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**Regulation of photosynthetic activity of crop species subjected to
abiotic environmental stresses**

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Abbreviations

A	Assimilation
ABA	Abscissic acid
ATP	Adenosine triphosphate
CET	Cyclic electron transport
Chl	Chlorophyll
3Chl*	Triplet excited state of chlorophyll
Ci	Intercellular CO ₂ concentration
Cyt b6f	Cytochrome b6f complex
DOE	days of experiment
E	Transpiration
EDTA	Ethylenediaminetetraacetic acid
Fd	Ferredoxin
FNR	Ferredoxin NADP reductase
Fv/Fm	Maximal efficiency of photosystem PSII
Fv'/Fm'	Maximal efficiency of photosystem PSII under light conditions
g _s	Stomatal conductance
LAI	Leaf area index
LHC	Light harvesting complex
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide
1O ₂ *	Singlet oxygen
3O ₂	Ground state oxygen
ΦPSII	Actual quantum efficiency of photosystem PSII
PARP	Poly(ADP-ribose) polymerase
PC	Plastocyanin
PC-DF	Principal component discriminant function
Pheo	Pheophytin
PLA	Plant leaf area
PPFD	Photosynthetic Photon Flux Density
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
PSII ETR	Primary electron donor of PSII
P680	Oxidised P680
P700	Primary electron donor of PSI
QA	Primary quinone electron acceptor of PSII
QB	Secondary quinone acceptor of PSII
ROS	Reactive oxygen species
RUBISCO	Ribulose 1,5 bisphosphate carboxylase oxygenase
RuBP	Ribulose bisphosphate
SWC	Soil water content
θ	Soil water content
Ψ _π	Osmotic potential
Ψ _l	Leaf water potential
TBS-T	Tris-buffered saline tween-20
WUE	Water use efficiency

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Chapter 1

General introduction

1.1 Effects of Abiotic stresses on plants

Abiotic stresses are able to affect, at a quantitative and qualitative level, crop yield in all cultivated land in the world (Boyer, 1982), because of causing changes in chemical and physical environmental conditions. Drought, salinity, extreme radiation and temperatures are the main factors limiting plant growth and productivity worldwide (Doupis et al., 2011). Although in most of cases different abiotic stresses have been deeply studied separately, crops are nearly always subjected to different combinations of them under field conditions (Mittler, 2006).

Plants responses to abiotic stresses consist of morphological, physiological and biochemical changes that reduce stress exposure and/or limit damages, supporting recovery of impaired systems (Potters et al 2007). Understanding mechanisms that underlie plant responses to stress is very difficult due to the complexity of processes and molecules involved, in addition plants responses to combinations of stress are often different from those observed when each stress is applied separately. (Mittler, 2006; Cramer et al., 2011).

Photosynthesis is the most important physiological process in plants, which has direct effects on plants growth and crops productivity, and is deeply affected by environmental stresses (Chaves et al., 2003; Flexas et al., 2004; Chaves et al., 2009; Lawlor and Tezara, 2009; Pinheiro and Chaves, 2011). Abiotic stresses reduce photosynthesis damaging photosynthetic pigments, thylakoid's membranes, the electron transport chain and CO₂ fixation. On the other hand, according to their tolerance level, plants show several kinds of responses to abiotic stresses. Thus, it is important to study the effect of these stresses in order to understand the mechanism of resistance and tolerance observed in many species, to develop agricultural practices designed to increase plants productivity.

In this thesis the effects on photosynthesis of two of the main abiotic stresses that threaten crop productivity and food supply around the world namely water stress and salt stress have been investigated.

1.2 Photosynthesis

Photosynthesis is the process by which light energy is captured and converted into chemical energy in the bonds of sugars and other compounds. Carbohydrates are synthesized from water and carbon dioxide and the oxygen is the waste product. In plants this fundamental process is carried out in organelles called chloroplasts, which contain the photosynthetic apparatus. Photosynthesis consists of two phases: the light-dependent phase and the light-independent phase.

1.2.1 Light Harvesting

Light is captured by light harvesting pigments, leading to the formation of an excited state. The absorbed energy, named excitation energy, can be relocated by resonance transfer to the RCs to drive charge separation. This process is named light harvesting. It is realized in green plants by the use of antennae, which ensure efficient energy transfer to the RCs. The antennae of higher plants are divided into a proximal antenna, which is part of the RC, and the light harvesting complexes LHCI and LHCII, which differ in dimension and composition (Dang et al., 2008; Keren et al., 2005; Picorel et al., 2004; Picorel et al., 2011). The pigments enclosed in the antennae are chlorophylls (chl a and chl b), xanthophylls and carotenoids (Li et al., 2004; Lince and Vermaas 1998). The LHCs act like a bottleneck in trapping the right wavelength of light needed to get the charge separation in the RCs (Demmig-Adams and Adams 1996, Zhu et al., 2010). The absorbed energy is moved from one molecule to another by resonance (Cheng 2006). The LHCs include chlorophylls a and b and xanthophylls, whereas the proximal antennae bind only chlorophyll a and carotene.

1.2.2 Photosynthetic electron transport chain

The photosynthetic electron transport chain drives the movement of electrons from water to NADP accompanied by the net transfer of protons (H^+) through the thylakoid membrane from the chloroplast stroma to the thylakoid lumen. The proton

electrochemical gradient achieved drives adenosine triphosphate (ATP) production by the chloroplast F₀F₁ ATP synthase (Andersson and Barber 1996, Barber and Tran 2013, Genty and Harbinson 1996, Kramer and Crofts 1996, Owens 1996, Takahashi and Badger 2011). The electron transport chain (ETC) in the chloroplast implicates 2

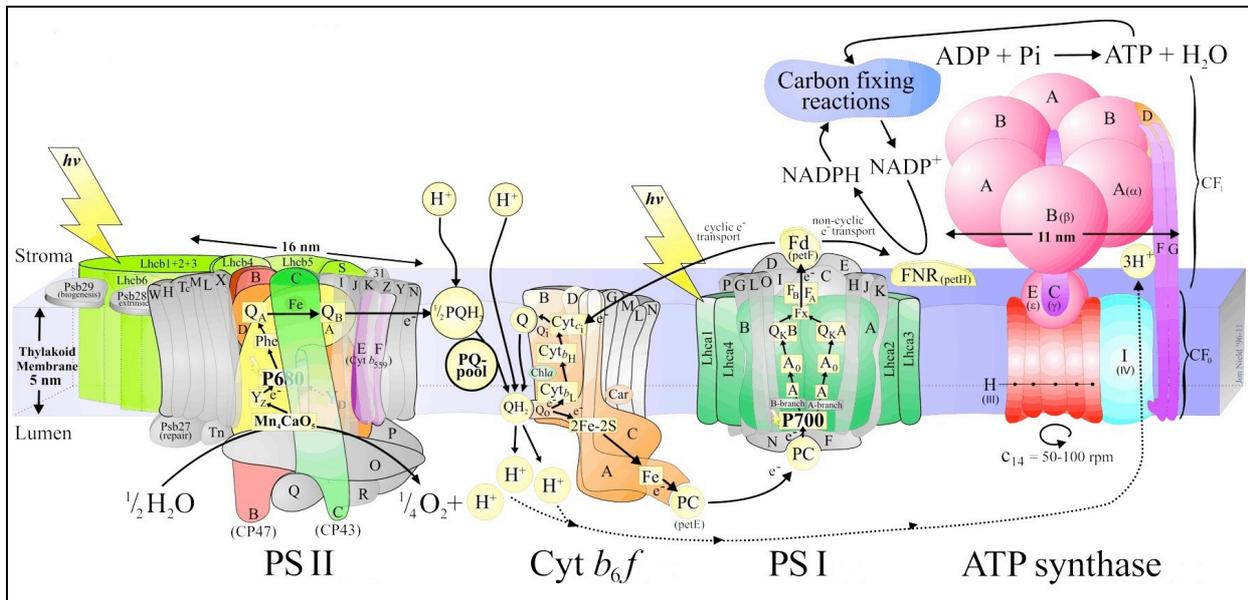


Fig. 1 Schematic model of the major protein complexes implicated in electron transport chain of photosynthesis.

reaction centers: Photosystems PSII and PSI and a cytochrome b₆f (cyt b₆f) complex. These are linked via the mobile carriers plastoquinone and plastocyanin (Fig. 1). PSII is the first complex in the ETC. It contains the primary electron donor chlorophyll P680 in the RC and moves energy from water to plastoquinone. The PSII core is built by a dimer of homologous peptides: D1 (PsbA) and D2 (PsbD) and co-factors like pheophytin and plastoquinone. PSI has chlorophyll P700 as primary electron donor in the RC and transfers light energy from plastocyanin to ferredoxin (Fd). Electron transfer in PSI includes chlorophylls (A₀, A₁) and FeS clusters FA, FB, Fx, Fd (Fromme and Mathis 2004; Vassiliev et al., 2001). The two photosystems have similar structures: they both have a core, which contains chlorophyll *a* and β -carotene, surrounded by LHCs (LHCII in PSII and LHCI in PSI) (Amerongen and Croce 2013; Blankenship 2002; Croce and Amerongen 2013; Demmig-Adams and Adams 1996; Gilmore and Govindjee 1999; Owens 1996; Romero et al., 2010; Rutherford and Faller 2003). When charge separation begins, P680⁺ is formed, a

strong oxidant that takes electrons from water, making oxygen, and transfers them to pheophytin. The electrons are then moved to plastoquinone, with the charge on P680 returning to a neutral state as a consequence of electrons obtained from a manganese center (Mn_4OxCa). This is included in the oxygen-evolving center (OEC) on the lumenal side of the PSII RC (Kulik et al., 2007). The synthesis of oxygen from H_2O , catalyzed by Mn_4OxCa , also outcomes in the increase of protons concentration (H^+) into the thylakoid lumen (Fig. 1). H^+ release generates a pH gradient throughout the thylakoid membrane, which is used in the production of ATP (Barber and Tran 2013, Renger and Renger 2008, Takahashi and Badger 2011). Water oxidation releases four electrons and that is the only known biological reaction where water is split into its component parts (Romero et al., 2010; Scott 2008).

The electrons accepted by plastoquinone are then transferred to the cyt b6f complex. This complex receives electrons from plastoquinol. The accepted electrons can be moved either throughout a linear or a cyclic (Q-cycle) electron transfer route. In the linear route, the electrons are transferred to the plastocyanin in the thylakoid lumen, through the rieske Fe-S protein and cyt f (Fig. 1). In the Q-cycle, instead, electrons from plastoquinol (PQH₂) are used to reduce a second plastoquinone (PQ) moved via two b cytochromes (cyt b563). For each PQH₂ oxidized, one electron is driven through each of these two route, so that, on average, each electron passes two times through the cyt b6f complex. For each oxidation of PQH₂, two protons are released into the thylakoid lumen. The Reduction of PQ to PQH₂ in the Q-cycle uptakes 2 protons from the chloroplast stroma. Thus, the cyt b6f is responsible for a net movement of protons to the lumenal side of the membrane (Andersson and Barber 1996, Barber and Tran 2013, Genty and Harbinson 1996a, Kramer and Crofts 1996, Owens 1996, Takahashi and Badger 2011). PSI gets light activated electrons from plastocyanin and moves them to Fd which is then oxidized by FNR to generate NADPH. NADPH synthesized is then used in the Calvin cycle (Foyer et al., 2012; Fromme and Mathis 2004). Reduced Fd can also reduce molecular oxygen to produce superoxide (O_2^-) instead of NADPH. While a small fraction of electrons moved to

PSI is used in the production of O_2^- , most is used to drive NADPH production (Foyer et al., 1994; Foyer et al., 2012).

1.2.3 The Calvin cycle

The Calvin cycle is a metabolic pathway that takes place in the stroma of the chloroplast. Carbon is incorporated in the cycle as CO_2 and exits as Glyceraldehyde-3-phosphate. The cycle can be divided into three phases: carbon fixation, reduction and regeneration (Fig. 2) (Klekowski, 1997). The ATP and NADPH needed for cycle reactions come from the light-dependent phase of the ETC.

During phase 1 (carbon fixation), CO_2 binds to a 5-carbon acceptor molecule, ribulose 1,5 biphosphate (RuBP) to obtain an unstable six-carbon compound, 3-keto-2- carboxyarabinitol-1,5-bisphosphate (3-Keto-CABP). This reaction is catalyzed by ribulose 1,5 biphosphate carboxylase/oxygenase (Rubisco) with the unstable product splitting immediately into two molecules of 3-phosphoglyceric acid (PGA) (Araújo et al., 2014, Bowyer and Leegood 1997).



Through phase 2 (reduction), ATP and NADPH from the ETC are used to transform PGA in glycerate-3- phosphate (G3P) (Bowyer and Leegood 1997, Klekowski 1997). The latter is a precursor for sugar production. The third phase (regeneration), in which G3P is converted back to RuBP using ATP, completes the cycle. For every molecule of G3P obtained, three molecules of CO_2 enter the cycle and the latter completes six times to generate 0.5 molecule of six-carbon glucose ($C_6H_{12}O_6$). Consequently the cycle needs nine molecules of ATP and six molecules of NADPH for the net synthesis of one G3P and 0.5 molecule of $C_6H_{12}O_6$ (Fig. 2) (Klekowski 1997).

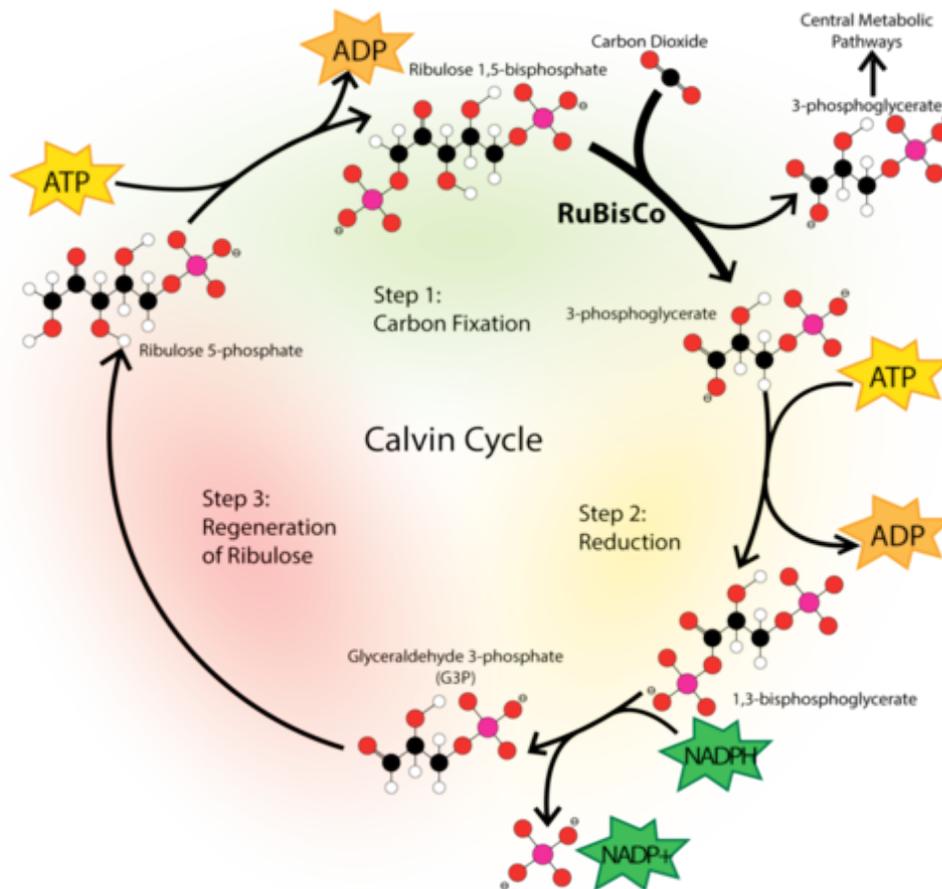


Fig. 2 Calvin Cycle. Stage 1 of the cycle: 3-phosphoglyceric acid (PGA) is formed. Stage 2: ATP and NADPH convert PGA to glycerate-3-phosphate (G3P). G3P must be produced six times to make 0.5 molecule of six-carbon glucose. Stage 3: G3P is transformed back to RuBP using ATP for the completion of the cycle.

1.2.4 Rubisco

Rubisco is the most abundant protein on earth and is located in the chloroplast stroma (Feller 1998). Rubisco includes two subunits, the large (RbcL) and small (RbcS) subunits. In plants, these subunits form a holoenzyme consisting of 8 large and 8 small subunits (Saschenbrecker et al., 2007; Spreitzer et al., 2005). The RbcL gene is contained in the chloroplast of plants and the resultant polypeptide has a molecular weight of 55-kDa. RbcL lead to the formation of the active site used for CO₂ fixation and has both catalytic and regulatory sites. On the contrary, the RbcS gene is present in the nucleus of plants and the protein produced has a molecular weight of 13-kDa (Berg et al., 2002). RbcS improves the catalytic activity and CO₂/O₂ specificity of the RbcL subunits (Berg et al., 2002).

Rubisco is an enzyme that catalyzes both the fixation of CO₂ (carboxylation) and O₂

(oxygenation) (Feller 1998). During carboxylation, from the reaction of RuBP and CO₂, PGA formed is used for either sugar production or renewal of RuBP for the

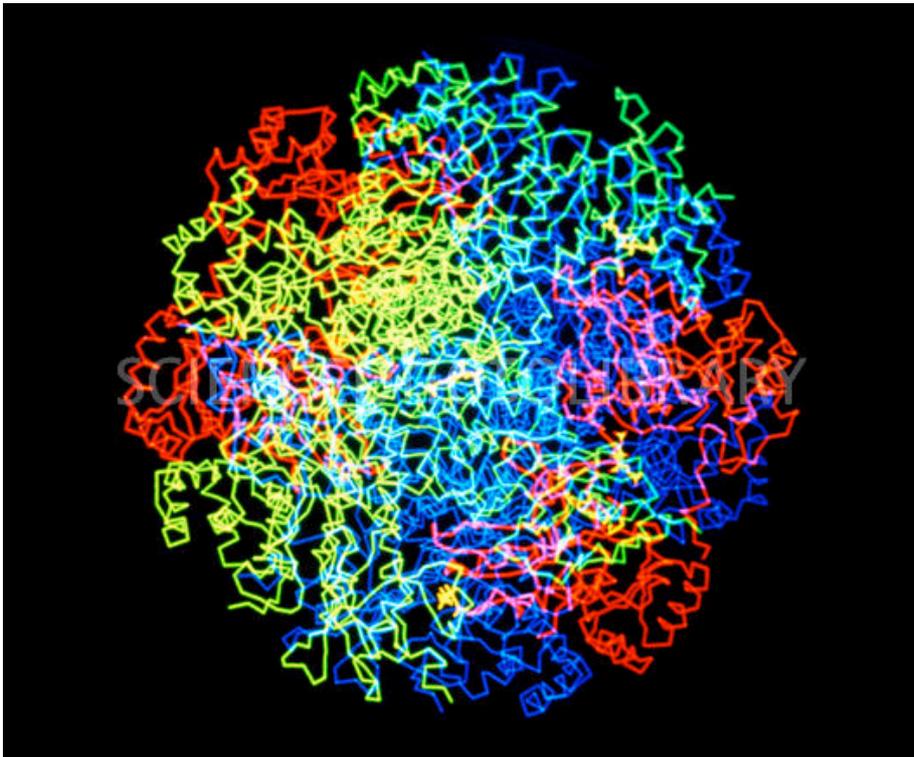


Fig. 3 Molecular graphic of Rubisco, the most abundant protein on earth.

continuation of the Calvin cycle (Berg et al., 2002). However, during O₂ fixation, Rubisco reacts with molecular oxygen instead of CO₂, generating one molecule of PGA and one of phosphoglycolate (PG). PGA re-enters the Calvin cycle while the carbon in PG is involved through a series of reactions termed photorespiration (Berg et al. 2002; Bowyer and Leegood 1997).

Rubisco is supposed to have evolved about 2.5 billion years ago in cyanobacteria when the atmosphere had a high concentration of CO₂ and limited O₂. Atmospheric CO₂ dropped in the late Miocene and into Pleistocene – (23-2 Mya) as a consequence of hot or warm climates often deficient in inorganic CO₂. This led to difficulty to discriminate between CO₂ and O₂ and adjustments in photosynthetic pathways as compensation. This resulted in the development of the photorespiration, CAM metabolism and C₄ photosynthesis (Barsanti and Gualtieri 2006, Christin and Osborne 2013). These mechanisms increase CO₂ concentration in cells where Rubisco is contained.

1.3 Plant responses to water stress.

On a global scale, drought limits plant growth and productivity more than any other environmental abiotic stress and it is well known to have detrimental effects on them (Boyer, 1982).

Water is one of the most important resources for the survival of plants and animals. Plants need it for photosynthesis, for nutrient supply and for cooling (Farooq et al, 2009). Plants are sessile organisms and, unlike the animal they cannot move when environmental conditions become adverse. Consequently they must be able to respond and adapt to environmental conditions changes. Since water is a key resource, the ability to tolerate water stress is crucial.

