2011). Fig and pistachio can be considered minor fruit crops in the Mediterranean countries but for their drought and salinity resistant, in the last years there is an increasing interest. (Caruso *et al*., 2017; Soliman and Alhady, 2017). Caruso *et al*., (2017) have studied salinity tolerance and physiological response of young fig tree and their results confirm that fig plants are moderately tolerant to salinity. Several studies have been conducted on pistachio, that is considered highly tolerant (Behboudian *et al*., 1986; Walker *et al*., 1987). Among Pistacia species, P. *atlantica* has been described as a highly tolerant to the salinity (Ferguson *et al*., 2005).

1.6 Underutilized crop and nutrition

Enhancing the use of neglected and underutilized species to better know their potential with regard to food and nutrition security, ecosystem sustainability and adaptation to climate change has been identified as an important strategic element for developing more productive, sustainable and resilient agricultural production and food systems (Padulosi *et al*., 2011).

Large parts of the world's population, especially in South Asia and Africa, suffer from nutrient deficiencies, because the affected people have an insufficient intake of vitamins and minerals. Half billion of the people in the world suffer from protein-energy malnutrition but over 1.6 billion suffer from iron deficiency, over 200 million from vitamin A insufficiency (Benoist *et al*., 2008). For this reason there is a greater demand for increased dietary diversity and for novel and nutritionally healthy foods, vitamins and other micronutrients are today being searched in crops and plant species. Underutilized crops provide essential micronutrients and thus complement staple foods, many of them have become popular in the developed countries due to their nutritional properties (Massawe *et al*., 2016).

This study is focused on two minor fruit tree crop diffused in the Mediterranean countries, Pistachio (*Pistacia vera L.*) and Fig (*Ficus carica L.*). In the last years, the interest for these two crops is increased for their nutritional values and for their economic importance due to their drought and salinity resistant. Pistachios and Figs are cultivated in the Mediterranean countries since early times (Zohary *et al*., 2012). They give a contribution to local diets, suppling some vitamins and minerals. Pistachio nuts are consumed roasted and salted, shelled or unshelled, and are used in ice creams, sweets, cakes. Pistachio is a nutrient-dense nut with a heart-healthy fatty-acid profile as well as protein, dietary fiber, potassium, magnesium, vitamin K, γ-tocopherol, and a number of phytochemicals. Among nuts, pistachios contain the highest levels of potassium, γ-tocopherol, vitamin K, phytosterols, and xanthophyll carotenoids. Experimental clinical studies suggest that pistachios help maintain healthy antioxidant and anti-inflammatory activity, glycemic control, and endothelial function (Dreher, 2012).

Figs fruits are consumed fresh or dried, used main for snack foods or as a confectionery ingredients. Also figs are an excellent source of minerals, vitamins and dietary fiber; they are fat and cholesterol-free and contain a high number of amino acids (Veberic *et al*., 2008). Some studies have described the existence of several phenolic compounds and phytosterols and fatty acid in fruits (Jeong and Lachance, 2001; Vaya and Mahmood, 2006; Veberic *et al*., 2008) *Ficus carica* latex is a rich source of natural anti-oxidant substances with established features of anti-carcinogenic properties that might be considered in biopharmaceutical industries (Hashemi *et al*., 2017).

The economic importance of fig production is likely to continue into the future with an increasing demand for both fresh and dried figs, and derived products.

1.7 Aim of the research

Aim of the research activity developed within this PhD dissertation was to improve knowledge of salt tolerance mechanism in Pistachio rootstocks and increase knowledge on the biodiversity of Fig (*Ficus carica L.*) in South Italy. Two minor crops that in the last years have an increasing interest for their resilience to environmental stresses. Particularly, in

pistachio has been carried out a study on the mechanism of salt tolerance. To this aim, researches have been conducted on “in vitro” plants to identifying of sodium uptake, ion compartmentalization and its effect on subcellular morphology. In Fig, the study has been focalized on the biodiversity that the species expressed in South Italy, an area in which, in the time course of century, has been developed a niche industry, for both fresh and dry processed fruits, today appreciated in the European market. In details, aim of the study has been to characterize main cultivar and clones, to collect the genotypes and evaluate it from both horticultural and quality point of view. It is very important to characterize and evaluate as wide a range of germplasm as possible for avoidance, resistance or tolerance to major stresses such as drought, heat, and soil salinity. It is also necessary for a better understanding of the physiological mechanisms, biochemical pathways and genetic systems involved in relevant genetic traits (Frison *et al*., 2012).

Experiment One

"Genetic diversity of fig (*Ficus carica* L.) genotypes grown in Southern Italy revealed by the use of SSR markers"

2.1 Introduction

The edible fig is one of the first plants that was cultivated by humans; its domestication came long before the domestication of other important fruit crops like grapes, olives, and dates. It is considered to be one of the oldest fruit trees in the Mediterranean basin and is widely grown and harvested for its dry and fresh consumption (Patil and Patil, 2011). Evidence for the first cultivation of figs has been found in the Lower Jordan Valley in an early Neolithic village known as Gilgal, where nine subfossil parthenocarpic fruits (and therefore sterile) were found (Zohary *et al*., 2012). The fig is originated from the Eastern Mediterranean region (Turkey, Syria, Saudi Arabia), from where its cultivation expanded to the whole of the Mediterranean region (Janick, 2005; Zohary *et al*., 2012). Spread to the Mediterranean, fig is now widely grown through the world, in typical areas with mild winters and hot dry summers (Tous and Ferguson, 1996). Recently fig tree (*Ficus carica L.*) has increased interest for its economic importance and medicinal benefits (Çalişkan and Polat, 2011). Wild forms of fig are found in Mediterranean countries such as in Algeria, Egypt, Syria, Tunisia, Turkey, as well as in the Arabian Gulf and in Central Asia (El-Rayes, 1995).

The common fig (*Ficus carica* L.; $2n = 2x = 26$) belongs to the *Moraceae* genus *Ficus*, a family with over 1400 species distributed in about 40 genera (Weiblen, 2000). The genus *Ficus L*. contains about 750 species of woody trees, epiphytes and shrubs, divided into six subgenera that share a unique inflorescence, the syconium (Ronsted *et al*., 2008). The sub-genus *Eusyce*, to which *F. carica* belongs, is characterized by having unisexual flowers only and gynodioecism (Flaishman *et al*., 2008).

Fig inflorescence is unique, consisting of a syconium, which encloses many unisexual flowers that can be accessed via the ostiole by pollinating wasps that may give the true fruits (Storey, 1975). All *Ficus* species are either dioecious, consisting of separate male and female plants,

or, in the case of *F. carica*, gynodioecious, consisting of hermaphroditic and female plants. Most of the edible fig varieties are parthenocarpic; these produce seedless fruit and due to this have been frequently propagated by cuttings (Starr *et al*., 2003). There are three types of female figs, grown commercially (Flaishman *et al*., 2008):

- The common-type that develops fruit parthenocarpically without pollination and can produce one (unifera varieties) or two (bifera varieties) crops;
- The Smyrna-type that requires pollination with pollen from caprifigs;
- The San Pedro-type that produces a first crop parthenocarpically (breba) and a second crop (fig) only after pollination with pollen from caprifigs.

Ficus pollination is dependent on the coevolution of *Ficus* species with pollinating wasps of the family Agaonidae (Herre *et al*., 2008). In the case of the common fig, pollination (caprification) is performed by a specific pollinating insect, *Blastophaga psenes L*. (Flaishman *et al*., 2008). For every *Ficus* species, usually a unique Agaonid wasp is associated with it. Fig wasps only lay their eggs inside the florets, pollinating the fig in the process (McLeish and Van Noort, 2012). Therefore, figs that require pollination cannot be cultivated or become naturalized without the presence of the associated pollinator wasp.

2.2 Economic importance of fig production

The fig tree is tolerant to a wide range of environmental conditions; it is tolerant to drought, although it grows most vigorously with abundant water. Figs can be grown on a wide range of soils, including heavy clays, loams, and light sands. The plant is moderately tolerant of high salinity (Tous and Ferguson, 1996). A recent study has investigated salt tolerance and physiological response of young *Ficus carica L*. plants irrigated with saline water and confirms that fig plants are moderately tolerant to salinity (Caruso *et al*., 2017). Despite the good adaptation of fig trees to salinity only few studies have been conducted to determine the effect of salinization (Abdoli Nejad and Shekafandeh, 2014; Okubo and Utsunomiya, 1996). Fig can stand in marginal lands, but before it is necessary to improve knowledge of its biodiversity as well its salinity mechanism of tolerance.

The economic importance of fig production is likely to continue into the future with an increasing demand for both fresh and dried figs, and derived products. The fresh and dried fruits are an important source of vitamins, minerals, carbohydrates, sugars, organic acids, and phenolic compounds (Çalişkan and Polat, 2011; Veberic *et al*., 2008). Thanks to the nutritional use of its products and employment in the confectionary industry, in some areas there is the increasing of the intensive cultivation of figs to produce fruits for fresh consumption, drying and processing. Therefore, there is the need to characterize local cultivars and clones for the vegetative propagation of the plants and its nursery certification.

2.3 Fig production in the Mediterranean countries

The production of the fig tree fluctuates in the Mediterranean countries: it is largely related to the annual rainfall, climatic conditions and soil properties (Flaishman *et al*., 2008). Among the Mediterranean countries, Turkey is the first in fig production with 300.282 tonnes (FAOSTAT 2014). In Italy, the common fig, usually present in family orchards, is considered as a minor fruit species (FAOSTAT, 2014). In the 2004 – 2014 decade there was a high decrease of fig production in Italy (Table 3), despite there is a great rise of interest in the drought resistant crop such as fig, which can produce sustainably in arid and semiarid area, is taking place.

Table 3 - Fig cultivation in the main producing countries of the Mediterranean basin in the decade 2004 - 2014 (Source: FAO, Statistics Division FAOSTAT 2014).

Area	***Year 2004***	***Year 2014***
Turkey	275000	300282
Egypt	160124	176105
Iran	80769	72672
Algeria	64940	128620
Morocco	60000	126554
Spain	41297	28896
Tunisia	27000	27000
Greece	21545	10160
Italy	21226	10788
Albania	15000	19350
Lebanon	9600	4829
France	3562	2780
Jordan	3468	927
Malta	209	84
TOTAL	**783.740**	**909047**

2.4 The Italian fig germplasm

Italy has the richest germplasm among the different countries where the species is traditionally cultivated (Condit, 1955). Pliny indicated the presence of twenty-nine varieties in Italy since the I century A. C. The richness of Italian diversity of fig varieties is reflected in the works of Della Porta (1583), Aldrovandi (1668) and Cupani (1696). In the early XIX century, Gallesio described numerous varieties in his monumental work "Italian Pomona" (1820) and, for some of them reported some wonderful botanical illustrations. Afterward several authors have dealt with the Italian varietal heritage of this species (De Rosa, 1901; Vallese, 1909; Guglielmi,

1908; Donno, 1948, Donno, 1951 a; b; Donno, 1952; Chessa and Nieddu, 1990; Ferrara and Vendola, 1990, Grassi *et al*., 1990; Grassi, 1997).

The most economically important regions for fresh and dried fig production in Southern Italy (ISTAT, 2013) are Apulia (Salento area); Calabria (Cosenza area); Campania (Cilento-Salerno area) and Sicily. In those areas the production of dried figs is deeply connected with local food traditional and cultural heritage and the industry, represented by local specialized companies, transforms dried figs in various traditional products (‘palloni’, ‘corolle’, ‘crocette’) (Grassi, 1997).

In Calabria, 86% of the cultivated area is in Cosenza province, where fig orchards occupy an area of 902 hectares in 2010 (6th General *Census of Agriculture*). In Calabria and Campania, the main production of dried figs is based on the cultivar Dottato, admitted in two different Protected Designation of Origin (PDO): “Fichi di Cosenza” (Calabria) and “Fico Bianco del Cilento” (Campania). The cultivar ‘Dottato’ (syns. = ‘Ottato’, ‘Uttato’, ‘Ortata’, ‘Fico Bianco’, ‘Kadota’) (Gallesio, 1820; Vallese, 1909; Condit, 1955; Grassi, 1990) has ancient origin. The Greek cultivated it since 450 B.C. for fresh or dried consumption; it was introduced in Southern Italy prior to the VI century B.C. Its name may derive from the Greek words *optào* or *optetéon* «to be dried or baked» (Grassi, 1990). ‘Dottato’ develops parthenocarpically both the first crop (breba) and second-crop (figs), which represent the main production. In Cosenza area, traditionally the growers prevent the caprification by eliminating wild figs near the orchards, to avoid that seeds get tough. This cultivar has been considered among the best for the production of dried figs (Eisen, 1901; Vallese, 1909; Casella, 1953; Condit, 1955). The ripening period is on August, in Cosenza area, fruits do not fall from the tree and they start to dry (passuluni) on the same plant (Grassi, 1991). Local growers, indicating that it may be a polyclonal cultivar or cultivar-population, have observed a certain amount of morphological and phenological variability within the ‘Dottato’ genotypes. Although several work of description of varieties of figs grown in Italy have been reported, there is a lack of description of ‘Dottato’ ecotypes and/or clones.