Water stress can be seen either as reduced water availability, or osmotic stress (high salt concentration). It can affect photosynthesis, respiration, growth and several other metabolic processes, and in more severe cases can lead plants to death (Jaleel et al., 2009). In nature, depending on the local climate, plants can be subjected either to long or short period of water stress and thus many species have developed several kind of response or adaptation to enhance growth and survival rates (Keeley and Rundel, 2003). Water stress effects can be studied at morphological, physiological or biochemical levels in plants (Ghannoum 2009, Medrano et al., 2002). At a morphological level plants show changes in root, xylem and leaf anatomy (Ahmad 2011) Generally plants exposed to drought condition exhibit an inhibition of root growth (Westgate and Boyer 1985). Xylem modification are reflected in reduction of vessel diameter, and with the onset of embolism (Lo Gullo et al., 1995; Tyree 2002), but the first evident effect the decrease of leaf water potential (Ψ_l), which expresses the free energy of Gibbs on molar volume basis. Ψ_l is compounded by osmotic potential and turgor pressure $\Psi_l = \Psi_\pi + \Psi_p$ (Taiz and Zeiger, 2002). Under intense water stress the accumulation of inorganic or organic solutes in cell, which decreases Ψ_π , may non be sufficient to compensate the reduction of Ψ_l so Ψ_p approaches the point of no turgor (wilting point). Turgor pressure pushes the plasma membrane against the cell wall of plant. This pressure is caused by the osmotic flow of water

from an area of low solute concentration outside of the cell into the cell's vacuole, which has a higher solute concentration (Campbell et al., 2008). Under water shortage, turgor pressure is reduced and the plant cell is called plasmolysed (Campbell et al., 2008).

It is well-known that water stress leads to inhibition of photosynthesis (Blum 2011, Chaves et al., 2003). However, the exact mechanism of this inhibition is still controversial (Chaves et al., 2009). In order to survive plants need to perform photosynthesis by absorbing water from the soil and CO₂ from the atmosphere. Both CO₂ uptake and water transpiration, are carried out in leaves through stomatal pores. Water transpiration in leaves drives water from the roots through the xylem. When stomata are open, at the same time, CO₂ is adsorbed in and water vapour is diffused out of leaf. When stomata are closed, both transpiration and CO₂ uptake are strongly reduced. In order to cope with water shortage and to prevent itself from dehydration, plants can regulate the amount of water lost by opening and closing stomata.

There has been debate as to whether the inhibition of photosynthesis induced by water scarcity is due to stomatal or non-stomatal limitation (Chaves et al., 2009).

Stomatal limitation causes a decline in photosynthesis as a result of stomatal closure, induced by the accumulation of high concentrations of abscisic acid (ABA), thus precluding CO₂ entry. This is often considered as the earliest response of plants exposed to drought (Ghannoum 2009; Medrano et al., 2002). In the chloroplast, the thylakoid and stroma are more exposed to damages. It is well known that severe drought causes damages to photosystem II (PSII) (Cornic et al., 2004; Cousins et al., 2002, Golding and Johnson 2003) but not to PSI (Golding and Johnson 2003, Shikanai 2007, Takahashi et al., 2009). Cornic et al. (2004) ascribed severe damage to PSII during water stress to a direct effect of the drop in the CO₂ uptake of the chloroplast following the closure of stomata. In the stroma, CO₂ shortage causes a decline in the activities of the Calvin cycle enzymes (Cornic et al., 2004; Cousins et al., 2002). Drought realizes this reduction by lowering intracellular CO₂

concentration (C_i) and so reducing CO_2 assimilation (Cousins et al., 2002). This causes the accumulation of ATP and NADPH and in turn to accumulation of reduced plastoquinol, which stimulates the formation of the reactive oxygen species singlet excited oxygen ($^1\text{O}_2^*$) in PSII.

1.4 Plant responses salt stress

Soil salinization is one of the most serious factors affecting the productivity of agricultural crops (Munns 2008). Salinity interests more than 800 millions hectares of lands all over the world representing about the 6% of the total world's lands (FAO 2008). Soils are classified as saline when the electrical conductivity of a saturated paste (ECe) is 4 dS m^{-1} (40 mM NaCl) or more (Brown, 2008).

Salt stresses plants in two different ways: high concentration of salt in soil make it harder for root to adsorb water, and high concentration of salt in plants can be toxic (Munns 2008). As a consequence of these two leading effects, many others can be observed: nutritional disorders, oxidative stress, alteration of metabolic processes, reduction of cell division and expansion (Hasegawa et al., 2000; R. Munns, 2002; Zhu, 2007). All these factors affect seeds germination, plant growth and crop yield (Munns 2008).

During the beginning and development of salt stress the main plant processes such as photosynthesis and protein synthesis are affected (Parida & Das, 2005). Plants respond to salinity stress in two phases: a rapid response to the increase in external osmotic pressure (starts immediately after the salt concentration around the roots increases to threshold levels, which decrease the new shoot growth) and a slower response due to the accumulation of Na^+ and Cl^- in leaves (salt accumulates to toxic concentrations and increase senescence of older leaves) (Munns and Tester, 2008).

The effect of osmotic stress can be observed soon after the beginning of salt stress imposition and it lasts for the whole time in which it is applied, manifesting itself in inhibited cell expansion and division, as well as stomatal closure (Flowers, 2004; Munns, 2002). During the long-term exposure to stress, plants are affected by ionic

stress that can result in a premature senescence of adult leaves, and consequently in the reduction of photosynthetic area available (Cramer & Nowak, 1992). Moreover the excess of chloride compromises the function of several plant enzymes, causing cell swelling, resulting in the reduction of energy production and in the occurrence of other several physiological damages (Larcher 1980). Ionic stress is revealed in premature senescence of older leaves and in several other symptoms of toxicity, such as chlorosis and necrosis in mature leaves.

Plant adaptations to salinity can follow three pathways: (I) osmotic stress tolerance, (II) Na⁺ exclusion and (III) tissue tolerance. Osmotic stress tolerance implicates a reduction in both root and leaf cells expansion causing stomatal closure (rapid response); Na⁺ exclusion by roots ensures that Na⁺ does not accumulate to toxic concentrations within leaves; in tissue tolerance, plants compartmentalize Na⁺ and Cl⁻ at the cellular and intercellular level, to avoid the toxic concentrations within the cytoplasm (Munns and Tester, 2008).

Photosynthesis is deeply affected by salt stress. Salinity in soil either causes short-term or long-term effects on this process (Parida and Das, 2005). Salinity in soil prevents water uptake by plants, leading to stomatal closure and reduction of water release from transpiration (Hsiao, 1973; Fricke et al., 2004; Munns and Tester, 2008; Negi et al., 2014). Stomatal closure under salinity occurs because of loss in leaf turgor (Munns and Tester, 2008; Chaves et al., 2009). As a consequence, photosynthesis is inhibited, due to CO₂ shortage. Although low photosynthetic rates initially occur due to the stomatal closure, as salt stress becomes more intense, photosynthesis is inhibited due to metabolic damages (Cornic et al., 1989; Sharkey, 1990; Cornic and Briantais, 1991; Panković et al., 1999). In addition salt seems to limit CO₂ diffusion through the leaf mesophyll (Flexas et al., 2004; Flexas et al., 2007). This could be caused by physical alterations in the structure of intercellular spaces caused by leaf shrinkage (Lawlor and Cornic, 2002) or changes in the biochemistry or membrane permeability (Gillon and Yakir, 2000; Flexas et al., 2008).

1.5 General Aim

This thesis aimed to study regulations of photosynthetic activity of plants under two of the most important abiotic stresses: water stress and salt stress. We mainly focused on the physiological and biochemical mechanisms that regulate growth and productivity under limiting conditions, in order to acquire a better understanding of the complex defense responses of plants to environmental abiotic stresses, which represent the greatest global constraints for agriculture.

In order to tackle such broad aim, an integrated approach was necessary. This study was carried out on some specific crops with the peculiar purpose to test cultivars that are tolerant to drought or salinity used under harsh environments. Although abiotic stresses have been largely studied for tomato, pepper and grape species, the specific behavior of many local and typical cultivars and landraces to cope with drought and salinity problems is still new. Hence, the general aim of this work was pursued through three main experimental activities, illustrated in chapters 2-4. Chapter 2 focuses on two typical long-storage tomato landraces grown in greenhouse under extreme ($g_s < 0.01 \text{ mol m}^{-2} \text{ s}^{-1}$) water stress conditions (Fig. 4); chapter 3 focuses on two pepper cultivars - grown in a soilless system differently behaving under severe (15.6 dS m^{-1}) long-term salt stress conditions (Fig. 5); chapter 4 focuses on a wine grape cultivar, typical in Southern Italy, grown in two soils with contrasting hydrological properties for retaining water (Fig. 6).

The three experiments have been part of multidisciplinary complex research projects, founded under umbrella of national and regional programs, as follow:

“Conoscenze Integrate per Sostenibilità ed Innovazione del Made in Italy Agroalimentare”, (CISIA) Legge 19/2009

“Valorizzazione di produzioni ortive campane di eccellenza con strumenti di genomica avanzata” (GenHort), Programma Operativo Nazionale Ricerca e Competitività 2007-2013.

“Zonazione Viticola alla Scala Aziendale” (ZOVISA), Misura 124 “Cooperazione per lo sviluppo di nuovi prodotti, processi e tecnologie nei settori agricolo e alimentare e settore forestale”, PSR Campania 2007/2013.



Fig. 4 Potted Tomato grown in glasshouse in Portici (NA). (EXP chapter 2)



Fig. 7 *Locale di Salina* berries (EXP chapter 2)



Fig. 8 *Pizzutello di Sciacca* berries (EXP chapter 2)



Fig. 5 Pepper grown in soilless system in a glasshouse in Pontecagnano (SA)(EXP chapter 3)



Fig. 9 *Cazzone giallo* berry (EXP chapter 3)



Fig. 10 *Quadrato D'Asti* berries (EXP chapter 3)



Fig. 6 *Aglianico* rain-fed vineyard grown on two different soils with different hydrological properties, in Mirabella Eclano (AV) (EXP Chapter 4)



Fig. 11 *Aglianico* grapes (EXP chapter 4)

Experiment	Chapter	Localization	Experimental conditions		Species	Analyses
I	2	Portici (NA) ITALY	Pots	Limitation H ₂ O	<i>Solanum lycopersicum</i> L.	Soil water content, leaf water potential, gas exchanges, chlorophyll a fluorescence, biochemical analyses (ABA & proline content, PARP activity)
II	3	Pontecagnano (SA) ITALY	Pots	Hi salt level	<i>Caspicum annuum</i> L.	Soil water content, plants water status gas exchanges, chlorophyll a fluorescence, ions content (Na ⁺ , Cl ⁻), biometric measurement
III	4	Mirabella Eclano (AV) ITALY	Open Field	Limitation H ₂ O	<i>Vitis vinifera</i> L.	Soil water content, leaf water potential, gas exchanges, chlorophyll a fluorescence, chlorophyll content, must characteristics

Table 1. Synopsis of the experimental activities

Chapter 2

“Photosynthetic and biochemical responses to soil water deficit and re-watering of two long storage tomato landraces”

(Solanum lycopersicum L.)

2.1 Abstract

Long-storage tomato landraces are niche crops traditionally cultivated in southern Italy for drought tolerance, high nutritional and organoleptic quality of fruits. Nowadays, there is a growing interest in these crops as for exploiting biodiversity and the “Made in Italy” food production. In this study physiological and biochemical performances of two landraces has been studied in response to soil water deficit and re-watering. Potted-plant experiments were carried out in a greenhouse during 2012 and 2013.

During the first year, an intense water stress determined a decrease of both stomatal conductance (g_s) and net CO₂ assimilation (A) along with an increase in proline and ABA content, without any effect on photochemistry. In the second year, a more severe water stress led to a greater decline of both g_s and A, and to a reduction in photochemistry. During the re-watering, the full recover of gas exchanges and photochemistry, indicated the occurrence of efficient stomatal control and photochemical regulation, rather than impairment of photosynthetic apparatus.

The activity of poly ADP-ribose polymerase (PARP), a key regulator of the energy homeostasis during stress conditions, showed during the first cycle of stress an evident increase, followed by a decrease. Re-watering, instead, produced a new non-expected increase of activity, which returned to control values during the second cycle of water stress.

The two tomato landraces succeeded to cope with water stress by a useful synergy of biochemical and physiological regulatory mechanisms.

2.2 Introduction

Sustainable agriculture is a primary global goal to provide food for the increasing human population (Somerville, 2001). Typical losses of crop yields due to biotic and abiotic stresses may be, in a near future, magnified by impacts of global warming. For this reason, providing adequate food supplies for humankind may become

progressively hard (Bray et al., 2000, Takeda 2008). Drought is the main abiotic factor that limits the global productivity of major crops and, thus, it has become a major target of plant research (Boyer 2010). The expression “*more crop per drop*” has diffused among scientific communities during last years as future target for research (<http://www.fao.org/english/newsroom/focus/2003/water.htm>).

The horticultural production in southern Italy plays an important role in the Mediterranean agriculture for the great number of vegetables, representing a precious source of biodiversity. In South of Italy long storage tomatoes are typical crops, which need to be preserved from genetic erosion. Recently, there is an increasing interest in them because of their beneficial nutritional properties and their low-inputs agronomic properties (e.g. the opportunity to cultivate them without irrigation) (Riggi et al., 2006; Siracusa et al., 2012).

Long storage tomatoes are traditionally cultivated in warm and dry climate under rain fed conditions, as in southern Italy and Spain (Galmes et al., 2011; 2013).

The C.N.R. CISIA project initiated a wide activity of research dedicated to the characterization and evaluation of these ecotypes. In this work a study of physiological and biochemical responses of two tomato landraces “Locale di Salina” and “Pizzutello di Sciacca” has been carried out, in order to study mechanisms of adaptation to preserve physiological activity of the plant under conditions of intense soil water deficit. This work could be used in genetic programs to improve the tomato resistance to water stress and, at the same time, to identify useful relationships between these mechanisms and crop productivity.

2.3 Material and methods

2.3.1 Plant material and growth conditions

Two Sicilian long-term storage landraces, *Locale di Salina* (Lc) and *Pizzutello di Sciacca* (Pz), of tomato (*Solanum lycopersicum*, L.) were grown in a greenhouse of the Institute of Biosciences and Bioresources CNR–IBBR in Portici (Italy) and subjected to water stress and re-hydration, during spring season in 2012 and 2013.

Seeds were germinated in a sandy soil mixture, and when seedlings developed 2 true leaves (25 days after sowing) they were transplanted into 22 cm diameter (10 liter) plastic pots (one plant per pot), which were filled with soil. Dr. Cristina Patanè from IVALSÀ-CNR in Catania (Italy) kindly provided the seeds.

2.3.2 Drought Irrigation treatments: water stress and re-hydration

For each genotype, 12 plants were watered every other day to field capacity (Control treatment, Ctrl) during all the trial, while in other 12 plants irrigation was interrupted when they were thirty-five days old (first inflorescence visible, growth stage code 51 in BBCH scale for solaneous fruit), and until stomatal conductance (g_s) dropped to less than $0.020 \text{ mol m}^{-2} \text{ s}^{-1}$ (Stress treatment, St). Afterward, Stress treatment plants were re-irrigated for some days before a second period of no irrigation was imposed. Pots were arranged in a completely randomized block design, (24 blocks with 2 plants per block, 1 per genotype).

2.3.3 Soil water content

The volumetric soil water content (θ , $\text{m}^3 \text{ m}^{-3}$) was estimated from dielectric measurements performed by a Time Domain Reflectometer TDR100 (Campbell Scientific Inc. Logan, UT) through a 14.2 cm three-steel-wire probe installed in 3 pots for each irrigation treatment and genotype, and applying the Topp's equation (1980). The measurements were carried out on the same days of leaf gas-exchange measurement (see below).

2.3.4 Leaf water status

On 3 days in both years, leaf water potential (Ψ_w , MPa) was assessed at daytime (10-12 h) by a Scholander pressure chamber (SAPS II, 3115, Soilmoisture Equipment Corp., Santa Barbara CA, USA) on the first fully-expanded and well-exposed leaf in three plants per treatment. Following cutting, the leaf was inserted into the pressure bomb within 30 s, and pressure was increased at a rate of 0.2 MPa min^{-1} .

2.3.5 Leaf gas exchanges and modulated Chlorophyll *a* fluorescence emission

Net photosynthetic CO₂ assimilation rate (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) at saturating light, stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) and leaf temperature were determined using a portable open-system gas-exchange and modulated fluorometer analyzer Li-6400XT (Li-Cor Biosciences, Lincoln, NE, USA.), with CO₂ inside leaf chamber set to 400 $\mu\text{mol mol}^{-1}$ air. A LED light source with emission peaks centered at 630 nm in the red and at 460 nm in the blue provided a PPFD equal to 1500 $\mu\text{mol (photons) m}^{-2} \text{s}^{-1}$ (90% red, 10% blue). The instrument was also used to measure the fluorescence parameters. The measuring beam was set at intensity 5 (according to the instrument manual) with a modulation of 20 kHz. After the measurement of *Chl a* fluorescence emission at steady-state under light conditions, F' , the maximum fluorescence emission, F_m' , was assessed upon induction by a 0.8 s super-saturating light pulse at 6000 $\mu\text{mol (photons) m}^{-2} \text{s}^{-1}$, with a modulation of 20 kHz; then actinic light was briefly switched off while a far-red light of 8 $\mu\text{mol (photons) m}^{-2} \text{s}^{-1}$ for 6 s was used to discharge the PSI photosystem to allow measurement of the minimum fluorescence emission under light conditions, F_0' . The software of the instrument (Li-Cor, 2011) calculated the gas-exchange parameters on the basis of von Caemmerer and Farquhar (1981) model, and the actual quantum efficiency (ϕ_{PSII}) or $\Delta F/F_m' = (F_m' - F')/F_m'$ (Genty *et al.*, 1989) and the maximal quantum yield under light conditions F_v'/F_m' (Baker, 2008). Measurements were taken on 6-10 replicate plants per treatment. Leaf gas-exchange and fluorescence data were taken between 10:00 and 13:00 on 11 days in 2012 and 8 days in 2013.

2.3.6 Transient Chl *a* fluorescence emission:

A continuous excitation Handy PEA fluorometer (Hansatech, Instruments Ltd., King's Lynn, Norfolk, England) was used. The excitation red light pulse for fluorescence induction (FI) was emitted by a (red) 650 nm light diode source, and applied for 1 s at the maximal available photosynthetic photon flux density (PPFD) of

3,500 $\mu\text{mol (photon) m}^{-2} \text{ s}^{-1}$. Leaves were dark adapted for 30 min by means of the equipped white leaf-clips, prior to the assessment of the basal, F_o , and the peak F_p as a viable approximation of the maximum Chl *a* fluorescence emission, F_m (Giorio 2011), from which the dark-adapted maximal quantum yield of PSII photochemistry was calculated as $F_v/F_m = (F_m - F_o)/F_m$ (Baker, 2008). Measurements were taken on 6 plants per treatment on 2 leaves per plant, chosen as above.

2.3.7 Biomass determinations

Plant leaf area (PLA, cm^2) was measured at the end of experiment on the excised leaves of three plants per treatment using a scanning planimeter (Li-3100, LiCor, Lincoln, NE, U.S.A.). The instrument was equipped with a fluorescent source and a solid-state scanning camera to measure the area of leaves as they moved on a transparent conveyor. Leaves, stems, fruits and roots were oven-dried at 70 °C for a few days until a stable weight was reached to assess dry biomass weight (D.W.).

2.3.8 Weather station

Air temperature (T_a , °C), relative humidity (RH, %) and solar radiation (R_s , W m^{-2}) were acquired every 15 min by a data logger from related sensors (Watchdog data logger, Spectrum Technologies, Plainfield, IL, U.S.A.).

2.3.9 Biochemical and molecular analysis

During the experiment, leaf samples of three replicate plants for each treatment were collected by excising the leaf at the petiole, and quickly freezing it in liquid nitrogen. The tissue was ground to a powder in a pre-cooled mortar with liquid nitrogen and stored at -80 °C.

2.3.10 Extraction and determination of leaf ABA content

For ABA extraction, 150 mg of fine powder were added to 1.8 ml of distilled and autoclaved water in 2 ml tube, shaken at 4 °C overnight in darkness, and then

centrifuged at 10000 g for 10 min. Supernatant was diluted 50-fold with TBS buffer (50 mM TRIS, 1mM MgCl₂, 150mM NaCl, pH 7.8). ABA content was analyzed by indirect enzyme-linked assay (ELISA) using the Phytodetek ABA test kit (Agdia, Elkhart, IN, USA) following the manufacturer's instructions. Colour absorbance due from the reaction with substrate was read at 405 nm using a plate autoreader (1420 Multilabel Counter Victor³_{TM}, PerkinElmer). The ABA concentration was determined following established procedures by the protocol included in the kit.

2.3.11 Free-proline content

Free-proline content was estimated according to the methods of Claussen et al. (2005). 250 mg of tissue powders were suspended in 3% sulphosalicylic acid 3% and filtered through a layer of glass-fiber filter (GF6 Macherey-Nagel). 1 ml of sample was mixed with 1 ml of glacial acetic acid and 1 ml of ninhydrin reagent (2,5 g ninhydrin/100 mL of a solution containing glacial acetic acid, distilled water and ortho-phosphoric acid 85% at a ratio of 6:3:1). The reaction mixture was incubated in a water bath at 100 °C for 1 h and after it was cooled for 5 minutes at room temperature. Absorbance of the samples was measured at 546 nm using a UV–Visible spectrophotometer. The amount of proline in the samples was calculated using a standard curve and was reported in $\mu\text{mol proline g}^{-1}$ of fresh weight.

2.3.12 Preparation of homogenate for protein determination

For samples preparation, 1 g of fine powder was suspended and homogenized by Ultra Turrax T8 (IKA WERKE, Staufen, Germany) in a buffer containing 10 Mm Tris HCl pH 7.0, 1 mM EDTA, 1mM, EGTA, 1 mM PhMeSO₂F, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, protease inhibitor cocktail (1%) and 0.5% NP-40 (1:4, w/v). After filtering through two layers of cheesecloth the homogenate was centrifuged at 1500 g for 30 min at 4 °C, This procedure was repeated four times. Finally, the pellet was suspended in a small volume of the same buffer without NP40. Protein concentration was determined according to the method of Bradford et al. (1976).

2.3.13 Poly-ADP-ribose polymerase standard assay

The enzymatic activity was assayed in 0.5 M Tris HCl pH 8.0, 50 mM MgCl₂, 10 mM DTT, and a defined amount (20 µg protein) of sample in the presence of 0.4 mM [32P]NAD (10.000 cpm/nmol) in a final volume of 50 µl as described in Faraone Mennella et al. (2009). After incubation for 20 min at 25 °C, the reaction was stopped by transferring it onto ice and by the addition of 20% (w/v) trichloroacetic acid (final concentration). The mixture was filtered through Millipore filters (HAWPP0001, 0.45 mm) and washed with 7% trichloroacetic acid. The activity was measured as acid-insoluble radioactivity by liquid scintillation in a Beckman counter (model LS 1701). One enzymatic milliunit catalyses the synthesis of 1nmol ADPribose/ min under standard conditions.

2.3.14 Statistical analysis

For soil water content, gas-exchange and fluorescence parameters, two factors (genotype and water regime) were analyzed together, according to a randomized complete block experimental design by Two Way ANOVA. Duncan test at 0.05 probability level was used as mean separation test. For ABA and proline, the statistical significance was assessed by the Student's t-test ($p < 0.05$).

2.4 Results

2.4.1 Soil water content

In both 2012 and 2013, volumetric soil water content (θ) before stress imposition (DOE 0) was $0.36 \text{ m}^3 \text{ m}^{-3}$ for both Pz and Lc (Fig. 1 a, b), a value quite proximal to the maximum soil water capacity. Afterwards, as irrigation was interrupted in the Stress treatments, θ decreased to lower than $0.1 \text{ m}^3 \text{ m}^{-3}$ within 10 days in the 2012 and within 6 days in the 2013. In the latter case, θ further decreased to a value as low as $0.02 \text{ m}^3 \text{ m}^{-3}$ five days later (DOE 11). In both years, the restoration of irrigation

quickly re-increased θ , up to the values of the Wet treatments. Such a re-watering was continued for a week, after which a second withdrawal of irrigation was imposed. During this second period of no irrigation in 2012, θ decreased within about a week, to a similar minimum value observed in the previous stress period. As regard 2013, measurements were stopped after 3 days of the second stress period, when θ in stress treatments was about $0.15 \text{ m}^3 \text{ m}^{-3}$.

2.4.2 Leaf gas exchanges

Stomatal conductance (g_s) followed similar patterns of soil water content (Fig. 1 c, d). In the first year, after about ten days of no irrigation in the Stress treatments, this parameter dropped to the quite low value of $0.021 \text{ mol m}^{-2} \text{ s}^{-1}$. However, in the second year, stomatal closure resulted in such a low g_s value, within 6 days only. Subsequently, stomatal conductance continued to decrease reaching $0.006 \text{ mol m}^{-2} \text{ s}^{-1}$ five days later (DOE 11) when θ reached the lowest observed value. Restoration of irrigation re-increased g_s , up to the control value in 2012, and slightly higher in 2013. Since the start of second stress imposition, stomatal conductance dropped down to $0.030 \text{ mol m}^{-2} \text{ s}^{-1}$ within 3 days in 2012 and in the same period to $0.140 \text{ mol m}^{-2} \text{ s}^{-1}$ in 2013 at a rate clearly lower than that observed for θ . However, most of g_s recovering occurred within a couple of days in 2012 (65% in Pz and 75% in Lc), while it was less than 50% in 2013. As occurred for θ , about a week after the second withdrawal of irrigation in 2012 and 2013, g_s dropped down to about same low values observed at the end of previous stress period.

Patterns of net assimilation (A) for each treatment reflected stomatal conductance (Fig. 1 e, f). As for g_s , average A was quite low, $2.3 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ after ten days of interrupted irrigation in 2012, and as low as $-0.5 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ after only six days in 2013, as compared with the control values of $15.8 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ in 2012 and $8.95 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ in 2013. Interestingly, mainly in 2013 at the end of re-watering period, the Stress treatments showed gas-exchange parameters recovered at higher values than

control treatments. As for stomatal conductance, the second irrigation withdrawal induced a quite strong reduction in assimilation rate.

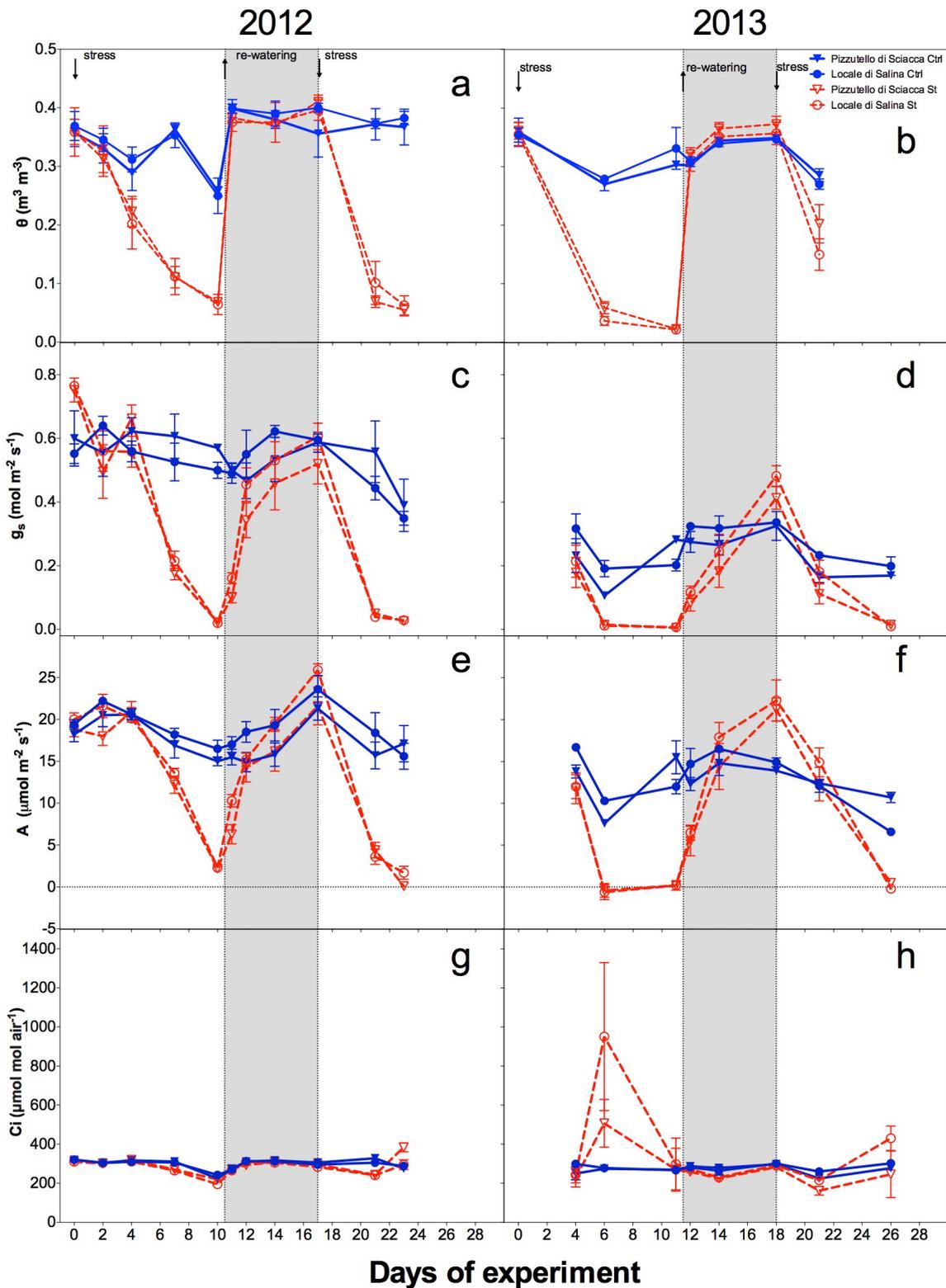


Fig. 1. Volumetric soil water content (θ , $\text{m}^3 \text{m}^{-3}$) a) and b), stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) c) and d), photosynthetic CO_2 assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) e) and f), intercellular CO_2 concentration (C_i , $\mu\text{mol mol air}^{-1}$) g) and h) of Stress and Control treatments of the two genotypes during the 2012 and 2013 trials. Each point is the mean of six replicates. Vertical bars indicate the standard error of the mean. Downward and upward arrows indicate the start of withholding and restoration of irrigation respectively; grey area indicates the re-watering period.

2.4.3 Chlorophyll *a* fluorescence

The (dark adapted) maximal quantum yield of PSII (F_v/F_m) showed a modest effect of irrigation in 2012 (Fig. 2, a), though with some variation throughout the experiment, but a clear effect in 2013 (Fig. 2 b). In the latter case, in the stress treatments of both genotypes F_v/F_m started to decrease on day 6 since interruption of irrigation. Such a decrease becomes more pronounced after 11 days (end of first period of no irrigation), when F_v/F_m in the Stress treatments was 0.596 in Lc and 0.652 in Pz, though a decrease was also observed in the control of Lc. The re-watering phase involved a full recovery of this parameter within a day, and after a week of rehydration it ranged 0.776-0.813 in all treatments and genotypes, with Stress treatments showing values slightly higher than Control treatments. After about a week of a second interruption of irrigation, F_v/F_m decreased to values comparable to those observed in the first stress period, as occurred for gas exchange parameters.

The effective quantum efficiency of PSII (Φ_{PSII}) in 2012 showed no appreciable difference between the Stress and Ctrl in both genotypes (Fig. 2 c), as occurred for F_v/F_m . In 2013, during first stress cycle Φ_{PSII} declined in both the two genotypes to 0.055 after 11 days of stress, as compared with an average of 0.130 in the Ctrl treatments (Fig. 2 d). After only 3 days of re-watering, the stressed plants rapidly recovered, and showed Φ_{PSII} higher than control plants as occurred for gas-exchange and F_v/F_m . During the second interruption of irrigation, Φ_{PSII} again decreased to about the minimum value observed in the previous stress period, similarly to both gas-exchanges and F_v/F_m patterns.

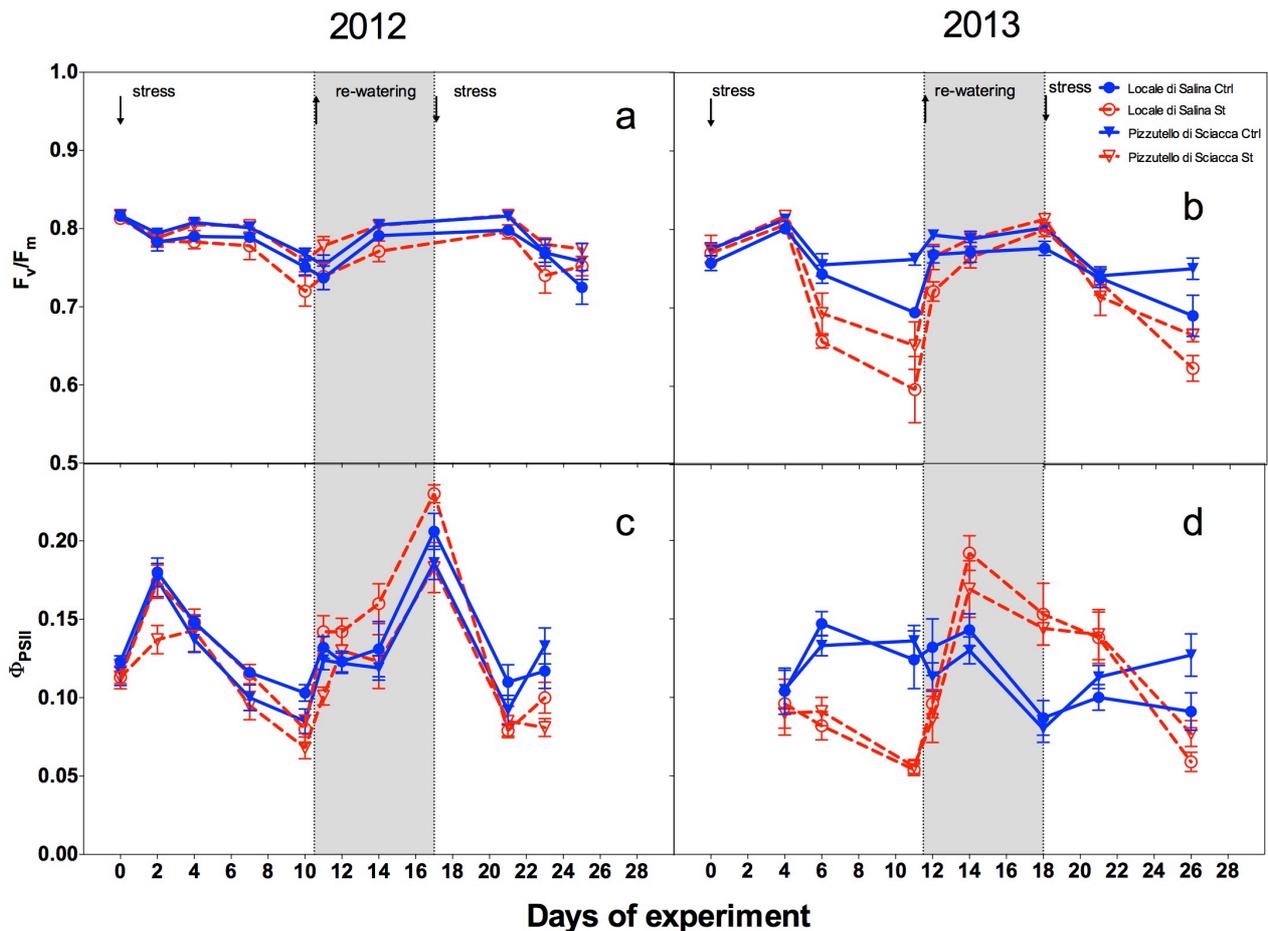


Fig. 2. Maximal quantum efficiency of PSII (F_v/F_m) a) and b), effective quantum efficiency of PSII (Φ_{PSII}) c) and d) for control and stress treatments of the two genotypes, during the experiments in 2012 and 2013. Each point is the mean of 40 measures for F_v/F_m and 6 measures for Φ_{PSII} . Vertical bars indicate the standard error of the mean. Downward and upward arrows indicate the start of withholding and restoration of irrigation, respectively. Grey area indicates the whole re-watering period.

2.4.4 Biomass determinations - Plant Leaf Area and Dry Weight

The biometric measurements were carried out at the end of the trials (DOE=25) when plants had developed fruits. Plant size was quite higher in 2012 than 2013. This was due to the environmental conditions in the glasshouse, which were quite different in the two years of experiment. In 2013, the low temperature since transplantation had a further detrimental effects on plant growth as compared with the previous year. In both years, there were no genotype effects on both PLA and total DW, whereas irrigation effect was statistically significant in both years for both Pz and Lc. As an average for the two genotypes in 2012, the Wet treatments had PLA and total DW,

respectively, 1.2 and 1.3 fold higher than Stress treatments, whereas in 2013 they were 2.1 for PLA and 1.6 for total DW (Table 1).

	Year	Treatments			
		Lc St	Lc Ctrl	Pz St	Pz Ctrl
PLA (cm ²)	2012	4123	6001	4437	5336
	2013	664	1242	724	1533
DW (g)	2012	34.7	59.3	34.1	43.9
	2013	15.2	19.2	16.1	24.8

Table 1 Plant leaf area (PLA) and total dry weight (DW) measured at the end of trials in the two treatments of the two genotypes. Each value is the mean of three replicates.

2.4.5 Biochemical and molecular analyzes

2.4.5.1 Foliar ABA and proline content

At the end of the first period of interrupted irrigation in 2012, ABA spiked 6160 pmol g⁻¹ in Pz St and 14440 pmol g⁻¹ in Lc St (Fig. 3 a). After that, in response to the restored irrigation in both genotypes, ABA decreased to the basal level of 800 pmol g⁻¹ observed for the Ctrl treatments throughout the experiment, and then at the end of the second period of no irrigation ABA re-increased to about previous peak values. In 2013, the peaks in the first stress period and the recovering during the re-watering were pretty comparable with the previous year (Fig. 3 b). As regard the second stress imposition, ABA response was almost absent in St of Pz and halved in St of Lc, as compared with the previous peaks.

As regard proline, in 2012 no response was observed after 10 days of stress in the first period of no irrigation, whereas peaks of about 5 μmol g⁻¹ (FW) in St of Lc and 9 μmol g⁻¹ (FW) in St of Pz were observed after 8 days in the second period of interrupted irrigation (Fig. 3 c). In 2013, proline leaf content in the stress treatment of both genotypes spiked to about 3 μmol g⁻¹ (FW) after 11 days of no irrigation, whereas in the second stress period there was almost no response in St of Pz after 7 days of stress while they both peaked around 5 μmol g⁻¹ (FW) 3 days later (Fig. 3 d).

Likewise ABA, proline content in the stress treatments decreased to basal level of the control treatments in response to the restored irrigation.

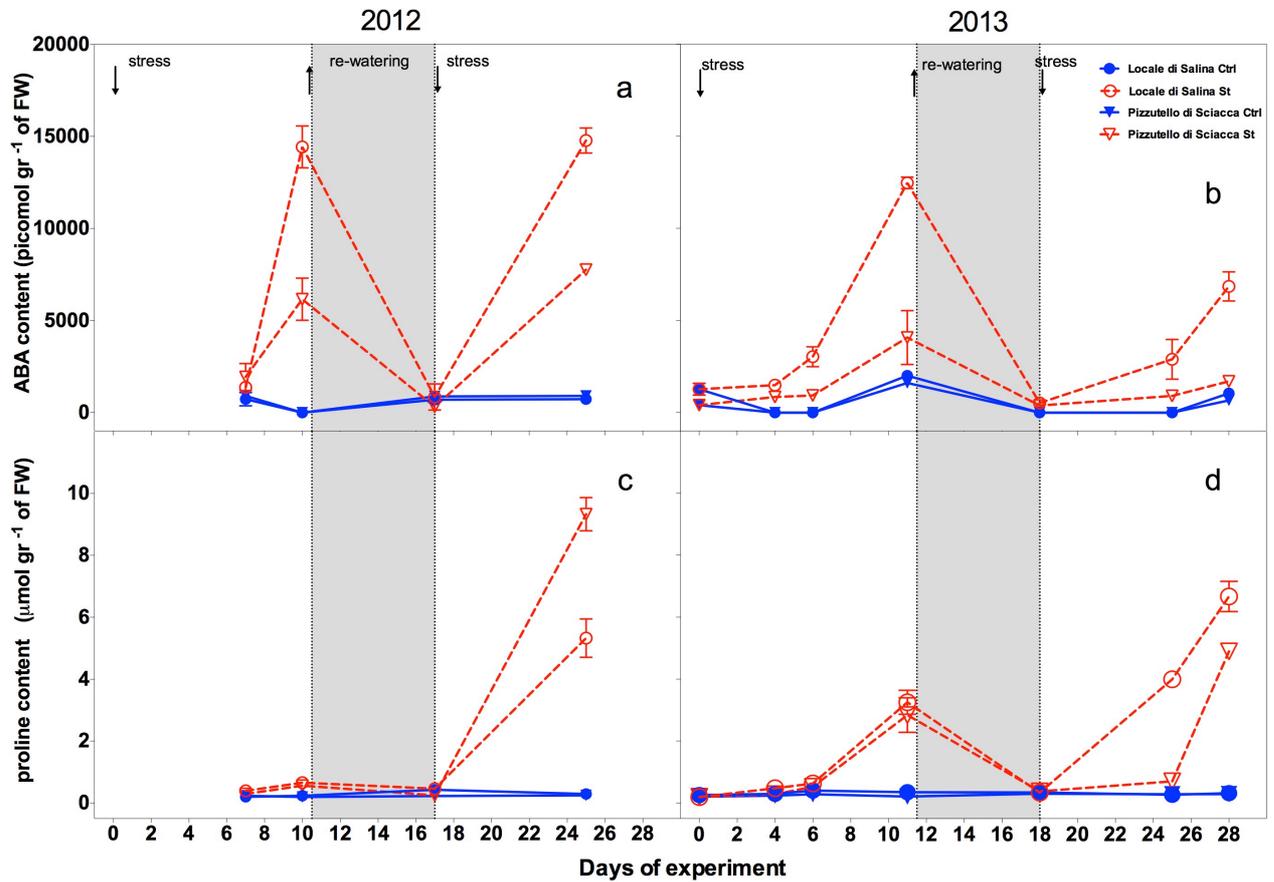


Fig. 3 Abscisic acid a) and b) and proline c) and d) concentration, in control and treated leaves, at different stages of the two stress cycle and re-watering. Values are the means of three biological replicates. Vertical bars indicate the standard error of the mean. Downward and upward arrows indicate the start of withholding and restoration of irrigation, respectively. Grey area indicates the whole re-watering period.