Although there are many descriptions of the varieties of fig grown in Italy, they are incomplete, lacking standardized method of characterization. In most cases, cultivars were described in their area of cultivation and therefore in different environments, which affected biometric and morphological characters, making difficult the comparison between genotypes,

especially among those that exhibit a high degree similarity. In addition, there are many traditional Italian cultivars with identity poorly studied and are often confused by the occurrence of homonyms and synonyms. Thus, there is the need to make order and clarity on the fig genetic resources present in Southern Italy. The denominations of the cultivars are usually based on the color, size and time of fruit ripening or geographical origin resulting in confusion in nomenclature. In future, appropriate characterization and differentiation among cultivars is necessary to optimize fig germplasm management and conservation, make vulnerable by intensive urbanization, cultivation of selected clonal varieties or biotic and abiotic stresses. Various studies have reported the use of morphological traits and isozyme markers for fig characterization (Çalişkan and Polat, 2008; Chatti *et al*., 2004; Chessa and Nieddu, 2005; Giraldo *et al*., 2010a; Papadopoulou *et al*., 2002). Since morphological and agronomic characters are influenced by environmental conditions and they can vary depending on the complex floral biology of fig, molecular markers are used as complementary tool to assess the genetic diversity. In fig, many genetic study have been conducted with various molecular methodologies, such as isozymes (Chessa *et al*., 1997), RAPDs (Khadari *et al*., 1995), AFLPs (Cabrita *et al*., 2001) and SSRs (Achtak *et al*., 2009; Giraldo *et al*., 2008; Khadari *et al*., 2005).

2.5 Microsatellites (SSRs) description and usage in fruit tree

Nowadays science gives us the opportunity to improve our knowledge of plants evolution with molecular data. Despite the progress that has been made using next generation sequencing technologies, molecular markers, and in particular microsatellites, continue to be developed and used (Achtak *et al*., 2009; Perez-Jiménez *et al*., 2012). Traditionally, plant germplasm characterization has been carried out using morphological and agronomic traits with fluctuations among years, environments and repetitions, creating difficulties in varietal identification of this species (Giraldo *et al*., 2010b). Microsatellites or SSR (SSRs) have become the markers of choice for fingerprinting and analysis of genetic diversity in most plant species (Gupta and Varshney, 2000) due to their high level of polymorphism, codominant Mendelian inheritance, reproducibility, and easy detection through PCR and electrophoretic

methods. SSR's markers can be used to define the population, and to define potential progenitor's origin (Feng *et al*., 2009).

Microsatellites (SSR) or simple sequence repeats are co-dominant high polymorphic DNA markers. In general, co-dominant markers are more informative than the dominant markers. Microsatellites are the commonly used highly polymorphic markers for assessment of population structure and differentiation. In addition, microsatellites are widely used in other fields of genetics: genetic conservation, molecular breeding, paternity testing, population genetics, etc. It is possible to track each individual and investigate the evolutionary history of species; also it can help to reveal genetic relationships between individuals and to estimate population genetic structure (Hoshino *et al*., 2012; Ikegami *et al*., 2009). Various molecular markers such as Simple Sequence Repeats (SSR) have been used for DNA fingerprinting studies in fig as well as for germplasm characterization and analysis of genetic diversity (Achtak *et al*., 2009; Aradhya *et al*., 2010; Ferrara *et al*., 2016; Khadari *et al*., 2005). These studies have conferred several advantages since they show that the markers are distinct and reliable and are of great assistance to manage important genetic resources.

2.6 Aim of the researches

In 2014, the Universities of Palermo, Reggio Calabria, Napoli and Bari and the region Basilicata started a collaborative study and conducted an extensive investigation in traditional fig growing areas to identify different fig cultivars and genotypes, in order to collect new knowledge on fig genetic resources originated in Southern Italy. The main objective of this study was to characterize and evaluate the genetic diversity of 181 accessions collected in small farms located in Campania, Basilicata, Apulia, Calabria and Sicily, using SSR markers in order to develop strategies to preserve the endangered genetic resources of this species, to organize conservation strategies and for the promotion of regional development.

2.7 Materials and Methods

Plant Material

The experiment was carried out at the department of Agricultural, Food and Forestry Sciences (SAAF) of the University of Palermo, thanks also to the collaboration with the Department of Agriculture of the University of Naples Federico II, the Department of Soil Science, Plant and the food of the University of Bari, the Department of Agraria, of the Mediterranean University of Reggio Calabria, and the "l'Azienda Agraria Sperimentale Didattica Pantanello Alsia" of Basilicata region and the "Azienda Agraria Didattica I giardini di POMONA" of Apulia. Young leaves were collected from 181 figs accessions originated in South Italy.
Moreover a total of 24 'Dottato' genotypes: 22 originated in Calabria, one in Cosenza area, one from Campania and one grown Apulia were added as controls (Table 4).
Samples were frozen in nitrogen liquid until use.

Table 4 - List of 'Dottato' genotypes analyzed. Reference genotypes in bold.

Dottato Amendolara	Dottato Rose
Dottato Cosenza	Dottato Trebisacce
Dottato Francavilla Marittima	Fico bianco Canna
Dottato Luzzi	Fico bianco Cosenza 1
Dottato Montalto Uffugo 1	Fico bianco Cosenza 2
Dottato Montalto Uffugo 2	Fico bianco Cosenza 3
Dottato Oriolo 1	Fico bianco Cosenza 4
Dottato Oriolo 2	Fico bianco Cosenza 5
Dottato Plataci 1	Fico Bianco San Demetrio Corone 2
Dottato Plataci 2	Ottato bianco Pedivignano
Dottato Plataci 3	Ottato bianco Zumpano
Dottato Rocca Imperiale 1	**Dottato (Apulia)**
Dottato Rocca Imperiale 2	**Dottato (Campania)**

DNA Extraction

Genomic DNA was extracted following the method of Doyle and Doyle (1987) with modifications. Leaves were homogenized in a 2 ml tubes, frozen in liquid nitrogen, by using a using a miller suitable for cryogenic grinding Retsch Mixer ball Mill. CTAB extraction buffer (800 µl/sample), containing polyvinylpolypyrrolidone 40 (2%), preheated at 65 °C, was added in the tubes, without beta-mercaptoethanol. Samples were incubated in a bath at 65 °C for 5 min, mixed with a vortex every minute. An equal amount of Chloroform: Isoamyl alcohol (24:1 v/v) was added to the supernatant and mixed by agitating the tubes several times for a 30 s. Samples were put in a centrifuge at 13000 rpm for 10 min. Supernatant was recovered and placed in a 1.5 ml tube, where 450 µl/sample of ice-cold isopropanol were added. Samples were stored in freezer at -20 °C overnight; then they were centrifuged at 13000 rpm for 5 min. Pellet was washed with ice-cold ethanol (70%), centrifuged at 13000 rpm for 5 min, air-dried for at least 4 h and re-suspended in 30µL of TE. DNA was quantified and checked with a Nanodrop spectrophotometer; usually 50-70 ng of genomic DNA were recovered.

SSR primers and PCR reactions

Genomic DNA was amplified with 18 fluorescently labelled microsatellite (SSR) markers (Khadari *et al*., 2001; Giraldo *et al*., 2005; Ahmed *et al*., 2007; Achtak *et al*., 2009; Bandelj *et al*. 2007) that have been successfully used in previous studies of figs. (Table 5).

PCRs were carried out in a reaction volume of 8 µL, containing 10 ng of genomic DNA, 1xMultiplex PCR master mix (Qiagen) and 0.2 µL of each primer pairs. The following cycling steps for touch down PCR were used: 95°C for 15 min; ten cycles: 94°C for 30 s, 60°C for 1 min 30 s, with 1°C of temperature reduction for each cycle, 72 °C for 1 min; 25 cycles: 94°C for 30 s, 50°C for 1 min 30 s; and finally 65°C for 30 min. Amplicons were separated on an automatic sequencer ABI3130 (Applied Biosystems) and alleles were sized using GeneMapper 4.1 software (Applied Biosystems).

Data Analysis of SSR Markers

Number of alleles/locus (No), observed and expected heterozygosity (*Ho* and *He*) and polymorphic information content (PIC) values were calculated with Power Markers v.3.25 (Liu and Muse, 2005). Genetic relationships among the fig accessions were represented by UPGMA cluster analysis of the similarity matrix obtained from the proportion of shared amplified fragments (Nei and Li, 1979) with the software Power Markers v.3.25, in order to illustrate the genetic diversity among genotypes. To evaluate the genetic structure of fig germplasm, the software Structure (Pritchard *et al*., 2000) was employed, using the 'admixture' model, assuming 1-10 populations (K), a burn-in length of 10,000, followed by 90,000 runs at each K, with 10 replicates for each K. The log likelihood for each K (L (K)) was used (Rosenberg *et al*., 2002) to determine the right number of populations (K). Genotypes having membership probabilities ≥0.80 were considered to belong to the same group, sharing a close genetic background.

Table 5 - *Ficus carica L.* microsatellite loci, references, repeat motifs and Nucleotide sequence accessions number.

Marker	**References**	**Repeat motif**	**Nucleotide sequence Accession No**
MFC1	*Khadari et al. 2001*	(CT)13	AF333696
MFC2	*Khadari et al. 2001*	*(AC)18(AT)7*	AF333697
MFC3	*Khadari et al. 2001*	*(AC)15TC(AC)8(AT)7*	AF33369
MFC7	*Khadari et al. 2001*	(AG)11	AF333702
MFC8	*Khadari et al. 2001*	(CA)9TA(CA)14(TA)6	AF333703
LMFC12	*Giraldo et al. 2005*	(CT)55	AY545926
LMFC13	*Giraldo et al. 2005*	(GA)28	AY545927
LMFC19	*Giraldo et al. 2005*	(AT)11(AG)12	AY545932
LMFC24	*Giraldo et al. 2005*	(CT)10	AY545937
LMFC26	*Giraldo et al. 2005*	(GA)15	AY545939
LMFC28	*Giraldo et al. 2005*	(CT)14	AY545941
LMFC30	*Giraldo et al. 2005*	(CT)18(CA)6	AY545942
LMFC32	*Giraldo et al. 2005*	(GA)23	AY545944
FCUP027_4	*Bandelj et al.2007*	(AC)19	EF198058
FCUP038_6	*Bandelj et al.2007*	(TG)23T(AG)11	EF198059
FM4-70	*Zavodna et al.2005.*	(GAA)20−1	AJ854076
MFC11	*Khadari et al. unpublished data*		
MFC9	*Khadari et al. unpublished data*		

Descriptors list for pomological analysis

For the morphological characterization, the biometrical traits were considered, following the descriptor list of Grassi and Pugliano (1984). The variable measured are:

- fruit weight (g);
- fruit length (mm);
- stalk length (mm);
- stalk thickness (mm);
- neck length (mm);
- fruit width (mm);
- width/length of fruit;
- maximum diameter position;
- ostiole width (mm);
- length (mm) and width (mm) of monolobate, trilobate or pentalobate leaves;
- petiole length (mm)
- petiole width of monolobate, trilobate or pentalobate leaves;
- monolobate, trilobate or pentalobate leaf length.

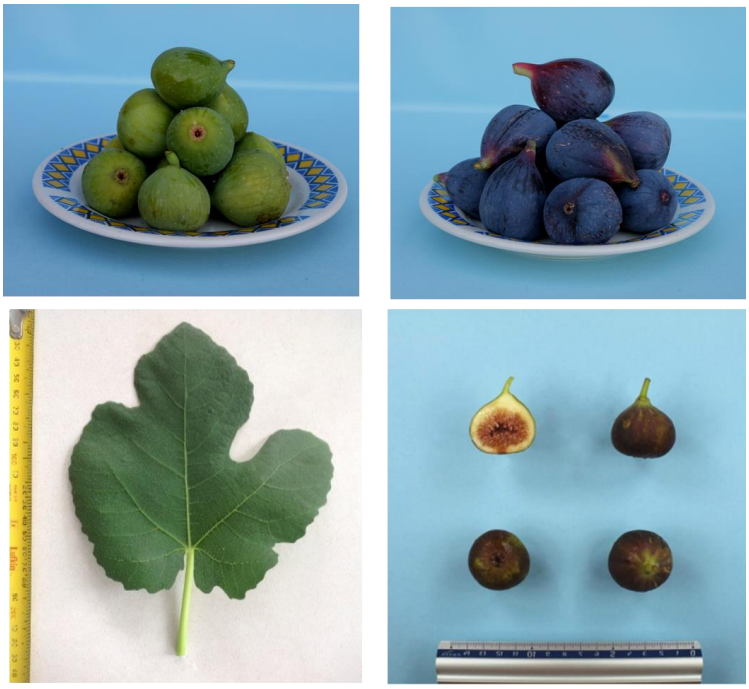

Figure 1 - Some of morphological traits analyzed

Data Analysis of Morphological Traits in the 'Dottato' genotypes

The results of the biometrical traits were used for computing the Multivariate Analysis. In particular, a Canonical Discriminant Analysis has been performed using Systat statistical software (Systat Software, Inc.).

2.8 Results

Molecular analysis of South Italian accessions

Regarding the molecular characterization, the SSR markers revealed a large genetic diversity among the accessions. A total of 117 alleles were detected with a mean of 6.5 per locus (Table 6). The average expected (He) and observed heterozygosity (Ho) were 0.56 and 0.66, respectively. The mean polymorphic information content (PIC) was 0.51, varying from 0.78 (LMFC30) to 0.21 (LMCF26), indicating a high molecular diversity among the fig accessions of the Southern Italy germplasm. The most polymorphic primers were FCUP038_6, amplifying 13 alleles and MFC3 detecting 10 alleles. The average allele per locus was 6.44.