2.4.5.2 Poly(ADP-ribose) polymerase (PARP) activity

PARP activity was measured in few occasions during the two experiments. In 2012, there was neither genotype nor stress effects on enzyme activity (Fig. 4 a). During the second year (2013), on sixth day after stress, the enzyme activity resulted about 8- and 2-fold higher than the control in Lc and in Pz leaves, respectively (Fig. 4 b). A more prolonged stress (DOE 11) induced a recovery of PARP activity to control levels. As seen for 2012, these values remained almost unchanged when leaves were subjected to the second cycle of water stress.

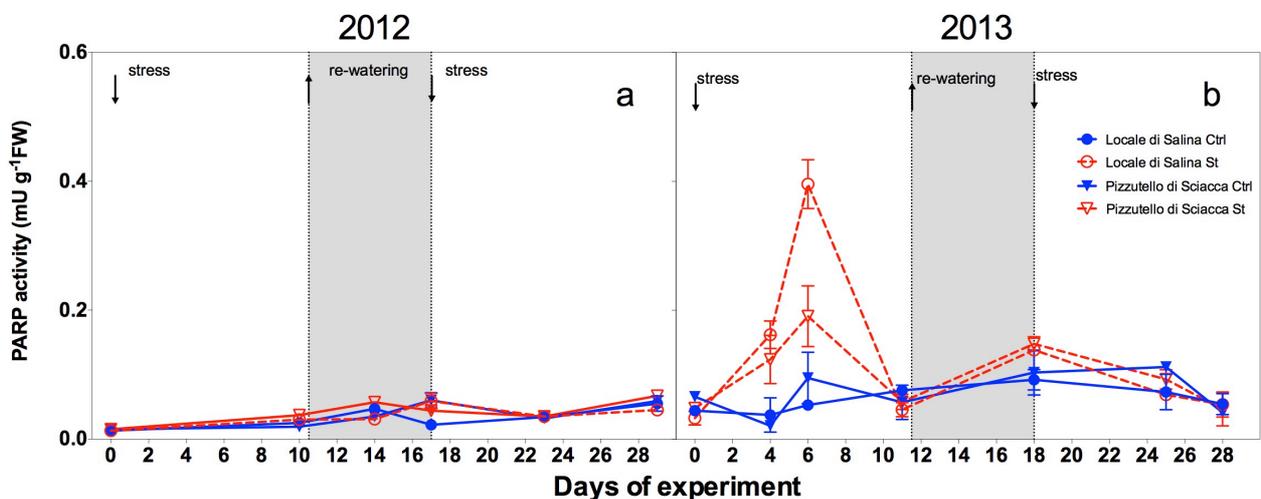


Fig. 4 Trend of poly(ADP-ribose) polymerase activity measured in control and stressed leaves of the two tomato genotypes in 2012, a) and 2013, b). Vertical bars indicate the standard error of the mean. Downward and upward arrows indicate the start of withholding and restoration of irrigation, respectively. Grey area indicates the whole re-watering period.

2.5 Discussion

2.5.1 Physiological responses

In this study during the two years of experiment we monitored the soil volumetric water content (θ) of potted plants of Lc and Pz, and found that there was no significant difference in θ between the two cultivars under control, drought, or re-watering conditions (Fig. 1 a, b). Therefore, this would indicate that in each year, water consumption did not differ between the two genotypes. However, in the second year experiment, the interruption of irrigation resulted in a more rapid decrease of θ if

compared to 2012. In fact, from Fig. 1 a) and b) it results that the slope of θ vs. DOE relationship in the first week of soil water deficit imposition was 3.43 and 5.22 $\text{m}^3 \text{m}^{-3} \text{day}^{-1}$ in 2012 and in 2013, respectively. Moreover, the minimum θ value measured on DOE 10 in 2012 was reached in 2013 already 5 days earlier. Therefore, the second year experiment was characterized by a stronger soil water deficit than 2012. It should also be highlighted that the exponential relationship between soil moisture and soil matric potential (Whalley, 2013) does imply that the water stress sensed by roots was even much stronger for plants in 2013 than 2012, as it can be deduced from data in Fig. 1 a) and b). As regard the re-watering, there were no significant differences in the patterns of soil moisture between the two years, whereas for the subsequent second water deficit imposition, a more rapid decreased of θ was observed for 2012 as compared to 2013. Those dynamics of soil moisture (the stressor) led to the occurrence of more stressful conditions sensed by plants in 2013 as regard the first period of no irrigation and vice versa in 2012. Such different stress conditions were reflected in the physiological responses of plants. A strong stomatal closure was induced by soil water deficit in both years with stomatal conductance following the decrease in soil water content availability (Fig. 1 c, d). As for soil water content, in 2013, g_s was quite low after 6 days of no irrigation, whereas in 2012 it required four more days to occur, confirming that plants were subjected to a higher degree of water stress in the first period of withdrawn irrigation as compared to the next year. Conversely, in the second period of irrigation withholding, g_s decreased faster in the first year than in 2013, reflecting a similar behavior as for θ . Similar relationships between θ and g_s were also evident during restoration of irrigation. Our data of stomatal behavior, reflecting patterns of soil moisture, are consistent with the well known mechanisms of roots sensing soil moisture, resulting in ABA produced in the root tissue, moving into the xylem and acting as a root to shoot signal to trig stomatal closure (Liu and Carns, 1961; Ohkuma et al., 1963; Cornforth et al., 1965; Patterson, 2001). Net photosynthetic rate (A) of control, and stress treatments as

shown in Fig. 1 e) and f) are comparable to the values reported for tomato grown under similar growth conditions (Liu et al., 2012; Rahman et al., 1999).

In the present study net photosynthetic CO₂ assimilation rate was strictly related to stomatal behavior. In fact similarly to g_s , this parameter showed a stronger decrease during the first stress period of 2013, as compared with 2012. At that time the stress was so intense that a negative value for net assimilation rate was observed. As a result CO₂ evolution was higher than gross CO₂ assimilation, resulting in substomatal CO₂ concentration (C_i) higher than ambient concentration (C_a) (Fig. 1, h) confirming that our un-irrigated plants suffered very stressful conditions. Interestingly, re-watering induced a recovering of gas exchange parameters above the values observed in the control plants (Fig. 1 c, d, e, f). Such a behavior was also reported by de Carvalho (2010) on maize. The decrease in stomatal conductance in response to soil water deficit evidently caused a restriction of CO₂ diffusion to the mesophyll, therefore limiting photosynthetic assimilation. However, a contribution of non-stomatal limitation may have occurred. Souza et al. (2003) in potted Cowpea submitted to water deficit, found a rapid complete recovery of assimilation, within 3 days of re-watering. This led the authors to argue that photosynthetic assimilation was not affected by either biochemistry or photochemistry impairments but only by stomatal closure. Similar plant responses to re-watering and same conclusions were reported by Rahman et al. (2007) in potted tomato cultivars submitted to soil water deficit in a greenhouse experiment. Our stressed plants also showed a rapid recovering in photosynthetic assimilation during re-watering. In fact, A of St plants rose to Ctrl values within just two days of re-watering both in 2012 and 2013 experiments. Such a rapid recovery would imply also in our case that most of photosynthetic limitations should be ascribed to stomata rather than to non-stomatal effects (Souza et al., 2003; Cornic, 2000). However, non-stomatal component of photosynthetic limitations should not be completely excluded. Souza et al. (2003) assumed that transient non-stomatal limitations occurred because of the complete recovering of C_i during the first day of re-watering, not associated to a similar

recovery in assimilation, which occurred few days later. In fact, a non-damaged photosynthetic machinery would lead to a clear reduction in C_i in response to stomatal closure caused by soil water deficit. On the contrary, in both two years of experiment (except for the first stress period of 2013 already mentioned above with C_i in St plants higher than Ctrl plants) we found no difference in C_i between the two treatments, in spite of much lower values of both A and g_s in Stress than Control plants (Fig. 1 g, h); this behavior suggests the occurrence of non stomatal photosynthetic limitations, implying that St plants had lower capacity to fix available CO_2 than Ctrl plants. However, similarly to Souza et al. (2003), such limitations were rapidly removed upon re-watering as confirmed by data of gas exchange parameters in the subsequent days.

As regard photochemistry, actual photosynthetic efficiency (Φ_{PSII}) in the first stress period of 2013 was clearly depressed by water deficit (Fig. 2 d). The impairments of PSII functioning (under-light-conditions), resulting in a proportionally reduction in electronic transport rate (ETR, data not shown) could not be recovered by dark adaptation, indicating a permanent damage to photosystems. In fact, F_v/F_m in St was quite low and lower than Ctrl plants at the end of first stress period. The maximal (dark adapted) photosynthetic efficiency (F_v/F_m) is about 0.83 in non-stressed plants (Bjorkman and Demmig, 1987), whereas a sustained decrease indicates the occurrence of photo-inhibitory damage, in response to many environmental stresses (Maxwell and Johnson, 2000). However F_v/F_m is a parameter quite resistant to abiotic stress, while impairments can be observed under very stressful conditions, e.g. as found for water stress in maize (de Carvalho et al., 2011) and in *Setaria sphacelata* (Da Silva and Arrabaca, 2004). We found that during the very strong water stress conditions in 2013, F_v/F_m clearly decreased in St as compared to Ctrl plants, whereas no stress effect was found in the previous year (cf. Fig. 2 a, and Fig. 2 b). Therefore, the intensity of stress in 2012 was sufficient to impair photosynthetic assimilation (due to both stomatal and non stomatal limitations), whereas, under the less stressful conditions of 2012 photochemistry was not affected.

2.5.2 Biochemical responses

The aminoacid Proline is considered a good indicator for water stress tolerance (Ahmed et al., 2009; Liu et al., 2011). Accumulation of proline in leaves under stress conditions permits osmotic adjustment, which results in water retention and avoidance of cell dehydration (Blum et al., 2005) without damaging enzymatic activity because of its osmoprotectant properties (Kishor, 2014). Moreover proline also acts like a scavenger of radicals (Szabados & Savoure, 2010) and it's known, that ABA is able to up-regulate their production (Abraham et al, 2003). In our study a peak in proline leaf content in St treatments was actually found under stressful conditions experienced by plants at the end of no irrigation periods (Fig. 3 c, d). However, this did not occur during first stress period in 2012 (Fig. 3 c) even if in a subsample of more stressed plants (data not shown) it was measured a clear increase in concentration of this aminoacid. Claussen (2004) studied the dynamics of proline as a measure of water stress in tomato plants submitted, in a long-term experiment, to a wide range of concentration of the nutrient solution, and therefore osmotic stress. The author found that leaf proline accumulation peaked after about a week of stress imposition, and that it ranged from about 0.5 to 5.5 $\mu\text{mol g}^{-1}$ (F.W.) in the most stressed tomato. Both time of occurrence and peak intensity found in that study are comparable with our results, especially for the second year experiment (Fig. 3 d). However, the author concluded that less than 3 $\mu\text{mol g}^{-1}$ (F.W.) is considered a threshold value above which tomato experienced a degree of water stress capable to impair productivity and fruit quality. Our tomato genotypes experienced peaks of proline accumulation behind such a threshold, indicating a high capacity of responding to intense water stress by biochemical mechanisms. Interestingly, Claussen (2004) reported that a sudden drop in proline content occurred even for the most stressed plants in response to a drop in the variable solar radiation. Therefore, it

could be possible that the low proline content in St plants during first stress period of 2012 could be caused by the effect of solar radiation or other unknown variable. However, as said before, in a subsample of plants we maintained the stress conditions for 4 days more, and found that in both genotypes there was an increase in proline content.

Abscisic acid (ABA) is a hormone discovered during the early 1960s. It was shown to have a role in many physiological processes such as seed dormancy, organ abscission and fruits maturation, but its most important function is the regulation of stomata opening. (Liu and Carns, 1961; Ohkuma et al., 1963; Cornforth et al., 1965 Patterson, 2001). In split-root experiments, the partial-root drying avoided the impairment of leaf water potential. In spite of this, growth and stomatal conductance (Gowing et al., 1990, 1993; Davies and Zhang, 1991) were impaired because of a (chemical) signal moved from the dried roots to the leaves. In fact, ABA content in roots is well correlated with both soil moisture and the relative water content of roots in many plant species (Dry, 1999). Moreover, ABA is synthesized in both roots and leaves (Thompson et al., 2007) in response to water stress. We actually found a quite significant increase in ABA content of leaves when plants were approaching the most stressful conditions imposed at the end of stress period (Fig. 3 a, b), when both soil water content and stomatal conductance were at their minimum values. Interestingly, in all four stress periods of the two-year experiment, *Locale di Salina* showed ABA increases higher than *Pizzutello di Sciacca* in spite of no appreciable differences were found between the two genotypes in soil moisture, stomatal conductance, net assimilation, actual and maximal quantum efficiency of PSII (Fig. 1 and Fig. 2).

Numerous studies suggest that in plants alike in animals, the poly(ADP-ribosyl)ation is linked to important physiological processes such as DNA repair and cell cycle (Doucet, 2001; Pellny 2009.), and it also plays an important role in abiotic stress responses (De Block 2005). In our experiment, during stressful conditions occurred in the first stress cycle (2013) there was a strong increase of PARP activity after 4-6 days from the interruption of irrigation (Fig. 4 b) with a spike of activity of about 0.4

mUg⁻¹ F.W. in LC. However, in response to more powerful stress conditions, PARP reduced its activity. This behavior is in agreement with several studies in which it was demonstrated that the reduction of PARP activity preserves cells from NAD⁺ and ATP depletion, and it enhances the energy use efficiency during stress (De Block, 2005, Arena et al., 2011). Another mechanism that could explain the reduction of PARP activity in our experiment, is its implication with ABA signalling pathway, (Vanderauwera, 2007) in fact NAD⁺ pool was used for cADPR synthesis which promotes the increase of Ca⁺⁺ responsible for ABA production (Xiong, 2003). Such a mechanism can explain PARP activity in the first stress cycle in 2012 and in the second stress cycle because changes in PARP activity, namely a decrease of PARP values, are accompanied by an increase of ABA content.

2.6 Conclusions

In conclusion, both genotypes showed a clear response to the imposed soil water deficit by regulation of stomatal closure and photosynthetic activity, and recovering upon re-watering. Physiological responses were strictly linked to biochemical mechanisms of response to water stress as both ABA and proline increased concomitantly with severe stress conditions. The activity of PARP was influenced by both stress imposition and ABA response. Although future confirmation is needed at the field level, the data revealed that the two landraces showed a wide physiological and biochemical plasticity to deal with water stress. Then combined investigation into the physiological and biochemical properties represents an essential approach for further studies, where many genotypes could be compared in terms of their tolerance to water stress.

Chapter 3

“Physiological responses to different levels of salinity of two sweet pepper genotypes grown in a soilless system”

(Capsicum annuum L.)

3.1 Abstract

Salinity is one of the most important environmental stress factors limiting plant productivity. Pepper (*Capsicum annuum L.*) is a widespread horticultural crop grown in the Mediterranean area where saline water is frequently used for irrigation. It is classified as moderately sensitive to salt stress, but limited information is available on salt tolerance mechanisms, due to the genetic and physiological complexity of the involved traits; moreover most of the available data are limited to a short period of exposure salinity.

In this experiment, two sweet pepper genotypes (*Quadrato D'Asti*, the most famous Italian pepper cultivar and *Cazzone Giallo*, a typical genotype of Campania Region) were grown in a closed soilless system and exposed from moderate to high salt levels (0-30-90-120 mM NaCl in nutrient solution) during the whole crop cycle. Data were collected at different stages of plant growth (vegetative and reproductive) on different organs including leaves and fruits. Plant phenotypic (biomass and yield) and physiological characterization (gas exchanges, total water potential and osmotic potential) were monitored. During the whole crop cycle ('shoot development', 'flowering' and 'maturity of fruit' growth stages) all the biometric and agronomic traits (leaf area, marketable yield, fruits size and weight) decreased with saline levels (significantly at 90-120 mM NaCl). Photosynthetic assimilation and photochemistry at high levels of salinity were dramatically impaired throughout two distinct phases. Stomatal closure caused photosynthetic limitations during first 30-40 days of salt stress imposition. Conversely, afterwards non-stomatal limitations had a relevant role with the high probable significant contribution of specific ion toxic effects. *Cazzone Giallo* resulted more salt sensitive for almost all performed analyses than *Quadrato d'Asti*.

3.2 Introduction

Bell Pepper or sweet Pepper, *Capsicum annuum*, L. like other species of *Solanaceae* family is one of the most important vegetable crops in the world. It is cultivated worldwide in temperate and tropical areas although most of the cultivars are thought to have originated from South America (Wien, 1997). It is only recently that peppers have been grown in greenhouses. As regard salt stress, in terms of yield, pepper is considered a moderate sensitive species with a threshold of 1.5 dS m^{-1} for the extractable saturated soil solution (Maas and Hoffman, 1977, Pasternak and Malach, 1994). However, several pepper genotypes with higher tolerance have been reported in more recent studies (Chartzoulakis and Klapaki, 2000; Aktas et al., 2006; Niu et al., 2010).

Munns (2002) highlighted that there are some common mechanisms of plant response between water and salt stress. Na^+ and Cl^- (as well as other ions) in the root zone decrease the free energy of water, and therefore through osmotic effect, its water availability for plant uptake. This mechanism is common to what occurs to plants under water deficit (Fricke et al., 2004; Munns and Tester, 2008). Moreover, salts enter root tissue and then further move through the transpiration stream to stems and leaves. As a result, finally sodium and chloride accumulate either on cell wall or vacuole where specific salt effects occur, not in common with water stress (Cramer and Nowak, 1992). Therefore, plants facing salinity in the root zone must face a Scylla and Charybdis dilemma, whereby uptake of water for transpiration, in order to overcome osmotic stress, will also imply that salts enter plant tissues, where they can cause toxic effects (Greenway and Munn, 1980). Moreover, these two aspects of salt stress are characterized by different plant response over time, as the osmotic phase is rapid while the ionic phase is slower (Munns and Tester, 2008).

Most of the available data on effects of salinity on pepper (and other vegetable crops) are limited to growth in hydroponic systems or commercial substrates that have been exposed to salinity for a short period of time. In a long-term experiment, potted plants

of two sweet pepper genotypes *Cazzone Giallo* (CG) and *Quadrato D'Asti* (QA) were tested at moderate (0, 30 mM NaCl) and high (90, 120 mM NaCl) salinity. The *Cazzone Giallo* (CG) in the 50th was one of the most common genotype in Campania Region, in the 60th, however, it was replaced by modern hybrids. *Quadrato d'Asti* (QA) is the most famous and widespread Italian sweet pepper (Barbagallo et al., 2012).

3.3 Materials and methods

3.3.1 Plant material, growth conditions and salt treatments

The experiment was carried out in a greenhouse of CRA-ORT (Pontecagnano, SA, Italy) equipped with an automatic, computer-controlled soilless system with drip irrigation system. The nutrient solution was pumped from independent tanks and delivered by means of two emitters per pot. The emitter flow rate was 2 litres h⁻¹. After application, solution returned to its tank for later recirculation (closed system). The two sweet pepper genotypes were 'Quadrato D'Asti' a widely diffused cultivar and "Cazzone Giallo" which is typical of Campania region. Transplantation occurred on 18th September 2013, one plant per pot (27 cm diameter, 10 L of capacity) adopting coconut coir dust as substrate. The averages height and weight of seedlings were 15 cm and 4 g, respectively.

The basic nutrient solutions used for irrigation (pH 5.6) had the following composition (meq/l): Na⁺, 0.25; NH₄⁺, 0.537; K⁺, 5.002; Mg⁺⁺, 3.998; Cl⁻, 0.505; NO₃⁻, 14.643; H₂PO₄⁻, 1.252; S-SO₄⁻, 3.543; HCO₃⁻, 0.502. The experimental design consisted of four saline (NaCl) levels: the control (0 mM), that was the basic nutrient solution, 30 mM, 90 mM and 120 mM NaCl (EC 2.6-5.7-12.0-15.6 dS/m, respectively; pH 5.6) obtained by adding proper amount of sodium chloride to the basic solution. Sixty plants were distributed in the saline treatments (15 plants per treatment). Salt treatment started after 13 days from transplanting ('vegetative' stage) and continued until the end of the experiment ('yellow fruit' stage). The pH and the electrical conductivity of the nutrient solution were daily controlled. The EC of the

nutrient solution for each treatment was kept constant through the culture cycle; the pH was kept within the range of 5.5 to 6.0 by adding HNO₃. During the experiment, the greenhouse temperature ranged from 18 to 30°C, and relative humidity from 60 to 85%. Plants were harvested on 6th mar 2013, DOE 158.

3.3.2 Physiological measurements

Leaf physiological parameters were assessed from 10:00 to 13:00 in well-expanded sunlit leaves in the middle of the plant, every seven days during the entire growing cycle ('vegetative', 'reproductive', 'fruiting' stages).

3.3.2.1 Plant water status

Total leaf water potential (Ψ_l , MPa) was assessed on three leaves of three plants per treatment using a Scholander type pressure bomb (SAPS II, 3115, Soilmoisture Equipment Corp., Santa Barbara CA, U.S.A). After cutting, the leaf was inserted into the pressure bomb within a maximum of 30 s, and the pressure was increased at a rate of 0.2 MPa min⁻¹.

Osmotic potential (Ψ_π , MPa) was estimated on the basis of the osmolality of expressed leaf-sap. The entire leaf was frozen in liquid nitrogen to break cell walls and squeezed at fixed pressure to extract the cellular sap. The osmo-moles of 100 μ l of this extracted sap was then measured by using a freezing-point micro-osmometer (13/13 DR-Autocal-Hermann Roebling Messtechnik, Berlin, Germany), and then converted to osmotic potential through the Morse equation (Morse, 1914): $\Psi_\pi = - (RT n_s/V_w)$, where: R = universal gas constant (m³ MPa mol⁻¹ °K⁻¹); T_s = solution temperature (°K); n_s= solute osmo-moles (mol); V_w= solvent volume (m³). Since no distinction between symplast and apoplast was possible, there is no way to identify the exact compartmentalization of the salt in the cells.

3.3.2.2 Gas exchanges

Net photosynthetic CO₂ assimilation rate (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) at saturating light, stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) and leaf temperature were determined using a portable open-system gas-exchange and modulated fluorometer analyzer Li-6400XT (Li-Cor Biosciences, Lincoln, NE, USA.), with CO₂ inside leaf chamber set to 400 $\mu\text{mol mol}^{-1}$ air. A LED light source with emission peaks centered at 630 nm in the red and at 460 nm in the blue provided a PPFD equal to 1500 $\mu\text{mol (photons) m}^{-2} \text{s}^{-1}$ (90% red, 10% blue). The instrument was also used to measure the fluorescence parameters. The measuring beam was set at intensity 5 (according to the instrument manual) with a modulation of 20 kHz. After the measurement of *Chl a* fluorescence emission at steady-state under light conditions, F' , the maximum fluorescence emission, F_m' , was assessed upon induction by a 0.8 s super-saturating light pulse at 6000 $\mu\text{mol (photons) m}^{-2} \text{s}^{-1}$, with a modulation of 20 kHz; then actinic light was briefly switched off while a far-red light of 8 $\mu\text{mol (photons) m}^{-2} \text{s}^{-1}$ for 6 s was used to discharge the PSI photosystem to allow measurement of the minimum fluorescence emission under light conditions, F_0' . The software of the instrument (Li-Cor, 2011) calculated the gas-exchange parameters on the basis of von Caemmerer and Farquhar (1981) model, and the actual quantum efficiency (ϕ_{PSII}) or $\Delta F/F_m' = (F_m' - F')/F_m'$ (Genty *et al.*, 1989) and the maximal quantum yield under light conditions F_v'/F_m' (Baker, 2008). Measurements were taken on 6-10 replicate plants per treatment.

3.3.2.3 Transient Chl a fluorescence emission

A continuous excitation Handy PEA fluorometer (Hansatech, Instruments Ltd., King's Lynn, Norfolk, England) was used. Leaves were dark adapted for 30 min by means of the equipped white leaf-clips, prior to the assessment of the minimum fluorescence emission F_0 [relative units, r.u.]. Then an excitation red light pulse for fluorescence induction (FI) was emitted by a (red) 650 nm light diode source, and applied for 1 s at the maximal available photosynthetic photon flux density (PPFD) of

3500 $\mu\text{mol (photon) m}^{-2} \text{s}^{-1}$ to measure the induced fluorescence emission. The instrument software calculated F_0 , the peak emission F_p [r.u.] as a viable approximation of F_m [r.u.] (Giorio, 2011), and therefore $F_v/F_m = (F_m - F_0)/F_m$. Data were taken on the adaxial lamina of 30-60 leaves of 10 plants per treatment in five occasions between DOE 71 and 156.

3.3.3 Agronomic measurements

Total leaf area of three plant replicates for saline treatment was measured at the end of experiment (yellow fruit stage) with an electronic area meter (Li-3100, LICOR Lincoln Nebraska USA). The plant samples (leaves, stems, mature green and yellow fruits) were then oven dried at 65 °C until constant weight was reached for biomass determination and subsequently ground for mineral composition (Na^+ and Cl^-) determination by ion chromatography (DionexIonPac CS12A and AS22 RFIC 4x250mm Analytical Columns for cations and anions determination, respectively).

Before final harvest, the number of fruits, mean fruit weight and total marketable yields were determined on three plants per treatments. Marketable fruit yield was determined according to the color and the normal shape; fruits with blossom-end rot or fruits lighter than 100 g were not taken into account for marketable yield.

3.3.4 Statistical analyses

Data were subjected to analysis of variance (Two-way ANOVA) with the null hypothesis rejected at $P < 0.05$ significance level, mean separations were done using Duncan's multiple range test using GraphPad Prism version 5.0 for Mac, (GraphPad Software, La Jolla California USA)

3.4 Results

3.4.1 Physiological parameters 3.4.1.1 Leaf water potential

Leaf water potential of control plants of the two genotypes showed a slight increase up to about -0.34 MPa during the first 17 days, whereas afterwards it slowly decreased, at a rate as low as 0.0031 MPa per day, reaching an average value of -0.62 MPa, 85 days later (DOE 102: end of Ψ_1 monitoring) (Fig. 1 A, B). The Ψ_1 in the salt treated plants was moderately affected in T30, which showed an average decrease of about 1 MPa. Conversely, a strong decrease was observed in both T90 and T120, which reached, as average of the two genotypes, a minimum of -1.03 in T90 and -1.25 in T120 at DOE 102 (Fig. 1 A, B).

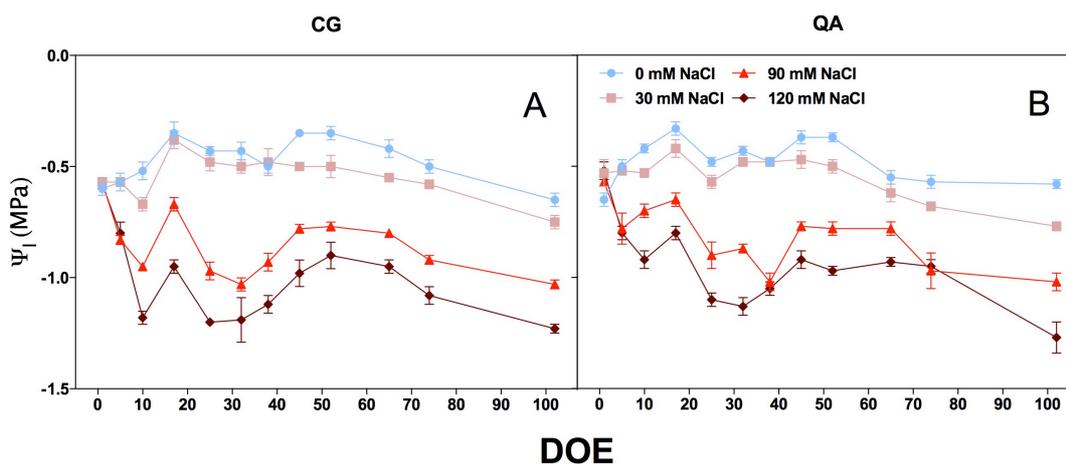


Fig. 1. Leaf water potential in 0, 30, 90 and 120 mM NaCl treatments of the two genotypes *Cazzone Giallo* (A) and *Quadrato D'Asti* (B). Each point is the mean of 5 measurements; vertical bars indicate the standard error of the mean.

3.4.1.2 Gas exchanges and fluorescence

In both genotypes, treatment T30 was not clearly affected by salinity resulting quite similar to the Control. In both these two treatments, as an average for the two genotypes, g_s showed a progressive decrease during the experiment from 0.63 mol m² s⁻¹ at the beginning of the experiment to the minimum 0.22 mol m² s⁻¹ observed at DOE 85 (Fig. 2 C, D). Conversely, during the first ten days of the experiment a rapid linear decrease was showed by the most saline treatment, T120, in both genotypes,

passing from the maximum value comparable to the control, to 0.25 in GC and 0.152 mol m⁻² s⁻¹ in QA. Afterwards, a smoother decrease led to minimum g_s of 0.04 in CG and 0.07 mol m⁻² s⁻¹ in QA, at the end of measurements. T90 plants showed stomatal conductance values ranging between Control and T120. (Fig. 2 C, D).

As regard photosynthetic CO₂ assimilation (A) in the genotype QA, all treatments, except of T120, which maintained a quite stable value around 15 μmol m⁻² s⁻¹, showed a clear increase of A during the first ten days from 15.0 to 21.7 μmol m⁻² s⁻¹ as an average among the three treatments (Fig. 2, B). Afterwards, as for stomatal conductance, a gradual decrease during the experiment was observed even for the Control plants, which showed a minimum of 13.5 μmol m⁻² s⁻¹ on DOE 102. Until DOE 52 no clear difference in A was evident among T0, T30 and T90. Conversely, since DOE 73, T90 showed assimilation rate lower than the other two treatments, which continued to maintain similar values. Salinity reduced assimilation rate in plants of T120 quite earlier than T90 (since DOE 25), reaching 5.5 μmol m⁻² s⁻¹ at the end of experiment when A was 8.4 μmol m⁻² s⁻¹ in T90 and about 11.4 μmol m⁻² s⁻¹ in both T0 and T30. The patterns of assimilation for the two genotypes were quite similar, with the only exception of T120 which was more affected in CG (Fig. 2 B) and started to decrease in this genotype earlier (since DOE 10) than in QA (Fig. 2 A). Conversely to QA, which showed no effect on T30 plants, this treatment in CG showed assimilation lower than the Control since DOE 85. At the end of gas exchange measurements (DOE 102), the three saline treatments of CG genotype showed A values lower than the control 15.4 μmol m⁻² s⁻¹ by 3.1 in T30, 7.1 in T90 and 12.7 in T120. During most of the trial, in both genotypes the intrinsic water use efficiency (A/g_s) of the Control was quite stable (around 34 μmol mol⁻¹) and did not statistically differ from T30 (Fig. 2 G, H). T120 showed a strong increase in A/g_s, reaching a maximum of 117 μmol mol⁻¹ after 32 days in CG, and 108 μmol mol⁻¹ after 10 days in QA. Afterwards, there was a significant decrease within 20 days, after which the values remained almost stable in both genotypes. Intermediate values were observed in T90 plants. In both genotypes, during the whole trial, the

substomatal CO₂ concentration (C_i) was quite stable in both Control and T30 (about 340 μmol mol⁻¹), without significant differences between them (Fig. 2 E, F). Within ten days of salt stress imposition, C_i in T120 plants showed a sudden drop to 264 in CG and to 234 μmol mol⁻¹ in QA, while no further comparable variations were observed afterwards.

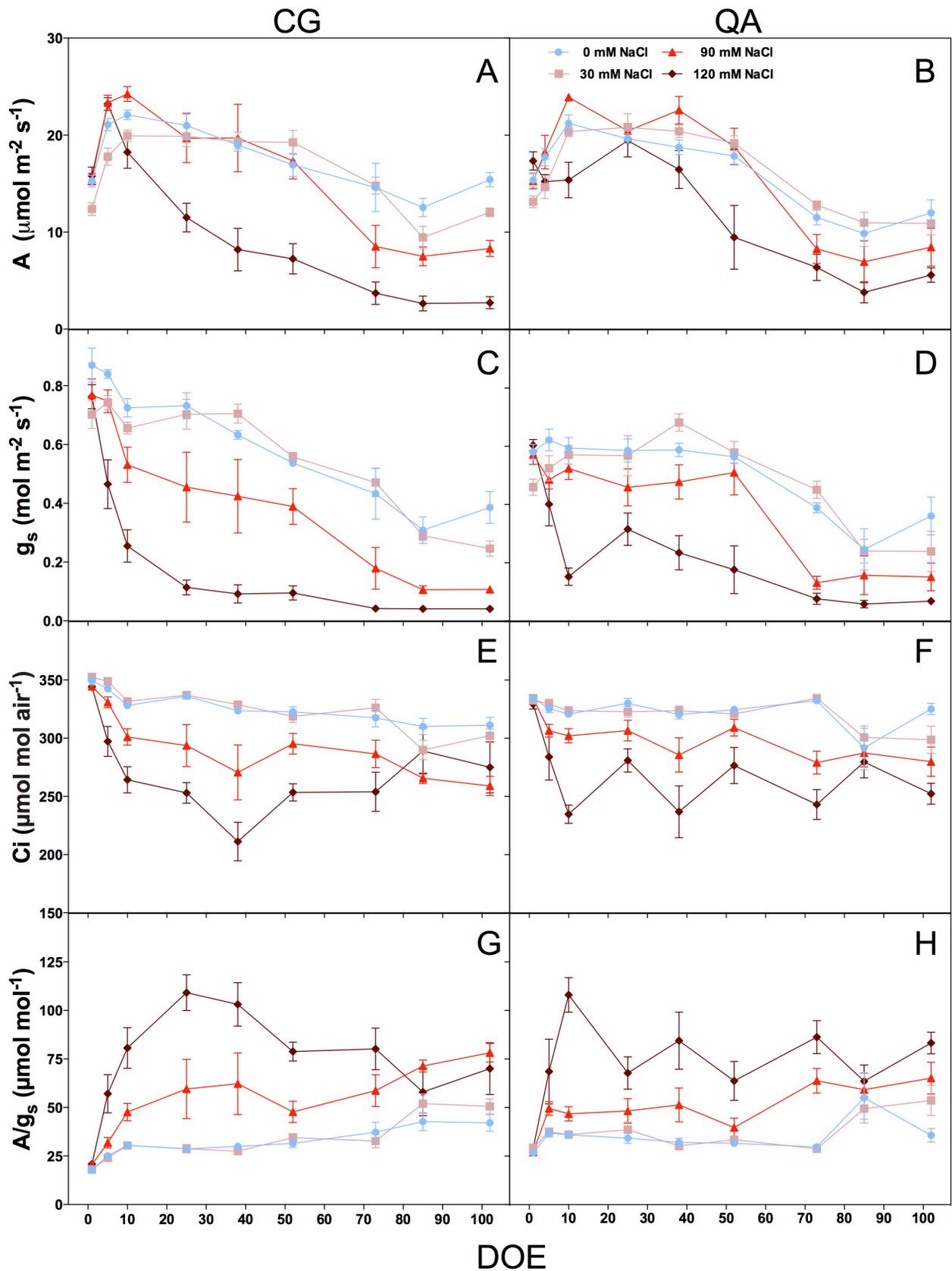


Fig. 2. Photosynthetic CO₂ assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$) A) and B), stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) C) and D), intercellular CO₂ concentration (C_i , $\mu\text{mol mol air}^{-1}$) E) and F), water use efficiency (A/g_s , $\mu\text{mol mol}^{-1}$) G) and H) in 0, 30, 90 and 120 mM NaCl treatments of the two genotypes *Cazzone Giallo* (CG) and *Quadrato D'Asti* (QA). Each point is the mean of 10 measurements; vertical bars indicate the standard error of the mean.

3.4.1.3 Fluorescence parameters

The maximal (dark adapted) quantum efficiency of photosystem PSII (F_v/F_m) in T0, T30 and T90 of QA genotype remained quite constant during all cycle near to the maximum value typical for healthy plants under no abiotic stress (Bjorkman and Demmig, 1995) (Fig 3 B). Conversely, the most saline treatment T120 showed F_v/F_m significantly lower than other treatments since DOE 122, decreasing to a value as low as 0.536 on DOE 156. Similar patterns were observed in CG genotype as compared with QA; however, T120 showed a stronger decrease since DOE 108, dropping to the quite low value of 0.427 on DOE 156 (Fig 3 A).

The PSII maximal efficiency under light conditions (F_v'/F_m') (the operating efficiency with all reaction centers open), as expected, was lower than F_v/F_m and higher than effective efficiency of PSII under light conditions (Φ_{PSII}) in all treatments and genotypes (cf. Fig 4 A, B, C, D). Especially for CG as compared with QA, the most salt treated plants had lower F_v'/F_m' than other treatments along the cultural cycle. On DOE 85 this parameter for T120 was 0.277 in CG and 0.331 in QA, whereas the average of other three treatments was 0.397 and 0.453 in the two genotypes, respectively (Fig 4 C, D). Φ_{PSII} was lower than 0.2 in all saline treatments and genotypes during all cultural cycle, with an average minimum of 0.057 in CG and 0.063 in QA (Fig 4 C, D).

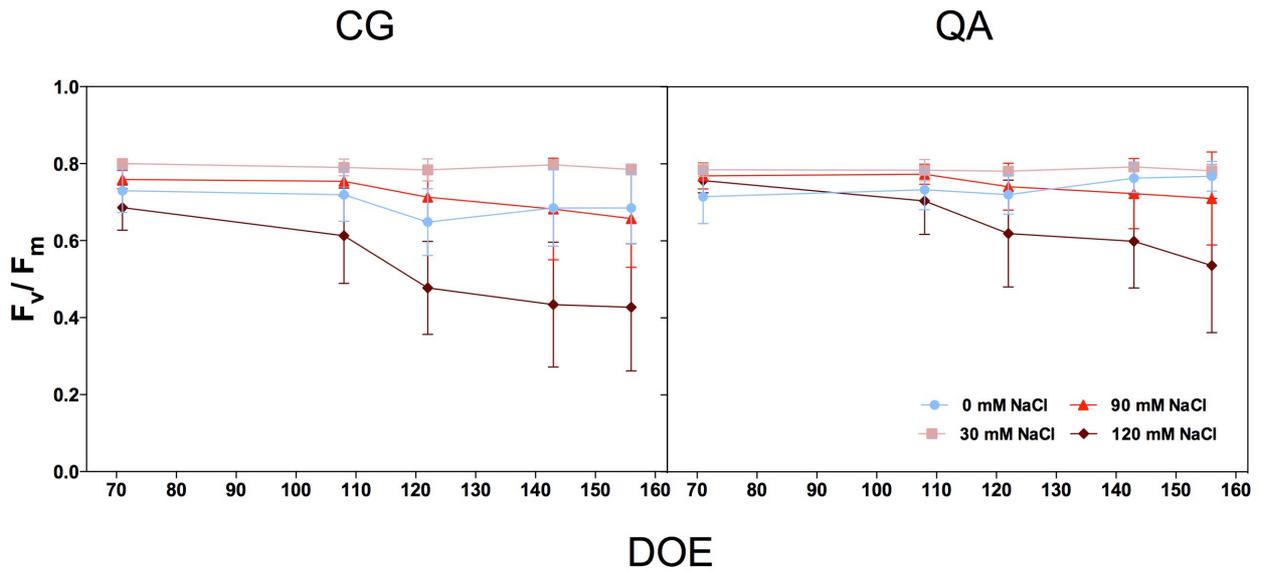


Fig. 3. Maximal quantum efficiency of PSII (F_v/F_m) in 0, 30, 90 and 120 mM NaCl treatments of the two genotypes *Cazzone Giallo* (CG) and *Quadrato D'Asti* (QA). Each point is the mean of 10 measurements; vertical bars indicate the standard error of the mean.