Cluster Analysis

The UPGMA cluster analysis allowed to discriminate 174 genotypes and to identify eight groups of identity (Figure 2). The accession 'Bianca d'Agosto' from Basilicata and the genotype"FCS138" from Calabria were the most diverse. This study showed that in some cases fig accession grouping followed the area of cultivation, but also many genotypes, especially from Campania, presented uneven distribution (data not shown).

In the Structure analysis, fig genotypes were grouped in four populations (Figure 3). The Dottato genotypes belonged to the yellow population, while most of the "Melanzana type" figs were included in the black population. The name "Melanzana" indicates the resemblance of the colour of these figs to that of an eggplant. The black population contained also the cultivar 'Paradiso'. The green population contained some late accessions such as 'Nvernitica', 'Natalina', 'Regina' and 'Troiano'. The red population was quite heterogeneous, as regards the phenology (ripening time), the type of fruit (brebas or figs) and the characteristics of the fruit. It included accessions producing big and good quality brebas ('Fiorone San Giovanni',

'Fiorone Sant'Antonio') and other accessions that need to be further characterised. Many accessions presented mixed genetic background.

This analysis is important for the "Dottato" genotypes that are included in the same population. A second UPGMA analysis was performed in order to understand the relationship within the 'Dottato' genotypes.

Table 6 - SSR statistics: loci names, number of alleles (No), references, values of observed (Ho) and expected (He) heterozygosity and polymorphic information content (PIC).

Marker	Allele No	He	Ho	PIC
FCUP027_4	6	0.68	0.87	0.63
FCUP038_6	13	0.72	0.81	0.69
FM4_70	8	0.50	0.43	0.46
LMFC12	6	0.53	0.70	0.44
LMFC13	5	0.48	0.58	0.42
LMFC19	7	0.40	0.37	0.38
LMFC24	4	0.36	0.41	0.31
LMFC26	4	0.22	0.24	0.21
LMFC28	6	0.64	0.63	0.58
LMFC30	9	0.80	0.90	0.78
LMFC32	5	0.37	0.20	0.34
MFC1	6	0.67	0.99	0.61
MFC2	8	0.67	0.91	0.61
MFC3	10	0.67	0.79	0.62
MFC8	4	0.49	0.76	0.38
MFC7	5	0.61	1.00	0.54
MFC9	5	0.51	0.36	0.44
MFC11	6	0.68	0.97	0.64
Mean	**6.44**	**0.61**	**0.66**	**0.51**

Figure 2 - Dendrogram illustrating the genetic relationships among 181 genotype using SSRmarkers by UPGMA algorithm.

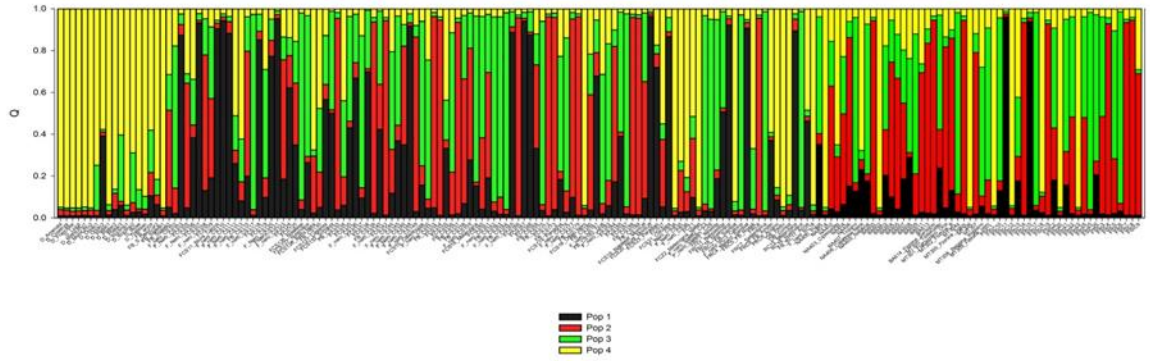

Figure 3 - Inferred population structure for K = 4 of 181 fig accessions from Southern Italy.

Molecular analysis of "Dottato" genotypes

The molecular characterization with the SSR markers of the 24 Dottato" genotypes, revealed a moderate genetic variability. The most polymorphic primers were FCUP038_6, amplifying 9 alleles and MFC1 detecting 5 alleles. The average allele per locus was 3.39; the average expected and observed heterozygosity were 0.42 and 0.62, respectively. The mean polymorphic information content (PIC) was 0.4, varying from 0.08 (LMFC26) to 0.67 (FCUP 38_6) (Table 7).

Cluster analysis

In the clustering analysis, illustrated by the UPGMA dendrogram (Figure 4), all genotypes were discriminated, a part from two groups: 'Dottato Montalto Uffugo 1' and 'Ottato Pedivigliano'; 'Fico bianco Cosenza 4', 'Dottato Cosenza', 'Dottato Amendolara' and 'Dottato Montalto Uffugo 2'. It was evident a high genetic similarity within genotypes due to the occurrence of somatic mutations, indicating that in Cosenza area 'Dottato' fruits, seem obtained from a population of clones and therefore 'Dottato' is polyclonal cultivar. The low level of genetic diversity, observed among 'Dottato' genotypes, may be due to clonal selection performed by local growers for fruit quality and parthenocarpy, and exchange of material of propagation.

Table 7 - SSR loci names, number of alleles (No), references, and values of observed (Ho) and expected (He) heterozygosity and polymorphic information content (PIC).

Locus	**Allele No**	***He***	***Ho***	**PIC**
FCUP027_4	4	0.68	0.87	0.63
FCUP038_6	9	0.71	0.87	0.67
FM4_70	3	0.44	0.39	0.40
LMFC12	3	0.52	0.65	0.40
LMFC13	3	0.47	0.52	0.42
LMFC19	2	0.04	0.04	0.04
LMFC24	3	0.12	0.04	0.12
LMFC26	3	0.08	0.09	0.08
LMFC28	3	0.33	0.39	0.30
LMFC30	2	0.50	0.96	0.37
LMFC32	3	0.23	0.26	0.22
MFC1	5	0.69	1.00	0.64
MFC2	4	0.54	1.00	0.44
MFC3	2	0.50	0.96	0.37
MFC7	4	0.59	0.96	0.51
MFC8	2	0.50	1.00	0.38
MFC9	3	0.12	0.13	0.12
MFC11	3	0.54	1.00	0.43
Mean	**3.39**	**0.42**	**0.62**	**0.36**

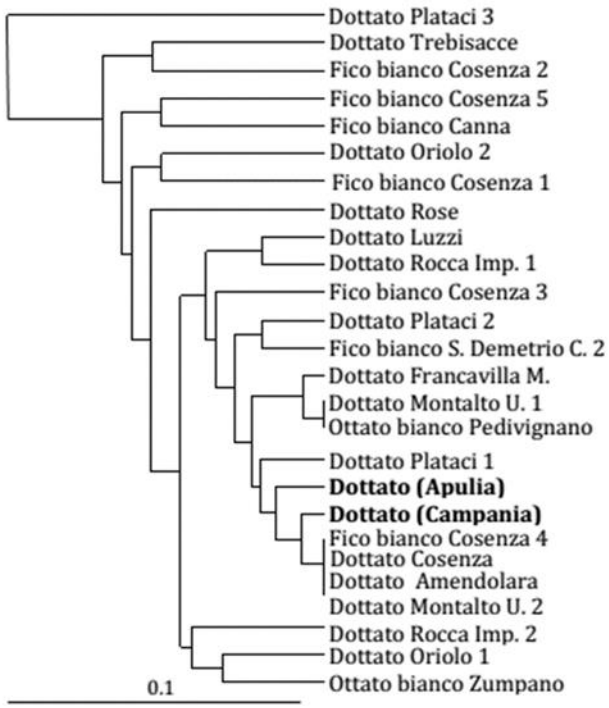

Figure 4 - Dendrogram illustrating the genetic relationships among 'Dottato' genotype using SSR markers by UPGMA algorithm. Reference genotypes in bold.

Analysis on morphological traits in the "Dottato" genotypes

The biometric data were statistically analyzed by Multivariate Discriminant Analysis or Canonical Discriminant Analysis CDA. In that analysis, the combination of the first three CDFs explained 55% of the total variance. CDF1 explained 29% of the total variance; CDF2 described 13% of the total variance and finally, CDF3 an additional 13% (Figure 5). Most different 'Dottato' genotypes from the standard 'Dottato Cosenza' resulted: 'Dottato Trebisacce'; 'Dottato Amendolara'; 'Fico Bianco Cosenza 1'; 'Fico bianco Cosenza 2'; 'Fico bianco Cosenza 3'; 'Ottato bianco Zumpano'; 'Dottato Oriolo 2'; 'Dottato Plataci 1' and 'Dottato Plataci 3'. Figs differed mainly for the width of the fruit and the ostiole width; in some cases for the ratio width/length of fruit and petiole length/leaf length (monolobate or trilobate or pentalobate). Since 'Dottato' genotypes were cultivated in different areas and under different growing conditions, some morphological differences within them may be

minimized when they will be re-analyzed in the ex-situ fig collection, recently established by the University Mediterranean of Reggio Calabria. Nineteen genotypes were uniform and this homogeneity is very important for industrial processing and for the protection of the PDO.

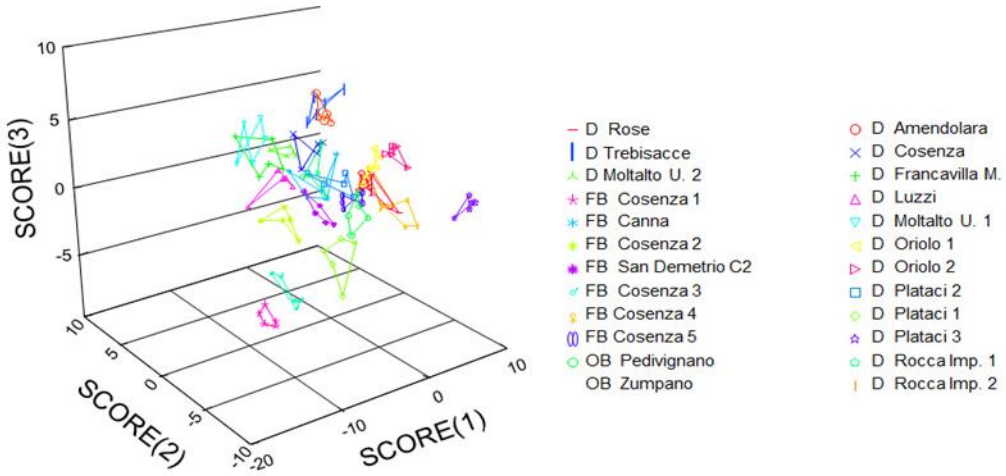

Figure 5 - Plots of first three canonical discriminant functions, calculated on the basis of the biometrical traits measured in 24 Calabrian 'Dottato' genotypes.

2.9 Discussions and conclusions

With the exception of the two PDO productions, in South Italy the fig industry is based on fruits produced from trees which genetic basis are still uncertain. In order to move the fig production from family enterprise to a sustainable industry, it is necessary the availability of fruits having a proper quality standard and this, in turn, largely depends on the genetic basis of the tree that have to be fixed. For this reason was examined the genetic diversity of 181 South Italian fig genotypes using 18 microsatellites. The 18 SSR loci were selected following three molecular and genetic criteria: 1) clear florescent peaks; 2) no ambiguous scoring data; 3) efficient detection of alleles in a large gene pool. Microsatellite markers are now widely used for molecular characterization in fruit trees: grape (Tessier *et al*., 1999), olive (Marra *et al*., 2013), pistachio (Pazouki *et al*., 2010), cheery (Marchese *et al*., 2007) and other fruit trees.

In the present study, the results of a microsatellites investigation suggest a large diversity that characterize the fig industry in South Italy. The average number of alleles (NA) per locus was 6.44, higher than the NA (5.2) obtained by Ikegami *et al*., (2009) using 13 microsatellites in a collection of 19 fig tree accessions, originating from Europe and China. In addition, it was higher than the 4.9 NA obtained by (Aradhya *et al*., 2010), analyzing 15 microsatellites in 194 fig tree cultivars from 11 different countries. And higher than the 3.6 NA found by (Perez-Jiménez *et al*., 2012) with 21 microsatellite markers in 57 Spanish fig tree accessions. Giraldo *et al*., (2008) observed a low polymorphism within an ex situ Spanish collection with 3.9 alleles per SSR locus and a relatively low genetic diversity (He = 0.38). However, the NA 6.44 of the present study (181 accessions) was lower than the 9.33 NA revealed with 6 microsatellites in 124 Tunisian genotypes by (Saddoud *et al*., 2007). South Italian fig germplasm presents a genetic diversity similar to that of other fig germplasm; for instance, the Mediterranean ex situ collection in the Porquerolles island, South of France, with a total of 807 fig trees and 383 accessions from several collections and traditional orchards of different Mediterranean countries. All these 807 fig trees accessions were analyzed and displayed an average of 6 alleles per locus and 0.54 of observed heterozygosity (Khadari, 2010). Similar value (6.8 NA was obtained also using 10 microsatellites in 76 Turkish accessions (Caliskan *et al*., 2012).

For all microsatellites utilized, the mean polymorphic information content (PIC) was 0.51, varying from 0.78 (LMFC30) to 0.21 (LMCF26) , showing that all loci were highly informative and suitable for genotype identification and were in agreement with (Perez-Jiménez *et al*., 2012) that found PIC values ranged from 0.239 (MFC2) to 0.798 (LMFC30).

The average expected heterozygosity (He) of 0.56 was higher respect to previously published works, 0.482 in 194 worldwide fig tree accessions (Aradhya *et al*., 2010), and 0.44 in 19 European and Asian fig tree varieties (Ikegami *et al*., 2009). This result indicated extremely large genetic diversity among the germplasm studied in South Italy, especially because the region analyzed is smaller than in others research. Moreover, similar data have been reported in Tunisian fig trees using different molecular markers (Baraket *et al*., 2009; Saddoud *et al*., 2007; Salhi-Hannachi *et al*., 2005). Overall, the gene pool of fig trees analyzed possesses substantial genetic polymorphism but at the same time, shows limited genetic differentiation

among the five different South Italian regions investigated probably due to a common origin of the cultivars.

UPGMA dendrogram revealed some cases of synonymy, but not as many as expected. In general, it seems possible that some of these genotypes are wrongly identified and named. It is common that genetically identical cultivars may have different names in different collections and, probably due to wrong use of names by growers, nurserymen, and traders, corruption in English transliteration of original names, the presence of variants within cultivars, and lack or poor documentation of passport data. Condit (1955), in his monograph on fig varieties, lists more than 700 cultivars and the majority have large numbers of synonyms. Many of the old and popular cultivars such as 'Kadota', 'Brown Turkey', 'Ischia Green' and 'Brunswick' often possess several synonyms and they generally possessed similar tree structure, morphology and fruit characteristics. Interpreting genetic identity and relationships among these cultivars is complicated due to occurrence of extensive synonymy and non-availability of reference cultivars for comparison.

The genetic structure observed in the present study should reflect a complex combination of natural evolution, genetic drifts and founder events during domestication, historical migration of cultivars along human migrations from the center of origin and diversity to secondary centers and regions of commercial production, and genetic modifications through modern plant breeding. Clonally propagated perennial species such as fig trees are known to carry relatively high genetic contents and tend to exhibit an excess of heterozygotes, as we have found in this study for the 181 accessions considered.

In the present study, morphological traits and microsatellite markers were used to detect for the first time the intra-cultivar variability of the cultivar 'Dottato'. This cultivar shows some peculiar agronomic traits that make it interesting for the potential use for the realization of orchards to improve the supply of commercial agricultural production niche. Twenty-four accessions of 'Dottato' from five regions of South Italy were collected and characterized. Both pomological and SSR markers are used for elucidating denomination problems and to find relationships among 'Dottato' cultivar.

The Canonical discriminant analysis was performed to identify patterns of variability among the putative clones and the relative standard 'Dottato Cosenza'. The combination of the first three CDFs explained the 55% of the total variance and it was considered statistically satisfactory in order to interpret our experimental results. The characters with greater discriminating ability were the width of the fruit and the ostiole, width/length of fruit and petiole length/leaf length (monolobate or trilobate or pentalobate). Thus, these characters should be preferred and prioritized in the complex activity of morphological characterization of the accession of the Dottato cultivar. Nineteen out of the Twenty-four "Dottato" genotypes analyzed show a certain uniformity; the differences found in the others five samples may be derive from difference in the environmental conditions, since they were cultivated in different areas.

The molecular characterization with the 18 SSR markers, revealed a moderate genetic variability, with several synonyms and homonyms that make difficult to identify the reference of 'Dottato' cultivar. The chosen SSRs revealed intra-cultivar diversity, caused by somatic mutations, leading to polyclonal cultivars, or by sexual reproduction, originated from natural crossing and subsequent seed dissemination leading to cultivar-populations. In the literature, high level of intra-cultivar genetic diversity has been reported in others species, for example a research conducted in the Sicilian olive germoplasm (Caruso *et al*., 2014). In their work 'Biancolilla', 'Giarraffa', 'Nocellara del Belice', 'Nocellara Messinese' and 'Moresca' represented a clear case of cultivar–populations, in which also clonal variants were present, whereas 'Cerasuola', 'Ogliarola Messinese' and 'Tonda Iblea' resulted a mixture of clonal variants (Caruso *et al*., 2014).

The study on morphological and genetic variability of 'Dottato' confirmed uniformity and homogeneity within this cultivar. The results could be further developed to verify authenticity and typicality of a plant product, and to mark and preserve the Protected Designation of Origin (PDO), Fichi di Cosenza. From the results, it seemed clear that the combination of the CDA with the cluster analysis of the molecular profiles is the most appropriate and reliable way to study relations among polyclonal cultivars and cultivar-populations and thus to verify intra-cultivar diversity.

The data proved that no geographical trends were observed among the cultivar ‘Dottato’ but also within the 181 accessions analyzed. Geographical closeness between Apulia, Basilicata, Calabria and Campania may have favored the exchange of plant material within the area and/or intercrosses.

As far as we know, this is the first study carried on fig germplasm in South Italy. Our results confirm the utility of morphological characters and molecular markers and their complementary information. The results also suggest the importance and the interest of taking into consideration the two types of approaches (morphological and molecular) to study genetic diversity within the wild inheritance of the fig tree. Molecular analyses in conjunction with morphological and agronomic evaluations are recommended, because these provide complementary information, increase the resolving power of genetic diversity analyses, and elucidate the domestication process. This has important implications for fig management and requires strategies to maintain longevity and genetic diversity of the species.

Knowledge of the genetic diversity range present in this germplasm will facilitate its use in breeding programs, improve the management of a core collection of fig, and guarantee this species' sustainability in the face of climate change predicted for the next years. Further analysis to determine possible associations between markers and quality characters should be carried out.

In conclusion, the research evidenced the richness of the available genetic resources in Southern Italy, resolved cases of synonymies and homonymies and helped to characterize fig accessions. It is an important preliminary work for establishing core collections or for planning on-farm conservation measures. The extensive molecular diversity found in the fig germplasm analyzed indicates a considerable potential for improving this crop. In the near future, the molecular characterization will be complemented with the morphological characterization of all accessions and thus we will have complete information on the real fig diversity in South Italy.

Experiment two:

"Characterization of salinity tolerance in Pistachio rootstocks"

3.1 Introduction

Pistachio is one of the most popular nuts tree around the world. Pistachios are as ancient as early civilization. It is believed to have originated in Central Asia 80 million years ago (Parfitt and Badenes, 1997), where it is still found growing wild in numerous dry lands and desert climate areas. It was introduced to Mediterranean countries at the beginning of the Christian era (Ferguson *et al*., 2005). Vavilov (1951) indicates two main centers of genetic diversification of *Pistacia vera L*.: one in the South of the Caspian Sea and another one in an area between Afghanistan and Kirgizstan. Some author consider Sicily a secondary center of diversification. Lucio Vitellio, the Roman governor of Syria in the year 30 A.D., as reported in the *Historia Naturalis*, introduced the Pistachio to Italy. During the Arabic domination (827-1060 A.D.), its cultivation was largely spread in Sicily, where the word "fastuca", synonym of the pistachio (from the Arabic name "fustuq"), appears in some books dating to the mid XVIII Century (Spina, 1982; Caruso e Sottile, 1999). Following Italy, pistachio was spread in Spain, France and Italy in northern Africa. The "USDA plant exploration service" introduced the pistachio plant in the USA (California, Texas, Arizona) for the first time in 1890. In California the pistachio has assumed cultivation interest only in the early 70s of the twentieth century (Hendricks and Ferguson, 1995).

Pistachio tree belongs to the *Anacardiaceae* family that includes 70 genera and over 600 species (Mitchell and Mori 1987). Numerous species of this family have an economic importance such as e.g. mango, cashew, pistachio, and pink pepper (Janick and Paull, 2008). There are eleven species within the genus Pistacia (*P. atlantica, P. cabulica, P. chinensis, P.falcata, P. integerrima, P. vera, P. kurdica, P. mutica,P. palestine, P. terebinthus* and *P. khinjuk),* but only *Pistacia vera L*. produce edible fruits (Zohary and Spiegel-Roy, 1975). The rest of the species are mostly used as rootstocks and, in some cases, for fruit consumption, oil extraction, or as a source of food for domestic animals in rural areas of different countries (Hormaza and Wünsch, 2011).

Pistachio is a diploid (2n=30, X=15) and wind pollinated tree (Basr Ila *et al*., 2003). Pistachio trees are dioecious, meaning that male and female inflorescences are produced on different trees. Inflorescences are small, brownish-green to yellow in color, apetalous, and without nectarines (Ferguson *et al*., 2005). Orchard plantings must include the appropriate ratio of females and males, 8:1 in older plantings, but up to 25:1 in more recently established orchards, (Kallsen *et al*., 1995). Pistachios are characterized by a long juvenile period and the trees require between 10 to 12 years having full bearing under good irrigated conditions (Ferguson *et al*., 2005).

The vegetative phase of the trees begins in March, blossom time occurs in April and harvesting period is September-October. During the flowering period, spring rains and dry winds limit the pollination process while, during vegetative growth and fruit ripening, high air humidity can result in fungal infections in both fruits and vegetables vegetation (Caruso *et al*., 2008).

It has been reported to grow well in dry climates with water shortage. Pistachio trees can survive temperatures ranging between −10 °C in winter and 48 °C in summer; they need a sunny position and well-drained soil. Long, hot summers are required for proper ripening of the fruit (Ardakani, 2015). The species has a cold demand of 600-1000 Chilling Units, variable with the cultivar (very high in Halebi cultivars in Turkey and Kerman in the United States). In areas with mild winters such as Sicily, the pistachio showed delays in leaf and flowering, as well as leaf malformations that cause productive decay (Caruso *et al*., 2008). Pistachio is a desert plant, and is highly tolerant to saline soil more than others nut crops (Grieve *et al*., 2012). Thanks to its distinctive features of xerophytic species, the pistachio lives and produces in areas with very low rainfall. The extended apparatus radical allows the plant to adapt to a wide variety of soils, with the exception of those where, even for short periods, there is water stagnation (Monastra *et al*., 1987).

The fruit is an edible drupe, commercially called nut, with a seed, or kernel. Fruits harvesting is done manually in the traditional area of cultivation because the fruits do not become mature at the same time but in modern orchards, harvest is done mechanically by trunk shakers. After harvesting, the fruits are dried naturally by sun, or with the aid of drier (Avanzato and Vassallo, 2008).

3.2 Pistachio Production

Pistachio cultivation play a vital role in the nutrition and agricultural economy of many poor communities living in the arid and semi-arid regions of Iran, Turkey, Syria and other pistachio producing countries due to the tree's adaptation at the drought typically of the desert where only a limited number of other Mediterranean species can be cultivated (Padulosi *et al*., 1997).

The production of pistachio in the world in the last decade has been arising, especially in the United States (California), Iran and several countries of the Mediterranean basin, particularly Turkey, (Table 8). However, there are still serious problems such as the alternate-bearing, the soil borne fungus *Verticillium dahliae*, and the long juvenility period (Tous and Ferguson, 1996). The production of pistachio in the main producing countries in the 2014 is around 743,627 tons (FAOSTAT 2014) and the main world producers of pistachio nuts are Iran, USA, and Turkey. However, there is an increase of interest in the drought resistant crop such as pistachio, also in countries such as Italy, where usually pistachio is an underutilized crop. In the 2004-2014 decade the pistachio production in Italy has been intensified (FAOSTAT 2014).

In Italy, pistachio cultivation is mainly located in Sicily (Barone and Marra, 2004) and 80% of its yearly production occurs in Bronte area (Gentile *et al*., 2007). Orchards are also located in the central part of Sicily (Caltanissetta and Agrigento areas, ISTAT, 2014). Few areas in Sicily are occupied by pistachio cultivation, so the production is very low compared to the other countries, as California and Iran, (FAO, 2014). In Sicily, pistachio has been traditionally cultivated in dry and marginal areas characterized by unsuitable conditions for applying modern management system (Barone *et al*., 1985), but in the recent years new irrigated orchards have been developed.

Worldwide, the majority percentage of pistachios are sold roasted with salt or not, and consumed as snacks and confections. During roasting process several thermal and chemical reactions occur, which finally change some aromatic characteristics of the nuts and the overall sensory quality (Hojjati *et al*., 2013; Saklar *et al*., 2001).

Table 8: Pistachio cultivation in the main producing countries in the decade 2004 - 2014 (Source: FAO, Statistics Division FAOSTAT 2014).

Area	Year 2004	Year 2014
Iran	184899	415531
United States of America	157397	233146
Turkey	30000	80000
Greece	7917	5700
Italy	2400	3555
Tunisia	1800	2500
Spain	2899	2468
Jordan	24	727
TOTAL	**387336**	**743627**

3.3 Pistachio response to salinity

Pistachio tree is drought tolerant and grows well in dry climates with suboptimal water supplies (Behboudian *et al*., 1986). Pistachio appear to be more tolerant in salinity than many other nut crops. Several studies have reported a positive influence of irrigation on photosynthetic assimilation rate (De Palma and Novello, 1997; del Carmen Gijón *et al*., 2011), yield and constant production (Goldhamer and Beede, 2004).

If the trees are continually irrigated with saline water in soil with inadequate leaching, salinity will build up and slowly effect on their growth rate (Grieve *et al*., 2012). Many studies have been conducted to analyze salt effect on pistachio and several effects of salinity on growth, photosynthetic rates, and morphological changes in the leaves of pistachio have been shown (Behboudian *et al*., 1986; Munns, 2002; Picchioni *et al*., 1990).

The pistachio has the morphological adaptation of multiple stomata on both sides of the leaf, which remain open during hot and dry conditions to allow photosynthesis to continue with reduced overall leaf water and osmotic potentials (Rundel *et al*., 1994).