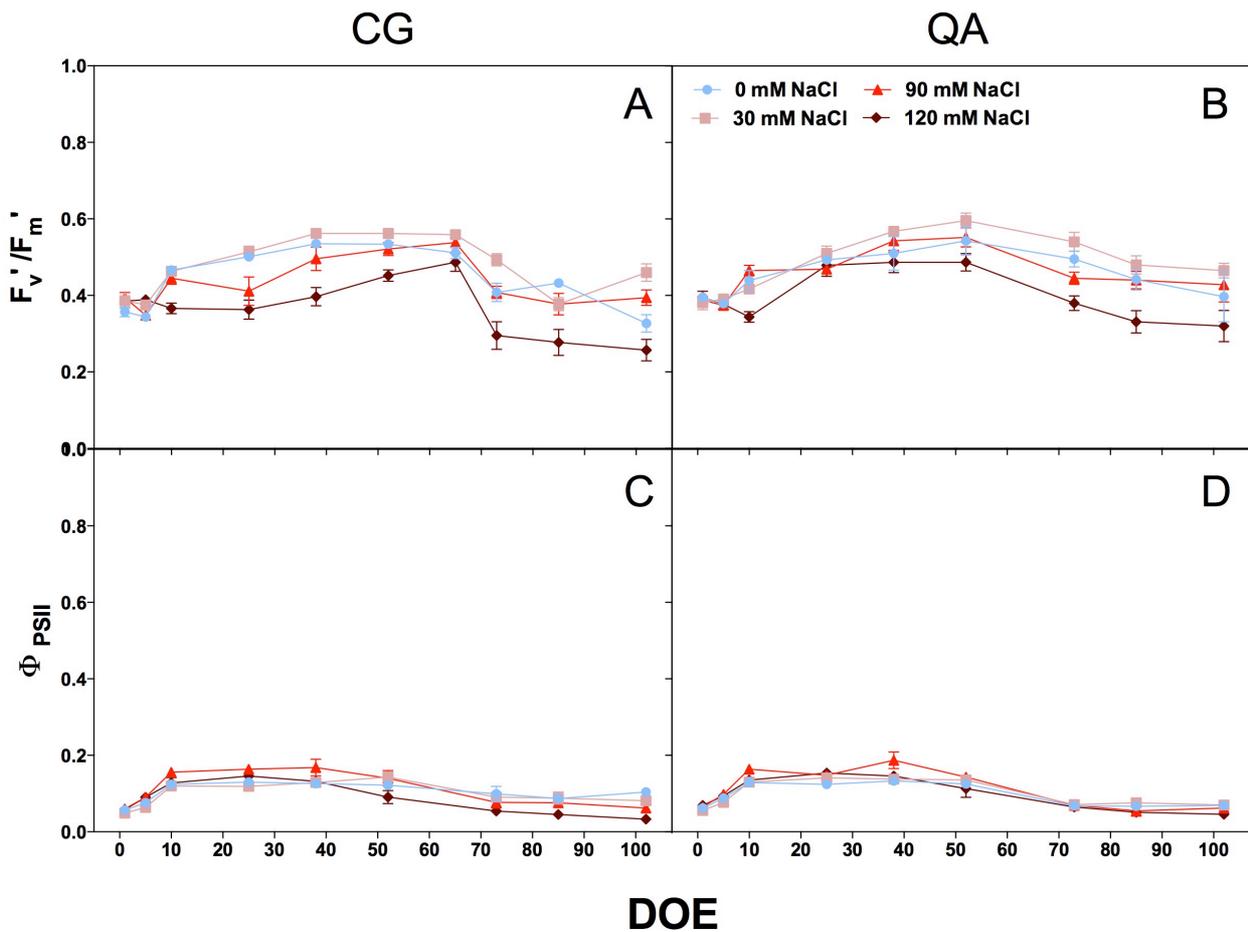


Fig. 4. PSII maximal quantum efficiency under light conditions (F_v'/F_m' A, B), actual quantum efficiency of PSII (Φ_{PSII} , C, D) in 0, 30, 90 and 120 mM NaCl treatments of the two genotypes “Cazzone Giallo” (CG) and “Quadrato D'Asti” (QA). Each point is the mean of 10 measurements; vertical bars indicate the standard error of the mean.

3.4.2 Long-term response

At the end of the plant cycle, further measurements of leaf water potential and osmotic potential on DOE 158, and gas-exchange and fluorescence parameters on DOE 148, were carried out to assess the very long term response to of plants to salinity.

In all treatments, as expected, $\Psi\pi$ was lower than Ψ_1 (Fig 5 A, B), the latter showing quite comparable values with those observed on DOE 109 in all salt treatments and genotypes (cf Fig 5 and Fig 1). As for Ψ_1 , in both the two genotypes, $\Psi\pi$ resulted significantly lower in T120 as compared to both T30 and Control, with values of about -1.97 and -1.32 MPa, respectively (Fig 5).

Similarly to Ψ_1 , treatments T0, T30 and T90 showed gas-exchange parameters comparable or slightly higher than those measured on DOE 102. However, the most saline treatment T120 showed a further decrease, especially in CG, as compared with values observed in the earlier dates (cf. Fig 6, Fig 2).

Treatment T120, as said above, showed a strong decrease in F_v/F_m from DOE 102 to 156 (cf. Fig. 4 and Fig 7). As regard F_v'/F_m' a decrease was observed in all treatments, whereas it was less evident for Φ_{PSII} .

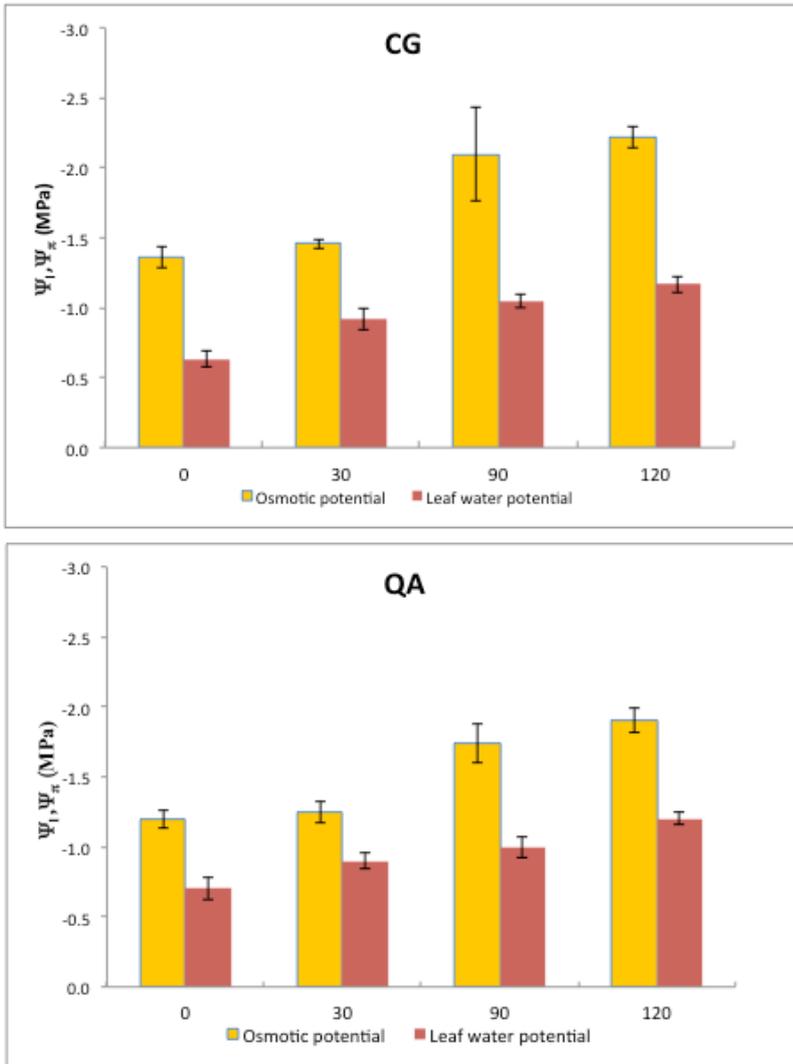


Fig. 5. Leaf water potential (Ψ_1 , red bars) and osmotic potential ($\Psi\pi$, yellow bars) measured on DOE 158 in *Cazzone Giallo* (CG) and *Quadrato d'Asti* (QA). Each point is the mean of 5 measurements; vertical black bars indicate standard error of the mean.

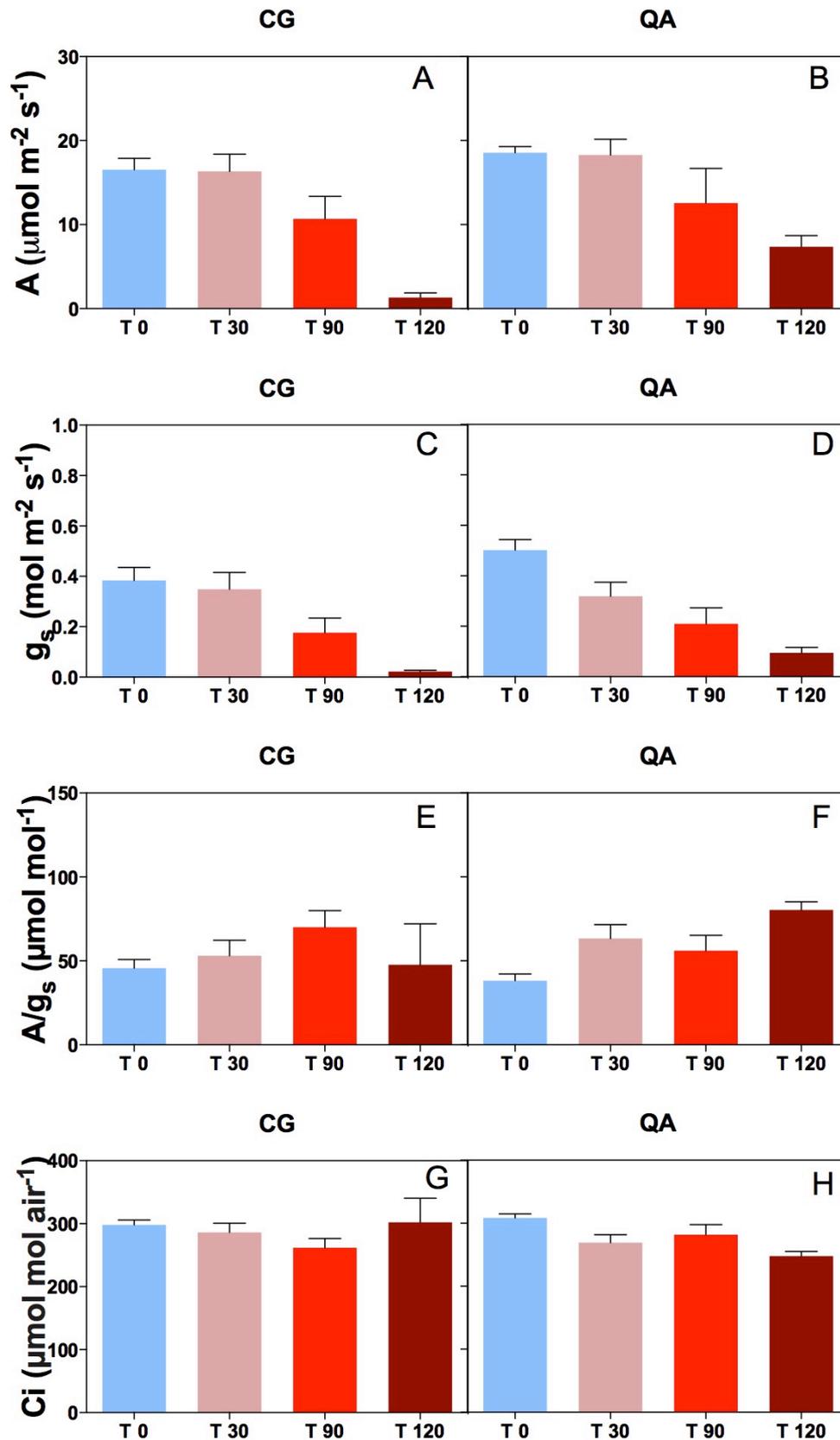


Fig. 6. Photosynthetic CO₂ assimilation (A, $\mu\text{mol m}^{-2} \text{s}^{-1}$, A, B), stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$, C, D), water use efficiency (A/g_s , $\mu\text{mol mol}^{-1}$, G, H) and intercellular CO₂ concentration (C_i , $\mu\text{mol mol air}^{-1}$, I, H) measured on DOE 148 in 0, 30, 90 and 120 mM NaCl treatments of the two genotypes *Cazzone Giallo* (CG) and *Quadrato D'Asti* (QA). Each point is the mean of 10 measurements; vertical bars indicate the standard error of the mean.

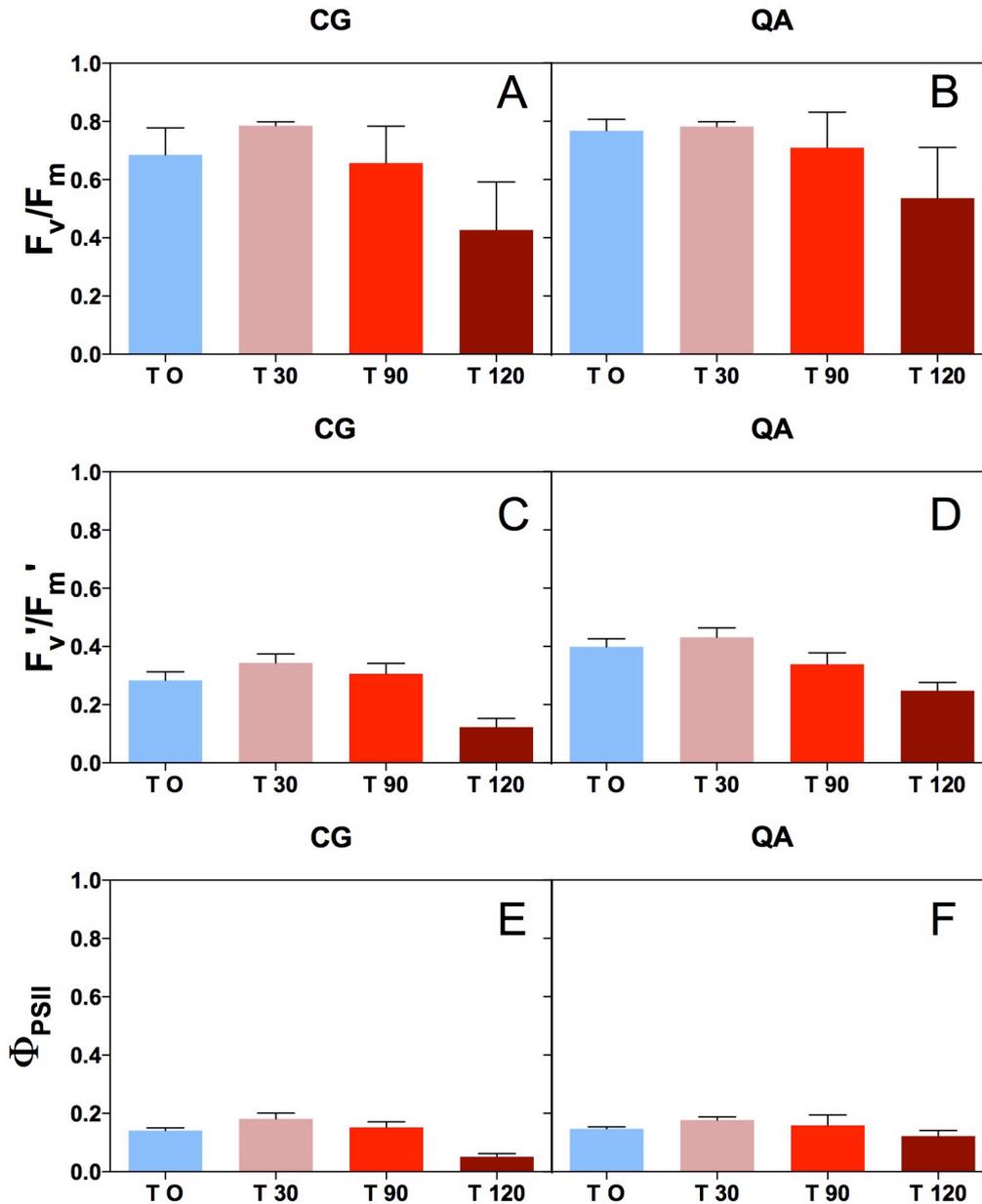


Fig. 7. Maximal quantum efficiency of PSII in dark adapted leaves (F_v/F_m , A, B), PSII maximal quantum efficiency under light conditions (F_v'/F_m' , C, D) and actual quantum efficiency on PSII (Φ_{PSII} , E, F) measured on DOE 148 in 0, 30, 90 and 120 mM NaCl treatments of the two genotypes “Cazzone Giallo” (CG) and “Quadrato D’Asti” (QA). Each point is the mean of 10 measurements; vertical bars indicate the standard error of the mean.

3.4.3 Growth and productivity

Plant leaf area (PLA) measured at starting of veraison (DOE 107) indicates for control plants a slightly higher size for QA if compared with CG: 0.71 vs. 0.52 m². Salinity induced a gradual decrease in both the two genotypes. However, T120 was decreased to 33% in CG and to 19% in QA (Fig 8). A clear reduction in marketable

yield of T30 (fresh weight of not discarded fruits, measured at the end of experiment on DOE 158) was observed in CG but not in QA. Such a yield in T90 and T120 was reduced to 40 and 18% in CG and to 80 and 48% in QA as compared to the respective Controls (Fig 9).

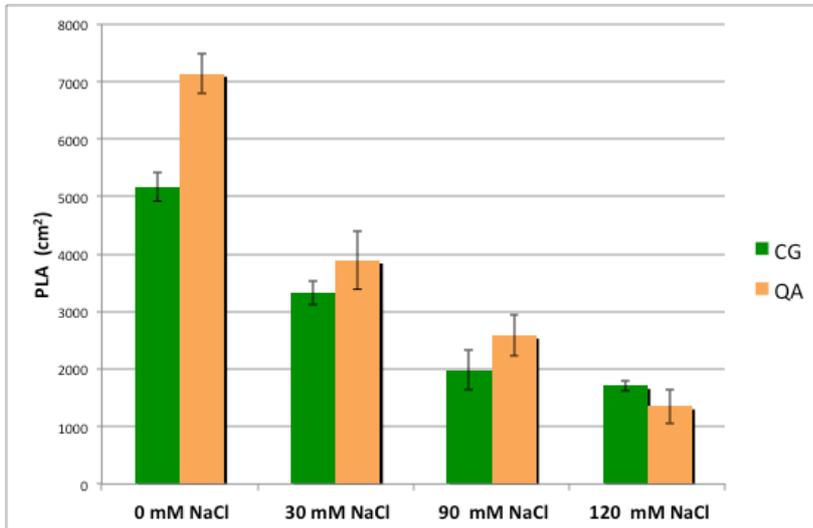


Fig. 8 Plant leaf area of the two genotypes “Cazzone Giallo” (CG) and “Quadrato D’Asti” (QA) measured on DOE 107. Each point is the mean of 3 measures; vertical black bars indicate standard error of the mean.

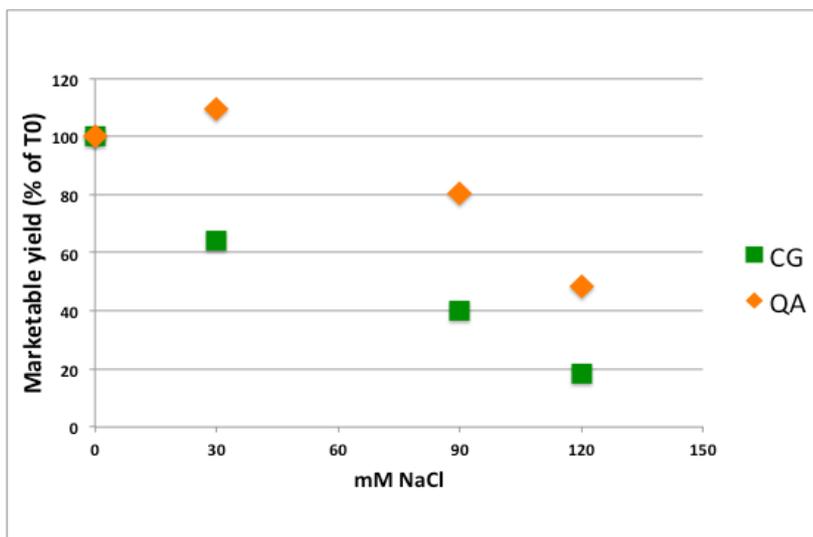


Fig. 9. Marketable yield of of the two genotypes “Cazzone Giallo” (CG) and “Quadrato D’Asti” (QA) measured at the end of experiment (DOE 158). Each point represents the normalized mean of 3 measures.

3.5 Discussion

Salinity affects plant growth, yield and photosynthesis (Maas and Hoffman, 1973; Greenway and Munns, 1980; Parida and Das, 2005).

Our data indicate that the treatment T30 ($EC=5.7 \text{ dS m}^{-1}$) in both CG and QA was actually slightly or not affected as compared with the Control T0 for almost all measured parameters. A clear separation of T0 and T30 from T90 and T120 was observed for leaf water potential already after 5-10 days since salt stress imposition (Fig. 1). As plants at that time were in a fast growing vegetative phase such an effect was related to the (rapid) osmotic phase of salinity (Munn and Tester, 2008) while it is unlikely that ion accumulation in leaves may have significantly contributed to water relations. However, during the entire experiment, salts certainly accumulated into leaf cells, significantly contributing to water relations. In fact, osmotic potential ($\Psi\pi$) measured after more than five months of stress imposition, significantly decreased in response to salt treatment, and it was lower than total water potential (Ψ_1). (Fig. 5 A, B). Such a decrease in osmotic potential could be due to increased leaf concentration of either inorganic ions or organic solutes (Taiz and Zeiger, 2006). Navarro et al. (2003) reported a reduction in Ψ_1 of about 0.5 MPa in pepper plants grown at 12 dS m^{-1} as compared with the control, not associated to a reduction in $\Psi\pi$ enough for turgor maintenance. Conversely, De Pascale et al. (2003) in field grown salt stressed pepper reported a better capacity for turgor maintenance by osmotic adjustment, however still with a turgor lower in stressed than control plants. In our case, as $\Psi\pi$ and Ψ_1 were measured with two different methodologies (osmometer and pressure chamber) and not on the same leaves, calculation of turgor component $\Psi_p = \Psi_1 - \Psi\pi$ would be biased, for example by dilution of leaf sap by apoplastic water. However, this latter source of bias would have induced an underestimation of leaf turgor. On the contrary, calculated Ψ_p from data of Fig. 6 indicates that this parameter even increased in the most salt treated plants. Bethke and Drew (1992) also found that in pepper plants grown in 100 or 150 mM NaCl nutrient solution, after 12 days, leaf turgor increased or remained constant in the two treatments, respectively. Moreover, in our plants both Na^+ and Cl^- accumulated in the leaves during the trial (data not shown; Venezia A., personal communication). At veraison (DOE 106) Na^+ strongly increased in the most saline treatments in QA (8.0 in T90 and 12.4 g kg^{-1}

D.W. in T120), whereas in CG a comparable increase was observed only in T120 (11.0 g kg⁻¹ D.W.), as compared with the control plants (4.4 g kg⁻¹ D.W. as an average). Conversely to sodium, quite high Cl⁻ leaf content was observed in the two genotypes of both T90 and T120. Cl⁻ leaf content in CG was 89.8 in T90 and 140.6 g kg⁻¹ D.W. in T120, whereas QA showed values about 20% in T90 and 30% in T120 lower than CG (Venezia A., personal communication). Such chloride leaf content was quite high as compared with data reported in several studies on pepper under salinity (De Pascale et al., 2003; Niu et al., 2010; Bethke and Drew, 1992) and certainly strongly contributed to osmotic adjustment for leaf turgor maintenance. Moreover, the aminoacid proline strongly accumulated into the leaves as organic compatible osmolyte with increasing salinity (data not shown). Therefore the two pepper genotypes showed in our long-term experiment a good capacity to maintain water relations even at higher level of salinity in the nutrient solution, comparably to what reported elsewhere by Navarro et al. (2003) and De Pascale et al. (2002). The much higher chloride content in the leaves as compared with sodium can be explained by the better capacity for compartmentalization in the different plant tissues. In fact, in Na⁺ content in stems of CG doubled as compared with leaves in both T90 and T120, whereas in QA such stem accumulation occurred at a lesser degree. In contrast to sodium, the content of Cl⁻ was lower in stems than leaves. Accordingly to the biphasic model of plant responses to salinity (Munns, 2002; Munns and Tester, 2008), accumulation of salt in the leaves pepper caused specific ion toxic effects, leading to a significant leaf senescence and abscission of basal leaves, quite evident in both T90 and T120 (visual inspection).

Similarly to leaf ionic and water relations, gas exchange parameters were not appreciably affected by the salinity imposed to treatment T30 (Fig. 2). In fact, both stomatal conductance and assimilation rate of T30 did not differ from the control. Chartzoulakis and Klapaki (2000) on pepper under soilless salinity treatment also reported no effect on both these two parameters for the two less saline treatments (25

and 50 mM NaCl), as compared with the Control. In the same experiment, after 5 weeks of 100 mM NaCl treatment, these authors found in two genotypes an average reduction in assimilation rate of 40%, quite comparable with the 50% reduction we found in T120 of CG after 25 days. As compared with CG, the genotype QA showed only a moderate reduction of A in T120 after 38 days, indicating a moderately less sensitivity to salt stress. Bethke and Drew (1992) also found that pepper exposed to 50 mM NaCl showed no decline in the photosynthetic capacity assessed through A/Ci response curves, whereas 150 mM NaCl strongly depressed such curve after 12 days. It is worth to highlight that the quick decrease we observed in g_s during first week was not followed by a decrease in A, which conversely increased in both genotypes (Fig. 2). It is known that the relationship between A vs. g_s in well-watered (unstressed) healthy plants saturates at high stomatal conductance (Farquhar and Sharkey, 1982). Therefore, a moderate g_s reduction actually would not imply a proportional reduction in A (but an increase in A/ g_s). However, in our case there was a significant increase in air temperature (about 5 °C) in the glasshouse during first week, which may have increased A independently from g_s (i.e. changing A vs. g_s relationship).

Subsequently to DOE 38, despite there was no appreciable change in stomatal conductance, which continued to slowly decrease to very low values, a gradual re-increase in Ci was observed, meanwhile assimilation continued to decrease, and it remained almost stable in the late measurement carried out on DOE 102 (Fig. 2 E, H). Lycoskoufis (2005) also found in pepper grown in a soilless system and exposed to 60 mM NaCl (8 dS m⁻¹) quite low g_s and A during a long period (from 50 to about 85 days since starting of salinization), similarly to our data. Moreover, a further measurement of gas exchanges carried out on DOE 148 (Fig. 6 G) showed that Ci in T120 of CG was even higher than the control. Therefore, the damage of salinity to the photosynthetic metabolism was enhanced as Ci continued to increase toward the external CO₂ concentration (Ca). Further than the dynamics of A/ g_s in T120, it is worth to note that also T90 showed higher A/ g_s than both Control and T30. It is know

that the increase of A/g_s can be considered an adaptive response of plants to salinity (Brugnoli, 1992), as it reduces water consumption per unit of fixed carbon, and it limits salt accumulation into the plant (Munns, 2002). All the above-discussed gas exchange response to salinity regarded essentially the CG genotype. However, although with some minor differences, the behavior of QA was quite similar to CG.

Photochemistry was also clearly affected by salinity. Maximum efficiency of photosystem PSII (F_v/F_m) was monitored in the late part of the experiment, from DOE 71 to 156. In the latter DOE this parameter was clearly depressed as it was almost halved in the most saline treatment of CG as compared with other treatments (Fig. 3 A). The effective PSII efficiency (Φ_{PSII}) was low in all treatments, and similarly to F_v/F_m it was the double in the control as compared with T120 (Fig. 4 C, D). Our results for F_v/F_m are comparable with data reported by Zhani et al. (2012) on potted chilly pepper irrigated with saline water up to 12 g/L NaCl. These authors found that the most saline treatments showed F_v/F_m around 0.5 whereas the much higher 0.79 was observed in the control plants, quite similar to our data (Fig. 3 A, B). Consequently, as compared with the control, the capacity of most saline treatment to generate electronic transport for ATP and NADPH production was impaired, reducing RuBP regeneration, and therefore contributing to the limitation of CO₂ fixation. Therefore, analyses of photochemical activity are in agreement with what discussed for gas-exchange.

Moreover, Cifre et al. (2005) elaborated for grapevine under water stress, a model to describe stomatal and non-stomatal limitations to photosynthesis, based on the relationships among gas-exchange parameters. It is well known, as above said, that conversely to water stress, salinity can induce ion specific toxic effects that impair photosynthetic performances through metabolic damages (Cornic et al., 1989; Sharkey, 1990; Cornic and Briantais, 1991). However, the analyses of Cifre et al. (2005) can be applied also to plants under salt stress.

As regard the most saline treatment (T120), during the first two weeks, a steep linear decrease in stomatal conductance was observed (Fig. 2), which probably was mainly

due to the osmotic stress induced by the nutrient solution. The concomitant reduction in assimilation was associated to a decrease in C_i . These relationships among g_s , A and C_i continued until DOE 38, when g_s was as low as $0.092 \text{ mol m}^{-2} \text{ s}^{-1}$. During the same period, we also observed a gradual increase in intrinsic water use efficiency (A/g_s) (Fig. 2) and an almost stable electronic transport rate (ETR) (data not shown). The latter, under our experimental conditions, was linearly proportional to Φ_{PSII} ($\text{ETR} = \Phi_{\text{PSII}} \cdot 0.5 \times \text{PPFD} \times \text{leaf absorbance}$), which was actually stable (Fig. 4). Such a down-regulation of photosynthesis characterized by a decrease in both g_s and C_i and an increase in A/g_s with stable ETR can be ascribed exclusively to a reduced CO_2 availability in the mesophyll caused by stomatal closure, and not to non-stomatal limitations (Cornic, 2000; Cifre et al., 2005). The stable ETR associated with a decreased assimilation could be explained by an increase in photorespiration as an alternative pathway for utilization of the captured light energy, induced by the shortage of CO_2 at carboxylation sites. With the exacerbation of stress conditions, stomatal conductance continued to decrease, reaching very low values ($<0.05 \text{ mol m}^{-2} \text{ s}^{-1}$) while assimilation also continued to decrease (Fig. 2). At same time, A/g_s started to decrease while C_i increased, despite the reduced assimilation rate, while F_v/F_m was reduced as well (Fig. 3). These relationships indicate that the role of non-stomatal limitations became highly relevant (Cifre, 2005). It is quite probable that the above-discussed salt accumulation into the leaves had a relevant contribution to the impairment of photosynthetic machinery, as indicated by the visual observed leaf damages. However, the transition between these two conditions described above (mild and severe stress) can be gradual with an intermediate condition where stomatal limitations are still prevailing but non-stomatal limitations are emerging.

Alike to water stress, the first effect of salinity on plants is the reduction of leaf expansion, which occurs within minutes (Passioura and Munns, 2000). It is known in fact that leaf growth is the earliest salt stress response in glycophytes (Munns and Termaat, 1986). Photosynthesis can be then reduced because of a negative feedback

caused by the reduced utilization of photosynthates resulting from the impaired growth. However, over long term, it is the reduced photosynthetic activity that becomes a limiting factor for growth and yield.

The genotype CG showed a higher sensitivity to salinity than QA as regard gas-exchange and photochemistry. In fact, A in T120 started to clearly decrease earlier in CG than QA (Fig. 2 A, B). As discussed above, there is a lot of evidences in literature that photosynthetic assimilation is not affected in pepper by moderate salinity, up to 25-50 mM NaCl (e.g. Beethke and Drew, 1992; Chartzoulakis and Klapaki, 2000; Lycoskoufis, 2005). On the contrary, we found that plant leaf area was significantly reduced even in T30 in both the two genotypes as compared with the control (Fig. 8). Similarly, also Chartzoulakis and Klapaki (2000) found an effect on leaf area at low salinity (25 mM NaCl). In addition to what monitored by the above-mentioned authors, we measured leaf water potential during the entire experiment. This parameter of water status resulted significantly lower (higher stress) even in T30 as compared to the Control (Fig. 1). Such a decrease in Ψ_1 inhibited leaf expansion in the T30, leading to the reduction in PLA as compared with the control plants (Hsiao, 1973). As regard the yield response to salinity, T30 was the sole treatment in QA that had no response as compared to the Control in terms of marketable yield (Fig. 9), resembling what normally occurs to moderate salt-sensitive field-grown pepper submitted to E_{Ce} values lower than 1.5 dS m⁻¹ (Maas and Hoffman, 1977). Conversely to QA, CG showed a gradual yield decrease with increasing salinity, which indicates that this genotype has a threshold for soilless salinity experiment below 30 mM NaCl.

3.6 Conclusions

In conclusion, the two tested pepper genotypes grown in a greenhouse on a soilless nutrition system in a long-term experiment showed great impairment of water status, and accumulated significant amount of both Na⁺ and Cl⁻ in stems and leaves. Photosynthetic assimilation and photochemistry at high levels of salinity were

dramatically impaired throughout two distinct phases. Stomatal closure dominated photosynthetic limitations during first 30-40 days of salt stress imposition. Conversely, afterwards non-stomatal limitations had a relevant role with the probable significant contribution of specific ion toxic effects. Moderate salinity did not affect physiological parameters in both genotypes. In several physiological aspects and in marketable yield response the genotype *Cazzone Giallo* resulted more salt sensitive than *Quadrato d'Asti*.

Chapter 4

“*Aglianico* grapevines grown in two nearby soils with distinct capability of water retention experienced different water stress intensities affecting vine water status and photosynthetic performances.”

(Vitis vinifera L.)

4.1 Abstract

Grapevine (*Vitis vinifera*, L.) is an important crop in the Mediterranean area for grape and wine production. This species is adapted to the Mediterranean climate, with hot and dry summers. Soil type is among the most important environmental factors affecting physiological and morphological performances and productivity of vineyards. Soil water content determines the dynamics and intensity of stress to which vineyards are submitted. A moderate water stress affects photosynthesis, yet it can be beneficial for grape yield and wine quality whereas intense water stress is deleterious. An *Aglianico* vineyard was grown under rain-fed conditions (with no irrigation) in two nearby soils with distinct capability of water retention and, therefore, soil moisture available to the vines, higher for the Cambisol (CAM) and lower for the Calcisol (CAL). In a two-year trial we monitored the effect of this soil characteristic on leaf water status, gas-exchange and Chl *a* fluorescence in order to highlight the mechanisms underlying physiological responses of grapevine under long-term water stress conditions under open field conditions.

Soil moisture showed a gradual reduction due to the depletion caused by vine transpiration and soil evaporation. Leaf water potential (Ψ_1) decreased to -1.64 MPa in CAL and -1.40 in CAM in late summer, typical values for the Mediterranean climate. Especially in the more stressed CAL vines, impaired leaf water status triggered a control of transpirative water loss, through marked stomatal closure. Stomatal conductance (g_s) in fact decreased to the minimum of $0.030 \text{ mol m}^{-2} \text{ s}^{-1}$ as a two-year average in CAL, whereas in CAM the average minimum g_s was significantly higher ($0.173 \text{ mol m}^{-2} \text{ s}^{-1}$), indicating for *Aglianico* a water saving (“pessimistic”) behavior. Nevertheless, the moderate differences in Ψ_1 between CAL and CAM vines also indicate a certain degree of near isohydric behavior, more evident in the second as compared with the first year.

Trends of assimilation rates (A) resembled those of g_s . The maximal difference between vines of the two sites occurred when was 16.1 in CAM and 5.7 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in CAL in the first year, and 13.9 and 2.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the second year for the two sites, respectively. Soil moisture, leaf water potential and gas-exchange recovered with the occurrence of some rainfalls.

Analyses of the dynamic of intracellular CO_2 concentration, and the relationship between intrinsic water use efficiency (A/g_s) and g_s , which is an integrative parameter of water stress, revealed information leading to relevant conclusions. The long-term gradual imposition of soil water deficit under field conditions resulted in a mild to moderate intensity of water stress imposed to the vines. Grapevines succeeded to bear water stress conditions by controlling water loss through stomatal closure. This markedly reduced photosynthetic assimilation, yet without significant impairments of photosynthetic machinery. Nevertheless, in the second year a severe water stress was imposed to CAL vines in a short-term period. A/g_s vs. g_s relationship and the dynamic of C_i indicate the occurrence of non-stomatal limitations to photosynthesis. Moderate water deficit reduced yield by improved berries quality especially in the first year.

4.2 Introduction

Grapevine (*Vitis vinifera*, L.) is one of the most important fruit crop in the world, with 7.8 million hectares cultivated in 2011, and a annual production of 67.5 million tons of berries with a production of about 29 millions of wine, two thirds of them in the Mediterranean area (<http://www.oiv.int/>).

Grapevine is a typical species of the Mediterranean area, which has a temperate climate with hot summers and mild winters (Williams et al., 1994). Environmental factors, namely climate and soil, cultivar and human practises, and their interactions affect yield and quality of grape and wine.

The concept of *Terroir* is adopted in viticulture to relate sensory attribute of wine to such a complexity of factors (van Leeuwen and Seguin, 2006; Wilson 1999).

Specifically, soil type is considered a crucial factor in the yield and quality performances of vineyards, as it affects among other factors the amount of water that plants can capture from soil.

During the grapevine growing season in the Mediterranean area, precipitations are really limited, increasing the risk of drought stress (Robinson 2006) in particular in soils characterized by a low water retain (Tramontini et al., 2013).

Intense water stress imposes excessive constraints to growth and photosynthesis, with negative effects on grape yield and wine quality (Griesser, 2015), especially if water deficit occurs before veraison (Smart, 1974; Hardie and Considine, 1976; Matthews and Anderson, 1988), yet negative effects in post veraison have been also reported (Poni et al., 1993). Wine grapevine cultivars are traditionally (or obliged by law) cultivated under rain-fed conditions where a moderate soil water deficit, e.g. imposed by deficit irrigation (DI) practices, can be beneficial for grape and wine quality (Hardie and Considine, 1976; Smart et al., 1990; Lovisolo et al., 2010). Chaves et al. (2010) reviewed “the rationale for deficit irrigation, (by asking) why mild to moderate water deficit may be favourable to grape berry quality”. Essentially, a moderate water deficit (regulated deficit irrigation, RDI) can be a favourable condition at specific phenological phases, normally late in the season after veraison, when fruit development is less sensitive. The effect of water deficit on fruit growth is higher and much irreversible during Stage I (ending at veraison) of the double-sigmoid growth curve than during the next Stages II and III (Williams et al., 1994, and references therein). Moreover, accumulation of sugar is less sensitive to water deficit than fruit growth, unless the stress is quite severe (Williams et al., 1994 and references therein). As a consequence of a moderate water deficit, yield is not much reduced while quality in some cases may be even improved (Chaves et al., 2010). Plant secondary metabolites are produced as a cellular response to abiotic stress and are involved in several mechanism of defense by ROS scavenging (Cramer et al., 2011). On the other hand, increase of their concentration of secondary metabolites

enhances grape quality, as they contribute to color, taste and aroma of fresh and dried grapes and they are crucially involved in wine stabilization and aging processes.

Zsófi et al. (2009, 2011) studied the effect of soil water deficit on water status, gas exchange, vegetative growth and berry sugar concentration on "Kèfrankos" grapevines grown on two soils, having different retention of soil moisture. The authors concluded that water deficit had a preeminent role on the different physiological responses, yield and quality showed by the vineyard in the two soil sites. However, those two vineyards were as far apart as 7 km.

We carried out a two-year similar experiment on a rain-fed (non-irrigated) "Aglianico" commercial red-wine vineyard. The peculiarity of the experimental conditions was that at the very short distance of 100 m, hydrological characteristics of the soil were quite different. This resulted in two soil sites having high and low availability of soil water content during the growing season, whereas, the short distance assured that all other environmental conditions were identical. The aim of the work was to assess how the different soil water availability induced plant water stress, affecting physiological responses and grape yield and quality.

4.3 Materials and methods

4.3.1 Study Area

The experimental site was located in Southern Italy (Mirabella Eclano-AV, Campania region: 41°02'48.3"N 14°59'26.1"E, elev. 368 a.s.l.), in a farm oriented to high quality wines production, named *Quintodecimo*. The trial was carried out in 2011 and 2012 in a rain-fed (not irrigated) *Vitis vinifera* L. cv "Aglianico" vineyard planted in the year 2000, grafted on *V. berlandieri* Planch. x *V. rupestris* Scheele (1103 P). Rows were oriented NW-SE (43° NW) and vines were spaced 2 m between rows by 1 m along the rows (5000 plants per hectare, espalier system, cordon spur pruning). A pedological characterization carried out by Bonfante et al., (submitted)

found two functional homogeneous zones, 100 m apart, with two soil types: Cambic Calcisol (Clayic, Aric) and Eutric Cambisol (Clayic, Aric, 357 Colluvic) (IUSS, 2014). The texture is quite similar and both soils can be classified as clay loam. Despite the similar texture, hydraulic properties measured in lab showed some important differences as the available water content (AWC) in the first 80 cm of soil depth resulted 80 mm in Calcisol (CAL) and 145 mm in Cambisol (CAM).

All the measurements were carried out in the two different sites of the vineyard, characterized by large different soil water retention under the same climatic conditions.

The long-term (2003-2013) mean daily temperature at the study area was 14.7 (± 0.9) °C, while the mean annual rainfall was 802 (± 129) mm (data from the Regional weather station of Mirabella Eclano – AV- at 1 km of study area).

The climate monitoring within the farm in the two years showed that during the cropping season (April-early October) the mean daily temperature was of 20.9 (± 1.2) °C while the precipitation was very variable during ranging from 200 to 285 mm.