Salinity tolerance of pistachio has been associated to:

- growth reduction (Picchioni *et al*., 1990; Sepaskhah and Maftoun, 1988);
- decrease in photosynthetic activity and transpiration rate (Karimi and Kuhbanani, 2015; Walker *et al*., 1988);
- ability to maintain relative high K+/Na+ and Ca2+/Na+ ratio needed for osmotic adjustment and enzymatic activities (Chelli-Chaabouni *et al*., 2010; Hajiboland *et al*., 2014);
- reduction of fruit production (Sanden *et al*., 2004);
- increase in ABA biosynthesis, which is responsible for stomatal closure (Bowler and Fluhr, 2000; Wilkinson and Davies, 2002).

The two earlier salinity tolerance trials conducted in California (Ferguson *et al*., 2002) and recent reports by Kallsen and Sanden (2012) have also demonstrated that there is a difference among the rootstock species and their hybrids in Na+ transport to the scion leaves.

Three sequential California salinity trials, one in greenhouse, and two in open field investigations have demonstrated that 'Kerman' pistachio trees grown on three different rootstocks, *P.atlantica, P.integerrrima* and 'UCBI' can be grown with irrigation water salinities up to 8.4 dS/m without yield loss (Ferguson *et al*., 2002).

Information from literature are sometimes contradictory about salt tolerance in pistachio. For (Picchioni *et al*., 1990; Sepaskhah and Maftoun, 1988) pistachio is considered moderately sensitive, but it is highly tolerant for Behboudian *et al*., (1986) and Walker *et al*., (1987).

Among Pistacia species, *P. atlantica* has been described as a highly tolerant to salinity (Ferguson *et al*., 2005). In the *in vitro* investigation experiment made by Chelli-Chaabouni *et al*., (2010), *P. atlantica* shows a higher tolerance to salt in comparison to *P. vera*. This variation is a result of many inter and intra-specific variations as well as the effect of different environmental conditions such as the characteristics of the soil and the temperature.

Nowadays the mechanisms used by pistachio to adapt to mild and high salinity are still unknown. Selecting salt tolerant rootstocks is an effective approach for sustainable development of pistachio in such salt areas. In this regard, understanding the effects of salinity on pistachio is of a crucial importance for establishing a successful rootstock-breeding program.

3.4 Aim of research

The relative saline tolerance of the pistachio tree provides great potential for future expansion into marginal soils with elevated salt levels (Sanden *et al*., 2009). Given the damaging effects of salinity on crop growth and productivity, application of genetic resources to the breeding of salt tolerant genotypes is a good tool for the development of sustainable agriculture. Several reports on species propagated by grafting often ascribe salinity tolerance to the rootstock (Huang *et al*., 2010; Okubo *et al*., 2000). Roots play a key role in the salt tolerance of plants, since they represent the first organs to control the uptake and translocation of nutrients and salts. Accumulation of Na+ in the roots is an adaptive response used by several woody species to minimize its toxicological effects on shoots (Gucci and Tattini, 1997; Picchioni *et al*., 1990; Walker *et al*., 1987). Accordingly, the control of the root-to-shoot transport of salt can serve as a criterion for tolerance (Chelli-Chaabouni *et al*., 2010). The uptake of large amounts of salt by the plant also leads to ionic imbalance. Notably, the disturbance of potassium (K+) nutrition under salt conditions by potassium–sodium (K+–Na+) interaction is a common feature in plants and is often associated with K+ deficiency (Cramer *et al*., 1987; Parida and Das, 2005). A selective uptake and transport of K+ into the shoots is necessary for salt tolerance in many higher plants (Cramer *et al*., 1987). Since a high K+/Na+ ratio in the cytosol is essential to maintain normal cellular functions (Chinnusamy *et al*., 2005), plant salt tolerance strongly depends on the K+ nutrition status (Maathius and Amtmann, 1999). A high K+/Na+ ratio in the leaves is, often considered as a salt-tolerance marker (Loupassaki *et al*., 2002; Maathuis and Amtmann, 1999). It is important to develop new technologies that can help to improve the knowledge of the mechanism of sodium, chloride and potassium uptake, transport and sequestration at the cellular and molecular level, to evaluate the mechanisms of salinity tolerance in pistachio rootstocks.

A new methodology using Confocal Fluorescence Microscopy (CFM) was developed to characterize the localization of sodium and potassium in the roots. Sodium detection is essential in plants subjected to salt (NaCl) stress, to understand the molecular mechanisms of tolerance. In detail, under salt stress, plants must regulate sodium and potassium (K+) cellular concentrations, adjusting activities of membrane transporters, channels, and co-transporters (Zhu, 2003). One of the greatest benefits of CFM is represented by its minimal invasiveness, which allows obtaining *in vivo,* live images with good spatial resolution of organisms, cells, and biomolecules, without alteration of their architecture, in a biologically relevant system. To visualize sodium (Na+) in cellular and subcellular compartments, different Na+ indicators are commercially available. To understand which molecular mechanisms is activated by the plants to resist to NaCl toxicity, sodium detection is essential, but also it's important to detect the variation of the potassium ion (K+), because the plant expends a major part of its metabolic energy maintaining the concentrations of Na+ and K+ within the cell (Zhu, 2003).

Further, the methodologies for the detection of sodium in leaf tissue has been developed. This technic permits to eliminate the notoriously chlorophyll auto-fluorescence signal. The Leica SP8 confocal microscope allows signal discrimination based on the lifetime of fluorophores, which are different for chlorophyll and our sodium stain. Structural differences observed in tissues within the different genotypes can be related to halo-sensitivity.

This work aims at the establishment of cellular and molecular methodologies to identify sodium, potassium and chloride uptake, ion sequestration and its effect on cellular morphology and viability for various rootstocks. The working hypothesis is that sodium and chloride sequestration in pistachio cells is an important and identifiable trait for salinity tolerance and that it is mediated by the activity of specific transporters. By observing sodium, potassium and chloride localization in live plants at the subcellular level with non-invasive fluorescence microscopy and saline induced structural/morphological cell and cell wall changes, this study aims in depth characterization of salinity tolerance. Application of these methods in larger genotype screening efforts affords a unique opportunity to assess rootstocks and elite cultivars in an efficient and cost effective way.

3.5 Materials and Methods

Plant Material and culture conditions

The experiment was conducted using *in vitro* germinated seedlings of the three most important Pistachio rootstocks *Pistacia atlantica Desf.*, *Pistachia integerrima and UCB*-1 an hybrid cross of a specific P. *atlantica* female with a specific P. *integerrima* male developed at UC Berkeley in 1960. The *in vitro* culture reduce experimental errors by increasing uniformity of growing medium. Absence of complex interactions of the soil, the environmental variation, and all the variability present in the field enhance repeatability of results and provide a clear understanding of the plant responses to salinity.

Mature seeds of *P. integerrima*, *P. atlantica* and P. UCB1 were collected from the foundation plant services, University of California. The seedlings obtained from in vitro germination were cultivated for 8 weeks on a ½ MS medium (Murashige and Skoog, 1962) supplemented with 7gr/L of agar and 15 gr/L sucrose. The pH was adjusted to 5.7 before autoclaving. Since sodium chloride (NaCl) represents the major source of salt in irrigation water and soil solutions, it was used as the source of salt throughout the experimental assays of this study. Several treatments, with different NaCl concentrations and culture durations, were performed on 2-month-old seedlings from both species cultured on MS media supplemented with NaCl. The treatments with NaCl concentrations (0 and 100mM) were conducted for 7 days. All cultures were incubated at a temperature of 24 ± 2 ◦C and a photoperiod of 16h provided by daylight fluorescent lamps.

A test of selectivity of the two dyes was performed on Arabidopsis. Seeds of Arabidopsis thaliana Col-0, wild-type were germinated in Petri dishes on half-strength Murashige and Skoog (MS) medium containing 1% (w/v) agar and 1% (w/v) sucrose. The plants were grown in growth chambers at a temperature of 24 ± 2 ◦C and a photoperiod of 16h provided by daylight fluorescent lamps. After 5 days, two different concentrations of NaCl 5 and 150 mM were applied, and 3 different concentrations of KCl 5, 75 and 150 mM. The confocal settings used during image acquisition are described below.

Tissue sectioning

At periodic sampling times, water rinsed roots and leaves of treated and control seedlings were excised 0.5 cm from the root tip, and leaves were separated at the base of the petiole. Root and leaf tissue were embedded in a 5% agarose and sliced into 1- to 2-mm segments with vibratome (Vibratome 1000 Plus Sectioning System). The sections were incubated in an incubation solution containing 20 mM Buffer MOPS pH 7.0, 0.5 mMCaSO4, and 200 mM sorbitol (incubation media) to recover from effects of excision and sudden changes in osmolality and salt concentration (Davenport and Tester 2000).

Confocal microscope analysis: Sodium staining

To visualize the Na^+ distributions in the root cells of treated and control of all the three pistachio rootstocks under evaluation, Na^+-specific fluorescent dye, CoroNa-Green AM (Invitrogen) was used. Segments were incubated in 10 mM of CoroNa Green AM fluorescent sodium indicator (Molecular Probes C36676; Invitrogen) in the incubation media above for 12 h. Tissue segments were washed three times to remove any excess fluorescent dye using the incubation solution without the CoroNa-Green. Acetoxymethyl (AM) ester of CoroNa Green (Molecular Probes, Inc., Eugene, OR) dye is a sodium ion indicator that exhibits an increase in green fluorescence emission intensity upon binding Na^+, with little shift in wavelength. The CoroNa Green indicator allows spatial and temporal resolution of Na^+ concentrations in the presence of physiological concentrations of other monovalent cations. CoroNa Green AM dye loads into cells more efficiently than does Sodium Green tetra-acetate, and the CoroNa Green indicator responds to a broader range of Na^+ concentration. The dye has excitation and fluorescence emission maxima of approximately 492 and 516nm, respectively (Meier *et al*., 2006). The excitation wavelength was set at 488nm, and the emission was detected at 510–520nm.

Confocal microscope analysis: Potassium staining

Root segments from treated and control pistachio plants were incubated with Asante Potassium Green, a fluorescent indicator for measuring cytosolic K^+ concentration. Segments

were incubated in 10 mM of Asante Potassium Green fluorescent in the incubation media above for 12 h. Tissue segments were washed three times to remove any excess fluorescent dye using the incubation solution without the Potassium-Green. The dye has excitation and fluorescence emission maxima of approximately 488 and 540nm, respectively. For Na+ and K+ compartmentation analysis for the root sections was used the fluorescent laser scanning confocal microscopy (ZEISS LSM710/700), microscopes as described (Lee and Drakakaki 2014; Park *et al*., 2014). The excitation wavelength was set at 488nm, and the emission was detected at 510–520nm for both dye used.

Sodium localization in leaves

Sections of leaves of UCB-1 and *P. integerrima* treated and control were incubated in 10 mM of CoroNa Green AM fluorescent sodium indicator (Molecular Probes C36676; Invitrogen) in the incubation media above for 12 h. Segments were labelled also with SNARF-1 (carboxylic acid, acetate, succinimidyl ester Molecular Probes, Inc., Eugene, OR), that have orange-red fluorescence that can be easily distinguished from that of cells loaded with green-fluorescent tracers. The fluorescent laser scanning confocal microscopy Leica SP8/ SP8 MP Microscope was used as described. The excitation wavelength was set at 488nm, and the emission was detected at 510–520 nm for CoroNa Green while for SNARF the excitation wavelength was set at 570 nm, and the emission was detected at 600-700 nm.

Micrographs were recorded on the ZEISS LSM710/700 for the roots sections and on Leica SP8/ SP8 MP for leaves sections. The imagines were analyzed with the software ZEN black edition and for Na+ and K+ compartmentation analysis in the roots sections as described (Lee and Drakakaki 2014). The software Leica Application Suite X (LAS X) was used for analyze the pictures from leaves sections.

Comparison of different levels of fluorescence between cells was carried out by visualizing cells with the identical imaging settings of the confocal microscope (i.e., laser intensity, pinhole diameter, and settings of the imaging detectors).

Mineral content

At the end of the experiment, Na+, K+, and Cl- concentrations in the leaves, stem and roots were measured. The method used to collect and prepare samples is given below.

Roots, stem and leaves from treated and control seedlings were briefly washed twice with DI water and then dried for 24 hours at 70^{o}C and ground to less than 2 mm for analysis by the UC Davis Analytical Lab. The concentrations of Na+, K+, were measured using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES; Thermo iCAP 6500, Thermo Fisher Scientific, MA, USA). Cl-measured using ion chromatography (Dionex ICS-2000, Dionex Corporation, CA, USA) based on the protocol by Liu (1998).

3.6 Results

Plant growth and ion concentration

All control seedlings appeared healthy and showed no signs of impaired growth. Salinized seedlings appeared chlorotic and had visible tip burns on most leaves after 1-week salt treatment, specifically the salinity treatment led to an accumulation of pigments in leaves manifesting itself in a "red-brown" leaf color. Differences in leaf browning were oserved between UCB-1 (Figure 6a), *P. atlantica* (Figure 6b) and *P. integerrima* (Figure 6c). The most severely affected was *P. integerrima* (Figure 6c), with a strong burn in leaves, and a faster mortality after only 7 days. UCB1 appeared the most tolerant of all three genotypes tested.

Further, the overall "robustness" and root architecture were considerably different between the various genotypes after 8 weeks of development, suggesting that this characteristic of the rootstocks may play a role in mechanisms of salinity tolerance (Figure 7).

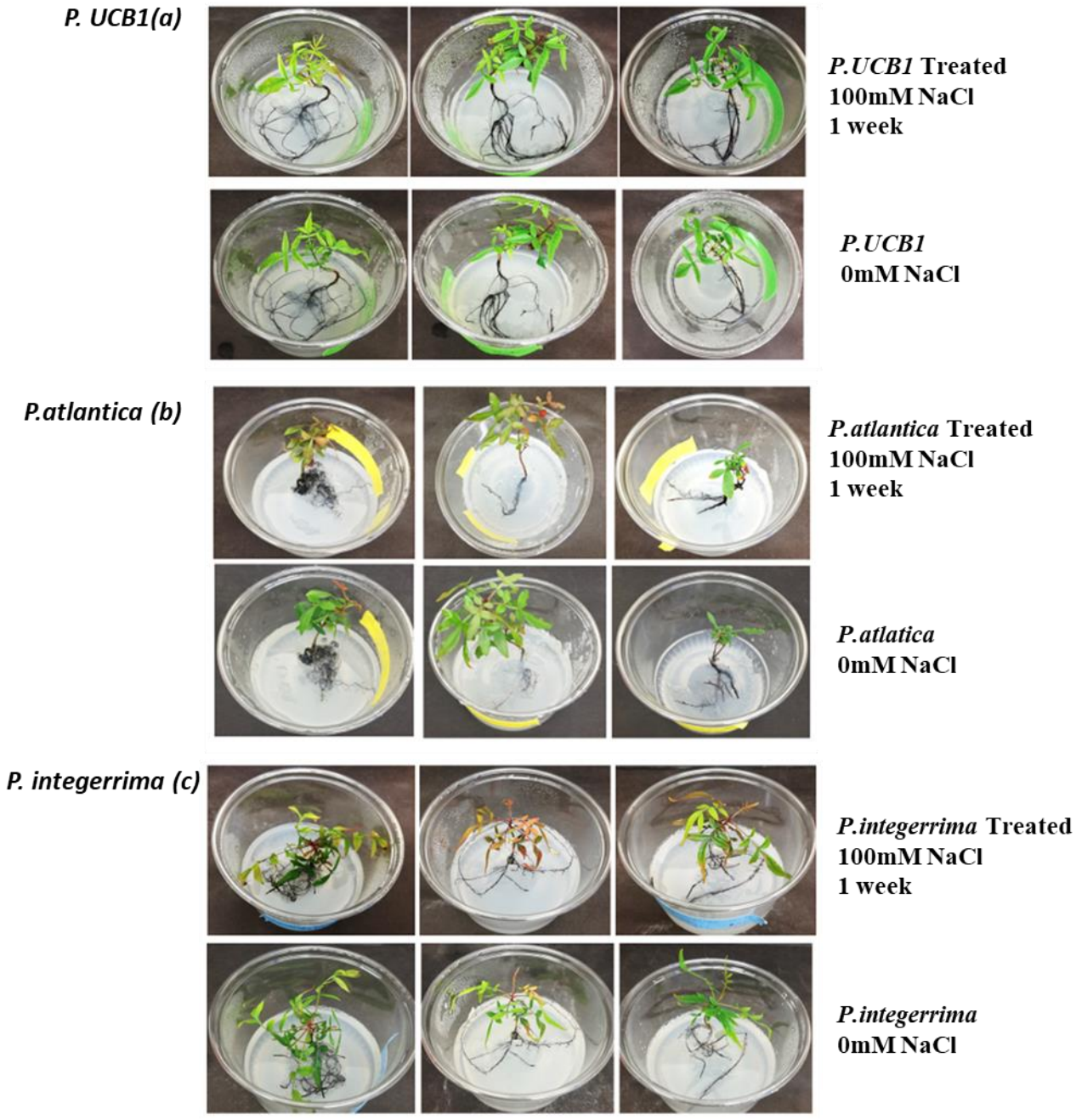

Figure 6 - Phenotypic differences between pistachio genotypes under salinity treatment. Differences in leaf browning were observed between UCB-1 (a) P. *atlantica* (b) and P. *integerrima* (c)

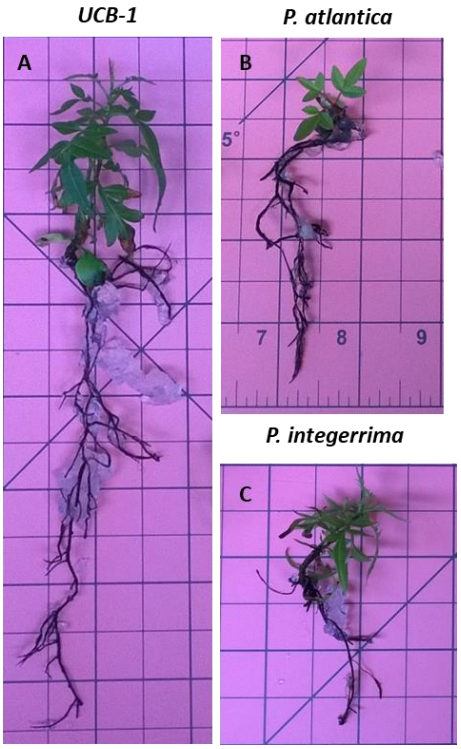

Figure 7- Differences in the root system of UCB- 1 (A) compared to P. *atlantica* (B) and P. *integerrima* (C).

Selectivity test of CoroNa Green AM and Asante Potassium Green

In order to establish a staining method of sodium and potassium we evaluated the specificity of the sodium stain CoroNa –Green and the potassium stain Asante green for their specificity. Arabidopsis roots of plants growth in vitro with 150mM of NaCl or with 150mM KCl were stained with the each of the two dyes. CoroNa-Green signal was detected in NaCl- treated plants but not in roots of plants supplemented with KCl (Figure 8). In addition, different concentrations of NaCl were tested, and CoroNa-Green signal increased proportionally to sodium concentration from 5 mM to 150 mM whereas no signal is observed at 5 mM (Figure 8 a,b). CoroNa Green is specific to Na+ and does not bind to K+, even at high concentrations of 150 mM (Figure 8 c,d), providing a reliable tool for the cellular detection of Na+. Altogether, the data showed that CoroNa green fluorescent staining provides a valuable means for non-destructive monitoring to detect the spatial and temporal distribution on Na+.

In addition, K Green, a specific stain for Potassium, was tested in Arabidopsis roots of plants grown with different KCl concentration (Figure. 9). K-Green intensity increases as potassium concentration increased from 0mM (B) to 75mM (A), whereas no signal was observed at 0 mM (Figure. 9B). Therefore, K-Green is specific to K+, providing a reliable tool for the cellular detection of K+.

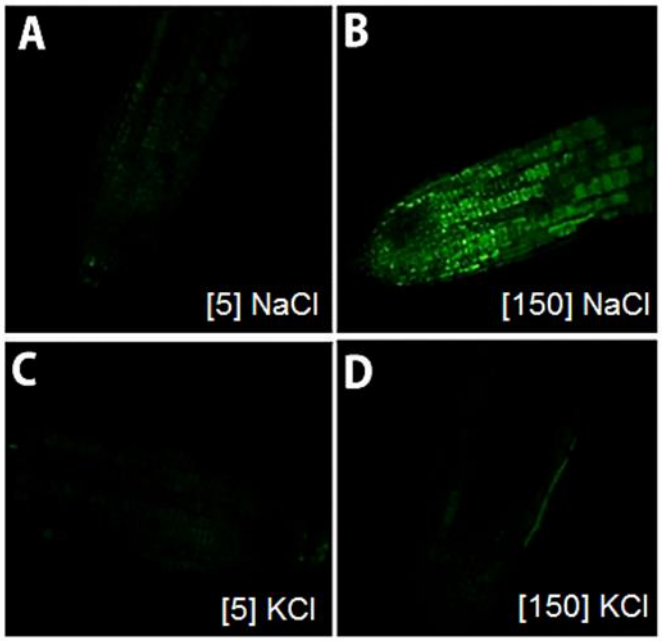

Figure 8 - CoroNa Green selectivity to Na+ in Arabidopsis longitudinal roots. A) Plant grown with 5mM of NaCl; B) Plant grown with 150mM of NaCl; C) Plant grown with 5mM of KCl; D) Plant grown with 150mM of KCl. Fluorescent laser scanning confocal microscopy (ZEISS LSM710/700) was used.

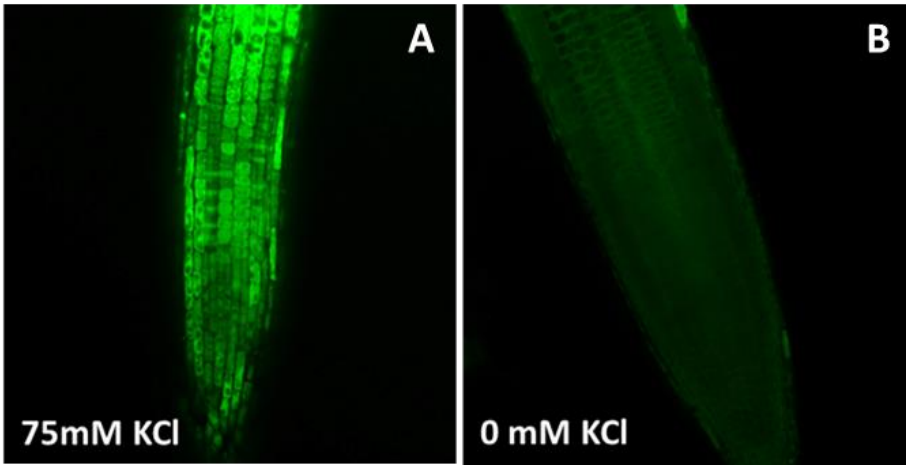

Figure 9 - K-Green selectivity to K+ in Arabidopsis roots. A) Arabidopsis treated with 75mM of KCl; B) Plant grown with 0mM of KCl. Fluorescent laser scanning confocal microscopy (ZEISS LSM710/700) was used.

Ion localization in pistachio root sections

Localization of sodium under salinity treatment was observed in root cells of UCB-1, *P. atlantica* and *P. integerrima.* Increased sodium accumulation was observed in the endodermal layer of UCB1 and *P. atlantica* under salinity treatment (Figure 10 b; d). Characteristic cell damage was observed in *P. integerrima* after seven days of salt treatment, manifesting itself in sodium dispersing throughout the cytoplasm. Additional localization of potassium showed an enhanced accumulation of fluorescence at the endoderm level of all rootstocks analyzed (Figure. 11). The vascular region of all three genotypes under saline stress for a week (100mM NaCl) and in the absence of salt control were observed. The ion dyes CoroNa + Green and Potassium Green were used for sodium and potassium localization respectively. Increased accumulation of sodium was detected within endodermal cells and in greater amounts in UCB1 (Figure 12 a). Minimal staining was observed in sections of roots from plants not exposed to salt stress (Figure 12). K Green signal was stronger in the endodermis of all 3 genotypes subjected to saline stress (Figure 13).

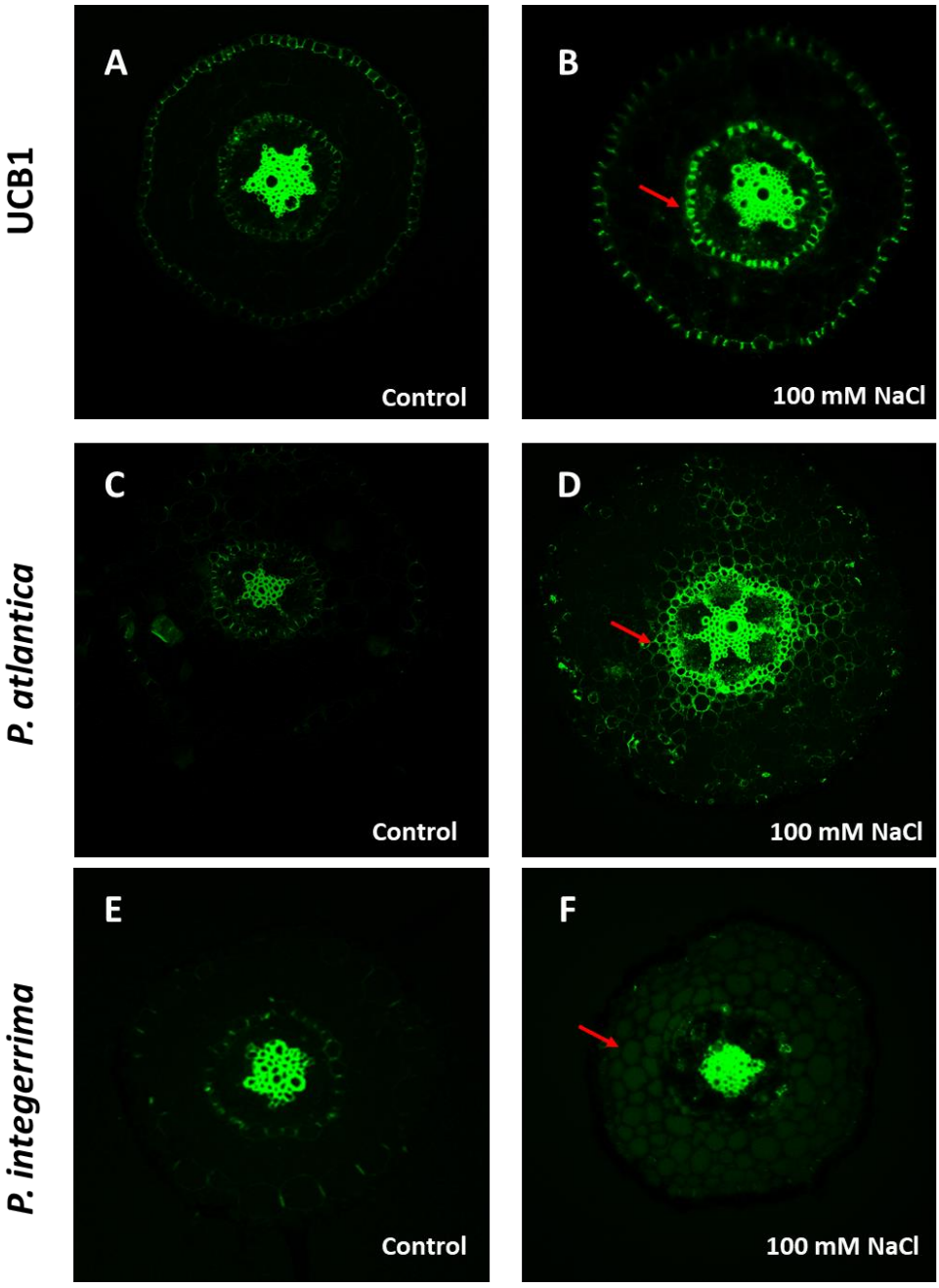

Figure 10 – CoroNa Green stain in UCB-1, in P. *atlantica* and in P. *integerrima* transverse root sections of control 0mM of NaCl (a,c,e) and treated for one week with 100mM of NaCl (b,d,f). Fluorescent laser scanning confocal microscopy (ZEISS LSM710/700) was used.

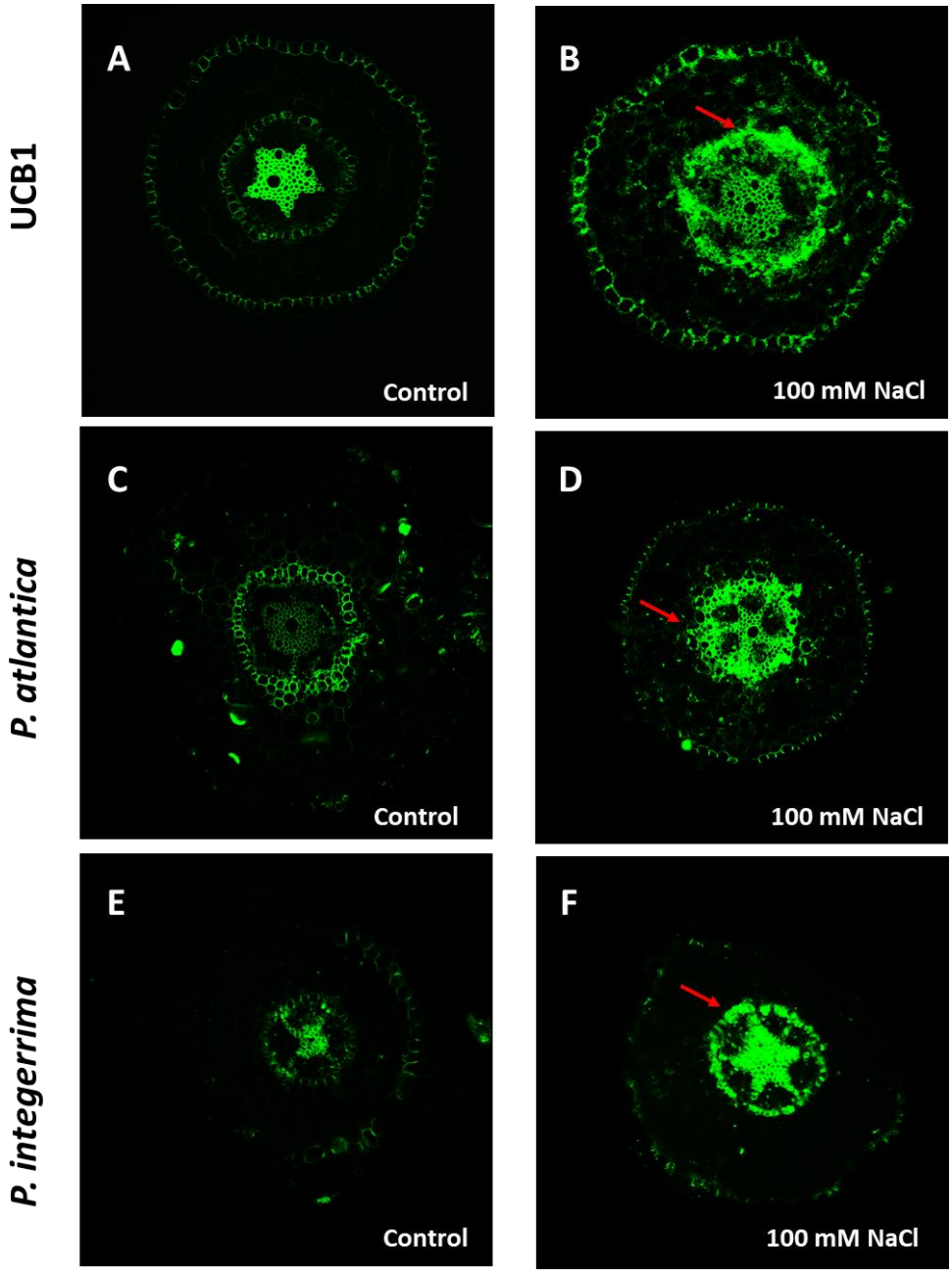

Figure 11 – Potassium Green stain in UCB-1, P. *atlantica* and P. *integerrima* transverse root sections of control 0mM of NaCl (a,c,e) and treated for one week with 100mM of NaCl (b,d,f). Fluorescent laser scanning confocal microscopy (ZEISS LSM710/700) was used.

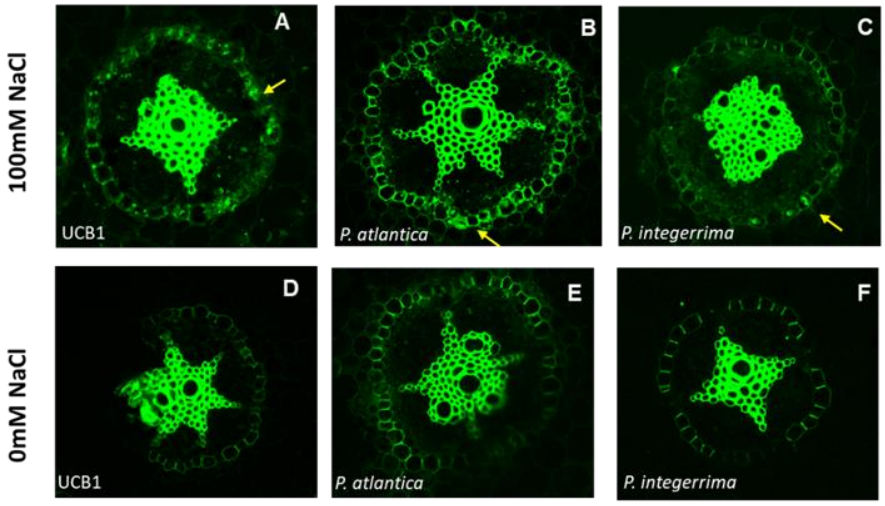

Figure 12 - Enlarged view of vascular bundle of *UCB1*, *P. atlantica* and *P. integerrima* treated and control, labelled with CoroNa green dye. Fluorescent laser scanning confocal microscopy (ZEISS LSM710/700) was used.

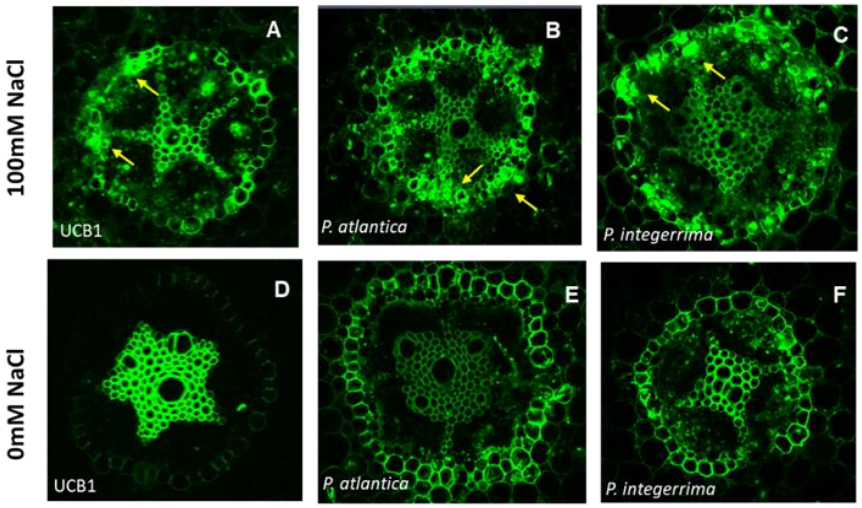

Figure 13 - Enlarged view of vascular bundle of *UCB1*, *P. atlantica* and *P. integerrima* treated and control, labelled with P green dye. Fluorescent laser scanning confocal microscopy (ZEISS LSM710/700) was used.

Na+ localization in leaf tissue

Confocal imaging of leaf tissue revealed differences between UCB-1 and P. *integerrima* in the leaf mesophyll cells (Figure 14). UCB1 shows a vacuolar compartmentation, that is a mechanism to prevent an excessive NaCl accumulation in the stroma of the chloroplasts and therefore it is important for the maintenance of photosynthesis. Further studies are necessary to complete the analyses in all genotypes and to study the impact of salinity on leaf growth, leaf morphology and long-distance ion transport.

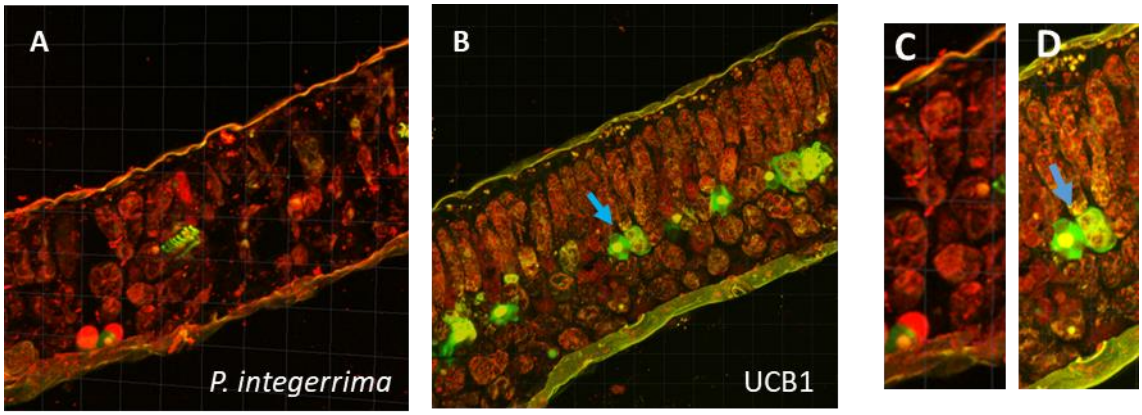

Figure 14 – CoroNa green stain in UCB1 and *P. integerrima* leaf sections of treated plants. Arrows indicate specialized cell localization of Na+. Red color indicates overall cell labelling by red fluorescent pH probe carboxy-SNARF to determine the structure of vacuoles. Fluorescent laser scanning confocal microscopy Leica SP8/ SP8 MP microscopes was used.

Analysis of the ion content in roots, stems and leaves

An analysis of the ionic content of sodium (Na+), potassium (K+), and chlorine (Cl-) was carried out. As shown in (Figure 14), all genotypes after one week of salt treatment showed increased accumulation of sodium and chlorine ions. The highest sodium concentration was detected in the leaves, compared to the roots and stem, suggesting that sodium is transported and accumulated in the aerial parts and this could lead to toxicity symptoms.

No concentrations of Na+ was found significantly increased in stems of UCB1 and P. *atlantica* after 7 days of salt stress. Notably, accumulation of Na in stems was less pronounced in P. *integerrima*, the genotype showing more accumulation of the ion in leaves.

Plant levels of Cl- were significantly affected by salinity with P. *integerrima* displaying the highest Cl- concentrations in both leaves and roots after treatment.

After treatment the leaf K+ concentration in UCB1 and in P. *integerrima* decreased in contrast with what observed in P. *atlantica.* The K+ concentration in stems of P. *integerrima* and P.*atlantica* and UCB1 were not altered by the treatments in any of the genotypes. Altogether, the data showed that P. integerrima was the most sensitive genotype to salinity stress with the highest accumulation of Na and Cl ions in the leaf tissue.

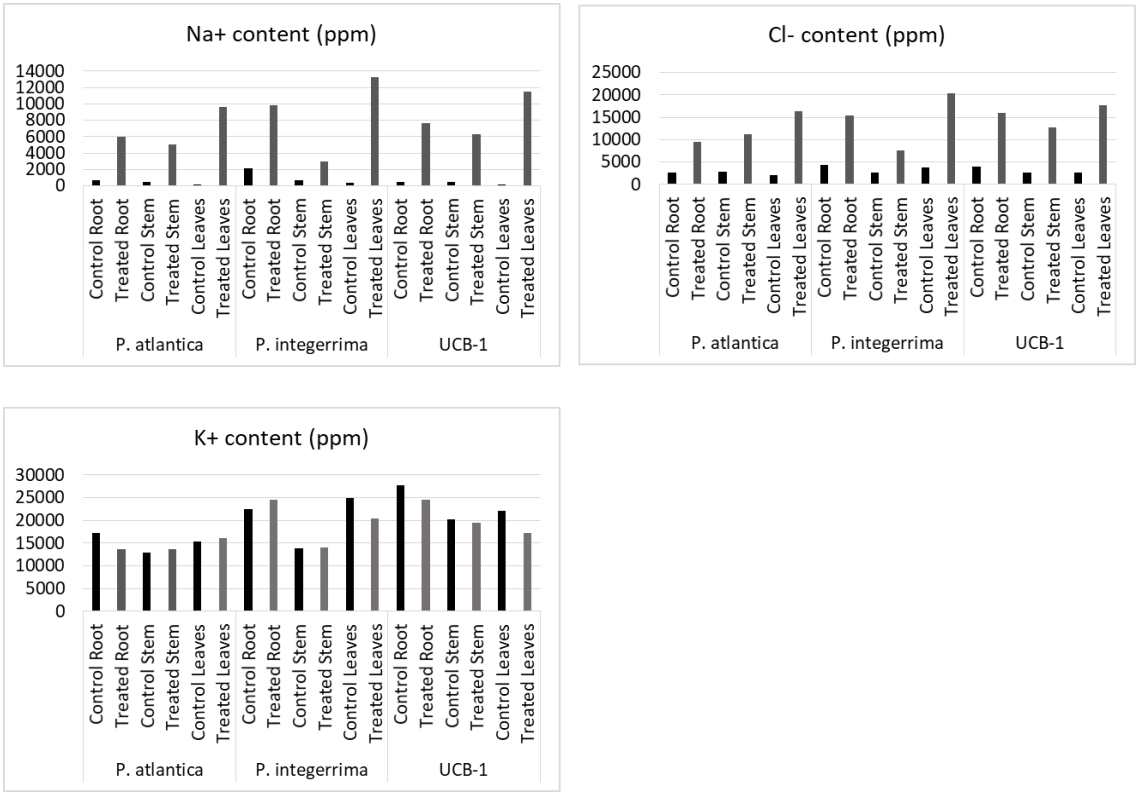

Figure 15 - Ion content of sodium (Na +) potassium (K +) and chlorine (Cl-) in UCB1, P. *atlantica* and P. *integerrima*, in grey treated in black control samples.

3.7 Discussions and Conclusions

Drought and salinity are two of the major constraint to vegetable crop production. It became particularly important to identify and select the best-adapted plants for improving yield in the more sensible areas. For these purposes, several investigations are conducted to improve knowledge on the drought and salinity tolerance phenomenon.

This is the first work aimed at analyzing salinity tolerant in pistachio rootstock's varieties using *in vitro* culture and confocal microscopy.

In vitro cultures have been used for selection of plant tolerance to abiotic stresses including salinity, drought (Chelli-Chaabouni *et al*., 2010; Metwali *et al*., 2014; Shibli *et al*., 2007). It provides a simple platform for the screening of plantlets under abiotic or biotic stress and permits to study many aspects of plant growth and development under well-defined conditions (Qrunfleh *et al*., 2013; Shatnawi *et al*., 2010). Therefore, the objective of the present study was to investigate the *in vitro* response of pistachio rootstocks to salt stress.

The in vitro responses of P. *integerrima*, P. *atlantica* and UCB1 to NaCl treatment were similar to those observed in vivo conditions (Sanden *et al*., 2004), in which P. *integerrima* appears more sensitive than UCB1. All stressed seedlings appeared chlorotic and had visible tip burns in most leaves. Symptoms of leaf necrosis have often been associated with high chloride (Picchioni and Graham, 2001) or sodium concentrations in the leaves (Ferguson *et al*., 2002). The study *in vitro* also indicate that some reactions are activated from early developmental stages and provide further information on the possible mechanisms employed by these species to avoid salt toxicity.

An observation of root architecture shows a great difference in the "robustness" between the various genotypes. UCB1 has more ramifications and length in the roots than others two genotypes. Some researches has highlighted the importance of root system (Passioura, 1988) in stress tolerance. The anatomy of the root system (length, root diameter, etc.) determines root performance, enabling plants to acquire water and nutrients and thereby increase the replacement rate of plant water lost (Passioura, 1988). Root systems can support shoot growth and improve plant yields, since roots serve as an interface between plants and the soil (Vamerali *et al*., 2003). A proliferated root system would therefore appear to be better for

plants, for it allows them to penetrate deeper layers of soil to acquire water and nutrients (Franco *et al*., 2011). Other root characteristics, such as the number and diameter of xylem vessels, width of the root cortex, number of root hairs, and the suberin deposition in both the root exodermis and endodermis, also determine the permeability of roots to water (Steudle, 2000). It is likely that the superior performance of UCB1 in salinity is also attributed to its overall root system.

The confocal studies showed an increase of the root cytosolic localization of sodium in P. *integerrima*, and increased concentrated localization of potassium in all rootstocks compared to non-treated controls. The specificity of staining was confirmed by our studies in Arabidopsis.

A higher zoom shows a conspicuous fluorescence localized in the cell walls, and in the xylem elements for both dyes used (Figure 12 and 13). This is in agreement with previous reports. A mechanism has been proposed, in which ions, after traversing the endodermis, continue to the xylem , following the movement of water (Peng *et al*., 2004). The mechanism of the movement of the Na+ from the roots into the xylem is still not clear (Flowers and Colmer, 2008; Kronzucker and Britto, 2011; Tester and Davenport, 2003).

Interestingly, the potassium specific stain has a well-defined fluorescence, with strong levels at the endodermis (figure 13)**.** This may be a critical mechanism for the salinity tolerance because the pericycle remains the last metabolic barrier before solutes enter the xylem stream to move toward leaves (Conn and Gilliham, 2010). The function of diffusion barriers in the endodermis is important when considering the uptake of toxic solutes into the root. In high-saline environments, the endodermis limits free apoplastic diffusion of sodium ions into the vascular stream , which leads to the accumulation of sodium ions in tissues peripheral to the endodermis (Lee *et al*., 2013). Previous researches have described the role of the endodermis in tolerance to salinity but further studies are necessary in pistachio rootstocks. Under drought and saline conditions, suberization in the endodermis can be accelerated and may prevent the desiccation of inner tissue layers (Enstone *et al*., 2002). Thus, plants react to different environmental factors (such as salinity and drought), reinforcing the level of their apoplastic barriers in roots (Chen *et al*., 2011). The Casparian strip and suberin lamellae not only prevent uptake of toxic compounds but may also prevent the loss of water under unfavorable

environments (Baxter *et al*., 2009; Chen *et al*., 2011). Further analysis are necessary to deepen the knowledge about these mechanisms also in pistachio. It is likely that differences observed under cellular and subcellular imaging are not detectable under whole root ion extraction, thus these differences may be masked in the ion analysis.

The CoroNa green dye was used for the first time in pistachio. Previous studies had been done in Arabidopsis, and wheat (Park *et al*., 2009; Wu *et al*., 2015). The Asante Potassium Green fluorescent has been used in plants only in Arabidopsis (Liu *et al*., 2017; Wang *et al*., 2016). Both dyes are used here for the first time together. In addition, the sodium localization in leaves with Leica SP8 microscope is an innovative methodology used for the first time in this field of research.

For the leaves analysis, there is a different compartmentation between the genotypes analyzed, with stain of vacuoles only in UCB1 and not in P. *integerrima*. This is another confirmation of the most sensitive behavior of P. *integerrima* that is not able to storage sodium in vacuoles. The accumulation of salt ions in the leaf vacuole may be used by species as a mechanism of salt tolerance (Chartzoulakis, 2005; Munns *et al*., 2016). This accumulation ensures osmotic adjustment required to maintain leaf turgor and activity of metabolic sites in the cytoplasm.

As storage compartment, the vacuolar membrane contains a variety of Na+-related transporters capable of removing toxic levels of Na+ from the cytosol (Apse and Blumwald, 2007). The relevance of this tonoplast-bound Na+ transporter to salt tolerance has been thoroughly demonstrated in several species such as tomato (Zhang and Blumwald, 2001) poplar and Arabidopsis where increasing expression of the Na+/H+ antiporter resulted in increased salt tolerance (Apse *et al*., 1999; Yang *et al*., 2017).

Under salt conditions, the results for Cl− and Na+ indicate that there was no inhibition of ion transport from root to leaves in all genotypes analyzed, since the Cl− and Na+ content increased more in leaf in both genotypes. The sodium transported and accumulated in the aerial parts was likely the cause of the observed symptoms of toxicity as described by Tester and Davenport, (2003). In previous reports, genotypes of pistachio with low Na+ concentration in the shoot were considered resistant to salinity stress (Banakar and Ranjbar, 2010; Karimi *et al*., 2011).

These results suggest that the higher tolerance of UCB1 was related to a higher capacity to compartmentalize toxic ions in vacuoles, minimizing the negative effect that these ions produce in the cells (Robinson *et al*., 1983; Blumwald *et al*., 2000).

Under salt conditions, the Na+ content in leaves of all *Pistacia* rootstocks analyzed was higher than in the roots. These findings are in disagreement with previous results on older seedlings cultivated in the field (Picchioni *et al*., 1990; Walker *et al*., 1987) and *in vitro* conditions (Chelli-Chaabouni *et al*., 2010) where Na+ concentration was described to be higher in the roots than in the shoots.

K+ is the major cation, able to counterbalance the negative charge of anions and thus contribute to the osmotic adjustment of cells in salt stressed plants. K+ stabilizes the pH, osmotic potential, and turgor pressure within cells. It also plays a crucial role in the activation of the enzymes involved in the metabolism and synthesis of proteins and carbohydrates, as well as in the regulation of stomata movement and tropism (Shabala and Pottosin, 2014). The results mineral content proved that salinity stress decreased the K+ concentration in shoot and root only in UCB1 in accordance with previous studies in pistachio seedling (Picchioni *et al*., 1990; Saadatmand *et al*., 2007) and pomegranate (Naeini *et al*., 2006).

Zaccaria *et al*., (2017) revealed that P. *vera* seedlings maintained higher K+ and Ca2+ concentrations than those of P. *atlantica* and irrigation with saline water induced a significant decrease in K+ content in shoots and roots of both species with a more pronounced reduction in roots. This is confirmed in our experiment *in vitro* for UCB1, in which a clear reduction of K+ concentration in roots and shoots was observed.

The findings presented in the current study demonstrate that, in terms of the parameters being investigated and in comparison to previous reports available in the literature, the *in vitro* responses of pistachio rootstocks (P. *atlantica,* P. *integerrima* and UCB1) to NaCl treatment reflected similar behaviors to those achieved under in vivo conditions. They also indicate that some reactions are activated from early developmental stages and provide further information on the possible mechanisms employed by these species to avoid salt toxicity. The higher salt tolerance of UCB1 observed in vitro seems to correlate with a higher survival rate, no damage in the leaves, the ability to store sodium in the vacuoles and a better control of salt ions (Na+ and Cl−) accumulation in the leaves. In contrast, P. *integerrima* appears more sensitive to salt

stress, evidenced by its lower survival rate, a great damage in the leaves with clear chlorosis and necrosis, and the inability to accumulate sodium in vacuoles.

In conclusion, this new methodogy used can help to complete the evaluation of different rootstock genotypes and characterize the mechanism contributing to salinity tolerance. Confocal microscopy has allowed acquiring in vivo imaging at the cellular and subcellular level. By observing sodium, potassium and chloride localization in live tissue at the subcellular level with non-invasive fluorescence microscopy and saline induced structural/morphological cell and cell wall changes, it is possible to have a rapid characterization of salinity tolerance. Thus, understanding the cellular sequestration of Na+ and Cl- and K+ in a quantitative manner can provide evaluation criteria for the identification of most salt tolerant rootstocks. Application of these methods in larger genotype screening efforts affords a unique opportunity to assess rootstocks and elite cultivars in an efficient and cost effective way.

General Conclusions

Pistachio and fig are two fruit crops that in the last years had a rising interest for their resilience to environmental stresses as drought and salinity, two abiotic stresses limiting crop production in the Mediterranean region. Due to climate changes, this geographic area, in the coming future, is expected to face increases in temperature, drought and soil and water salinity. The results presented in this thesis contributed to develop new tools to improve knowledge on the biodiversity and salinity tolerance.

Biodiversity studies based on morphological and molecular markers approaches contributed to characterize the diversity of germplasm, providing a powerful tool to discriminate the identity of genotypes thus, to develop, quickly, systems of traceability for product standardization, food security and, eventually, preserve Protected Designation of Origin (PDO).

Soil salinity causes intense modification including morphological, biochemical, physiological and metabolomics that significantly affect plant growth and development resulting in reduced productivity. In the plant model species such as Arabidopsis, detailed studies have been conducted to explore ion uptake, transport, storage and detoxification mechanism and gene expression. However, in fruit tree crops, limited studies have been carried to understand mechanisms of salt tolerance. The methods developed in the present studies is a non-invasive one and allows a rapid characterization of salinity tolerance, analizing the cellular sequestration of Na+ and Cl- and K+ in a quantitative manner.

The two techiniques developed could be tranferred to other fruit crop species to identify, within biodiversity generated in breeding programs, genotypes with salinity tolerance.

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