4.3.2 Soil measurements

In both the two sites, volumetric soil water content (θ , $\text{m}^3 \text{m}^{-3}$) was assessed by Time Domain Reflectometry (TDR) technique using two programmed automated systems, each one equipped with a solar panel and battery, a CR1000 data-logger for storage of data taken by a TDR 100 instrument and AM multiplexers (Campbell Scientific Inc., Logan, UT, USA) to which self-built three-steel wire TDR probes were connected. Probes were horizontally installed at 0.1, 0.3, 0.7 and 0.9 m depth along the soil profile.

4.3.3 Leaf water status

Leaf water potential (Ψ_l , MPa) was assessed on a leaf of 10 plants per site using a Scholander type pressure bomb (SAPS II, 3115, Soil moisture Equipment Corp., Santa Barbara CA, U.S.A). Well-expanded sunlit leaves were chosen at breast.

Following cutting, the leaf was inserted into the pressure bomb within 30 s, and pressure was increased at a rate of 0.2 MPa min⁻¹.

4.3.4 Leaf gas exchange

Photosynthetic CO₂ assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$), stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$), intercellular CO₂ molar ratio (C_i , $\mu\text{mol mol}^{-1}$) and effective quantum efficiency of PSII photochemistry (Φ_{PSII} also known as $\Delta F'/F_m'$) in light-adapted leaves, were measured by means of a portable photosynthesis and modulated fluorescence system (model either Li-6400-40 or Li-6400XT, Li-Cor Biosciences, Lincoln, NE, U.S.A.). Actinic light was provided by an artificial red and blue LED source with 630 and 470 nm peak emissions, respectively. The light source was set at a saturating photosynthetic photon flux density (PPFD, 10% blue light) of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. An external bottled CO₂ source was used to maintain the leaf chamber CO₂ molar ratio at 400 $\mu\text{mol mol}^{-1}$. The built-in modulated fluorometer was used to measure the fluorescence parameters on the adaxial side of leaves. After the measurement of *Chl a* fluorescence emission at steady-state under light conditions, F' , the maximum fluorescence emission, F_m' , was assessed upon induction by a 0.8 s super-saturating light pulse; then actinic light was briefly switched off while a far-red light was used to discharge the PSI photosystem to allow measurement of the minimum fluorescence emission under light conditions, F_0' . The software of the instrument (Li-Cor, 2011) calculated the gas-exchange parameters on the basis of von Caemmerer and Farquhar (1981) model, and the actual quantum efficiency Φ_{PSII} or $\Delta F'/F_m' = (F_m' - F')/F_m'$ (Genty et al., 1989) and the maximum quantum yield under light conditions F_v'/F_m' , where $F_v' = F_m' - F_0'$ (Baker, 2008).

4.3.5 Transient chlorophyll *a* fluorescence emission and chlorophylls content

Transient fluorescence induction (FI) (Kautsky effect) was assessed according to Strasser et al. (2000, 2004) and Lazár (2006) by a continuous-excitation *Pocket PEA*

or, alternatively, by a *Handy PEA* fluorometer (*Hansatech, Instruments Ltd*, King's Lynn, Norfolk, England) equipped with leaf-clips. The excitation red light pulse for FI was emitted by a red light diode source, and applied for 1s at the maximal available intensity for PFFD of $3500 \mu\text{mol m}^{-2} \text{s}^{-1}$. The basal (F_o [relative units, r.u.]) and peak (F_p , [r.u.]) fluorescence emissions were assessed in the adaxial lamina of leaves after 40-60 min of dark adaptation. F_p was assumed as a viable maximum fluorescence emission (F_m [r.u.]) approximation according to Giorio (2011) in order to calculate F_v/F_m . Before initiating dark adaptation, the same protocol was used to obtain steady-state (F' [r.u.]) and maximum (F_m' [r.u.]) fluorescence emissions in light adapted leaves, and consequently $\Phi_{\text{PSII}} = \Delta F'/F_m'$. See Giorio (2011) for detailed methodology. Measurements were carried out on 15 young fully expanded sun-exposed leaves, chosen as for gas exchange from the middle region of 15 vines. Chlorophyll content of leaves was optically estimated as CCI, an optical index of the actual chlorophyll content, by a handheld meter (CCM200, Chlorophyll content meter Apogee Instruments, Inc., Logan, UT), which is the ratio of the fractional leaf transmittances at 653 and 931 nm.

4.3.6 Canopy light interception and LAI estimation

A linear Accupar LP-80 PAR-LAI ceptometer (Decagon Device Inc., Pullman, WA, USA) was used to measure incoming light intercepted by the vineyard to estimate leaf area index (LAI, m^2m^{-2}). The ceptometer had 80 photosynthetic photon flux density (PPFD) sensors spaced at 1 cm interval, and it was programmed to average readings of every 10 sensors before logging data. The PFFD transmitted through the canopy (PPFD_T) was measured at 0.25 cm above soil surface over a grid of $0.1 \times 0.1 \text{ cm}^2$ across an area of 2 m along and 2 m between the rows. The measurements were carried out in 3-4 replicates in both CAL and CAM sites, while the measurements taken in a clear area near the two sites were taken as the PFFD incident over the canopy (PPFD_I). Intercepted light (PPFD_{Int}) was calculated as the difference between incident and transmitted PFFD, whereas the fractional light interception (f_i) was calculated as the ratio between PPFD_{Int} and PPFD_I .

The instrument software calculated LAI (one-side green leaf area (m^2) in a canopy per unit ground area.) by following the Norman/Jarvis/Campbell-Norman model (Campbell and Norman, 1989) which requires the measurement of both incident and below-canopy radiation. Assuming a random distribution of leaves within the canopy, the software adopts a simplified version of the complete model of Norman and Jarvis (1974) for transmission and scattering of light in a canopy. It assumes a spherical leaf angle distribution ($x=1$), which implies the extinction coefficient $K = 1/(\cos \Theta)$, where Θ is the zenith angle of the sun. On this basis, it can be shown that $\text{LAI} = [(1 - 1/2K) f_b - 1] \ln \tau / (A (1 - 0.47 f_b))$ (Decagon Devices, 2006), where f_b is the ratio between direct radiation coming from sun and total radiation coming from all ambient sources and $\tau = \text{PPFD}_T/\text{PPFD}_I$, whereas A depends on leaf absorptivity which was assumed to be 0.9 in the photosynthetic radiation band.

4.3.6 Statistical analysis

For each data, differences between two sites were evaluated performing Student's t -test, with the null hypothesis rejected at $p \leq 0.05$ by using the software package *GraphPad Prism ver. 5.0* for Mac (*GraphPad Software Inc.*, San Diego, CA, U.S.A.).

4.4 Results

4.4.1 Soil moisture

Volumetric soil water content gradually decreased during the growing season in both the two soil sites. Interestingly, at starting of measurements in spring, θ in CAM site (0.43 in the first and 0.35 $\text{m}^3 \text{m}^{-3}$ in the second year) was 0.1 $\text{m}^3 \text{m}^{-3}$ higher than CAL in both years (Fig. 1 A, B). The abundant rainfall of 61 mm occurring on 23 July of second year (day of year, DOY 205) significantly increased soil moisture to about 0.34 $\text{m}^3 \text{m}^{-3}$ in both two sites. A very low value of soil moisture (0.15 $\text{m}^3 \text{m}^{-3}$) was reached on 9th September (DOY 252) in first year and on 1th October (DOY 275) in the second year.

4.4.2 Leaf water potential

In the two-year trial, leaf water potential (Ψ_l) was about -0.7 MPa in spring and then it decreased afterwards with the occurrence of more stressful conditions. The average minimum in both years was reached in late summer, -1.64 in CAL and -1.40 MPa in CAM vines. During both seasons, drops in Ψ_l were followed by a re-increase with the occurrences of rainfalls. In the first year, no differences were found between sites until the middle of July (DOY 196). Afterwards, the worsening of vine water status was more pronounced in CAL than in CAM site, with a maximum significant difference of 0.58 MPa occurring at the end of Aug (DOY 242) (Fig. 1C). In the second year, Ψ_l was lower in CAL than CAM from DOY 177 to 202 (mid July, Fig. 1D). The abundant rainfall of 61 mm then recovered water status in both sites. Afterwards, Ψ_l decreased again until DOY 242 (end of August), with a following partial recovering. As an average during the season Ψ_l was -0.86 in CAM and -1.06 MPa in CAL in the first year, whereas in the second year it averaged -1.01 in CAM and -1.19 MPa in CAL.

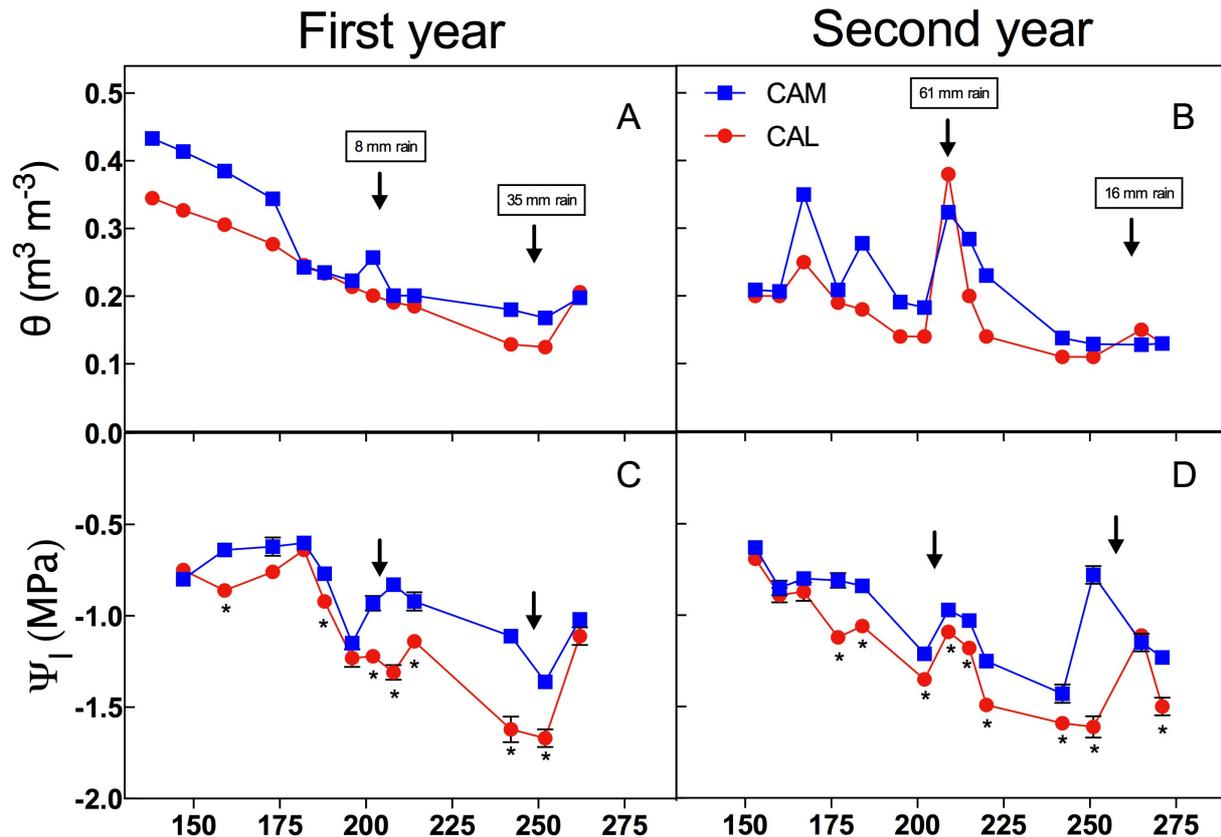


Fig. 1 Volumetric soil water content (θ , $\text{m}^3 \text{m}^{-2}$) (A, B), leaf water potential (Ψ_l , MPa) (C, D), in the two theses Calcisol (CAL) and Cambisol (CAM) during the first (left) and second (right) year of trials. Each point is the mean of 15 measurements. Vertical bars indicate the standard error of the mean. Downward arrows indicate rainfalls.

4.4.3 Gas exchanges

Stomatal conductance during the two years agreed with Ψ_l , with the CAL plants experiencing lower values than those of CAM. During first year, in both two sites g_s increased from May to early July up to about $0.350 \text{ mol m}^{-2} \text{ s}^{-1}$ (DOY 138 to 182, Fig. 2 A). Afterwards, there was a gradual decrease to quite low values of $0.042 \text{ mol m}^{-2} \text{ s}^{-1}$ in CAL on DOY 242 (end of August), when in CAM vines g_s was as high as $0.206 \text{ mol m}^{-2} \text{ s}^{-1}$.

Subsequently, a partial recovery was observed especially in CAL vines, leading to a difference in g_s between the two sites of $0.050 \text{ mol m}^{-2} \text{ s}^{-1}$ (Fig. 2 A).

In the second year, in both sites g_s raised during June reaching an average of $0.250 \text{ mol m}^{-2} \text{ s}^{-1}$ on DOY 177 (Fig. 2 B), then a steep drop occurred and it continued until DOY 202 (mid July) when the very low value of $0.038 \text{ mol m}^{-2} \text{ s}^{-1}$ in CAL was significantly different from $0.157 \text{ mol m}^{-2} \text{ s}^{-1}$ measured in CAM vines. At the end of

July (when an abundant rain occurred) stomatal conductance recovered to about previous higher values, before a decreasing started again on DOY 209. The minimum g_s observed a month later (DOE 242, end of August) was 0.018 in CAL and 0.140 $\text{mol m}^{-2} \text{s}^{-1}$, significantly higher in CAM vines (Fig. 2 B). In the late summer, a moderate re-increase followed by another drop of g_s was observed in both sites. Assimilation rates (A) showed trends quite similar to those observed for stomatal conductance in both years (Fig. 2 C, D). In fact, in the first year, the increase observed during June was followed by a period of progressive reduction until late summer, before the occurrence of a recovering (DOY 242). The maximal difference between vines of the two sites occurred in the first decade of September (DOY 252) when A was 16.1 in CAM and 5.7 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in CAL. Subsequently a moderate re-increase was observed in CAL.

In the second year, alike stomatal conductance, in both the two sites assimilation rates increased during June, then it decreased until DOY 202 (mid July), and re-increased during next 13 days (Fig. 2 D). Afterwards, it decreased again during August, until DOY 242, when the minimum assimilation in CAL, 2.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was significantly lower than 13.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$ found in CAM. Finally, assimilation moderately recovered again.

As regard water use efficiency (A/g_s) in both years this parameter remained almost constant and without significant differences between the two sites since spring until the end of June. During the first year, from DOY 182 to DOY 242 a clear increase to 150 μmolmol^{-1} was observed in CAL plants. Afterward this parameter quickly decreased to previous values of 50 μmolmol^{-1} . During the second year, differences between the two theses were lower, with the exception of two periods, in July, and late August in which CAL plants showed slightly higher values than CAM plants.

Intercellular CO_2 concentration C_i , during the first year was almost constant in CAM plants for the whole cultural season to a value of 250 $\mu\text{mol mol air}^{-1}$, conversely a steep decrease to 120 $\mu\text{mol mol air}^{-1}$ was observed in CAL plants from DOY 182 to DOY 242. Afterward in late summer a significant increase was observed to the same

value of CAM plants $290 \mu\text{mol mol air}^{-1}$. In the second year the only differences between the two theses occurred on DOY 220 when CAM plants were $50 \mu\text{molmolair}^{-1}$ higher than CAL, and on DOY 271 (end of September) when CAL plants reached the minimum value of $126 \mu\text{mol mol air}^{-1}$.

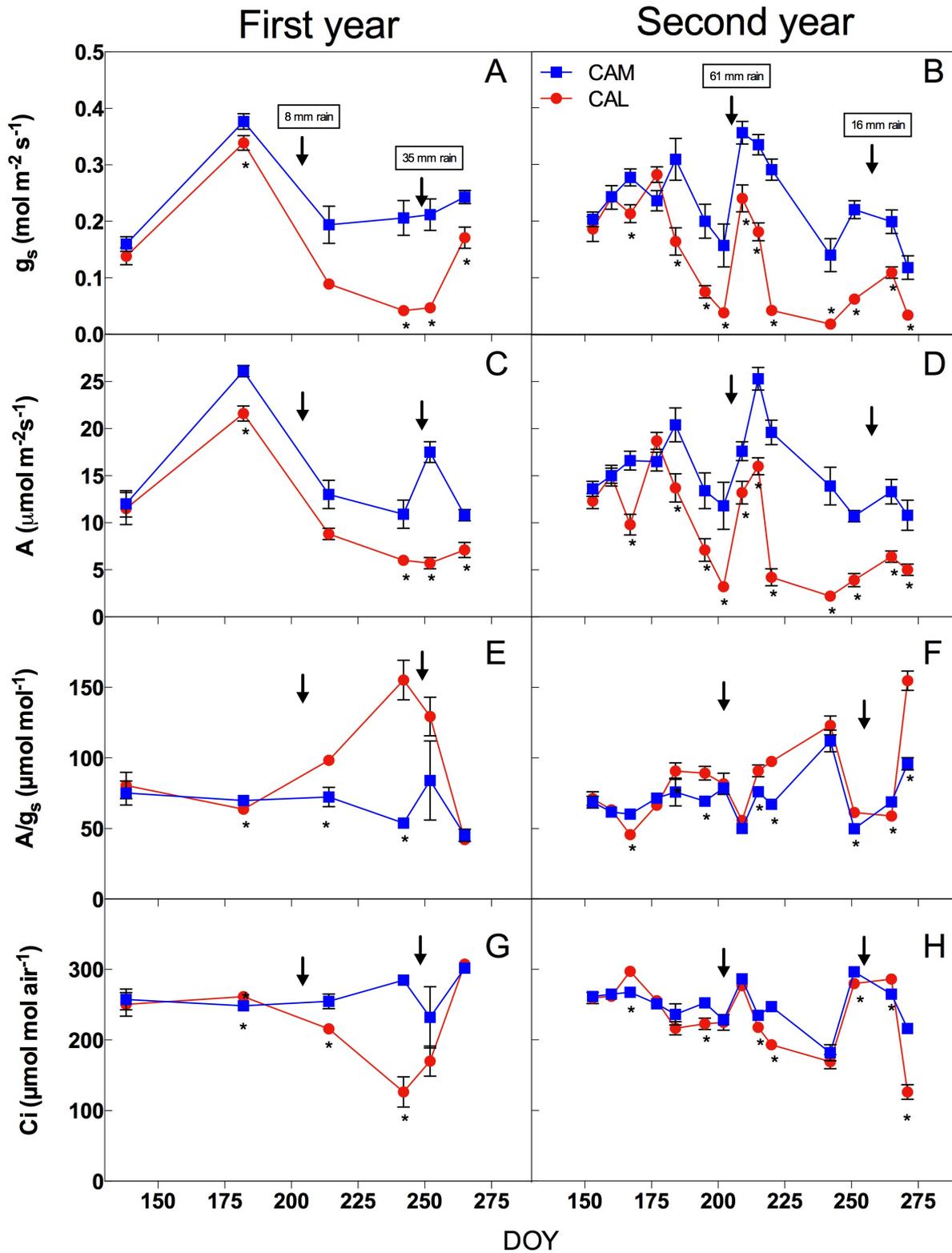


Fig. 2. Stomatal conductance to water vapour (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) A) and B) Photosynthetic CO_2 assimilation (A , $\mu\text{mol m}^{-2} \text{s}^{-1}$, C, D), water use efficiency (A/g_s $\mu\text{mol mol}^{-1}$, E, F) intercellular CO_2 concentration (C_i $\mu\text{mol mol air}^{-1}$, G, H), in the two theses Calcisol (CAL) and Cambisol (CAM) during first and second year of experiment. Each point is the mean of 15 measurements. Vertical bars indicate the standard error of the mean. Downward arrows indicate rainfalls.

4.4.4 Fluorescence parameters

The maximal quantum efficiency of photosystem PSII (F_v/F_m) in the first year did not show appreciable differences between CAL and CAM leaves with the exception of DOY 159 (early June) and 182 (start of July). F_v/F_m showed a moderate increase since spring until early July in both years. Afterwards, it remained pretty constant in both the two years and the two sites. With the exception DOY above mentioned, F_v/F_m ranged from 0.750 to 0.790 in the first year and from 0.683 to 0.816 in the second year, in which the difference was statistically significant in several occasions.

The effective quantum efficiency of photosystem PSII (Φ_{PSII}) decreased during most of the two growing seasons (Fig. 3), with higher values in CAM than CAL, and with more oscillations in the second year as found for gas exchange parameters. In the first year, a minimum of 0.069 occurred in CAL leaves on DOY 252 (late August), with a doubled value as compared to CAM. In the second year, Φ_{PSII} in CAL gradually decreased to about 0.082 in late summer. CAM maintained pretty stable values until the end of July when it was significantly higher than CAL. Afterwards, an increase from 0.143 to 0.220 was observed within 10 days, reaching a doubled value as compared with CAL leaves, and then followed again by a progressive decrease.

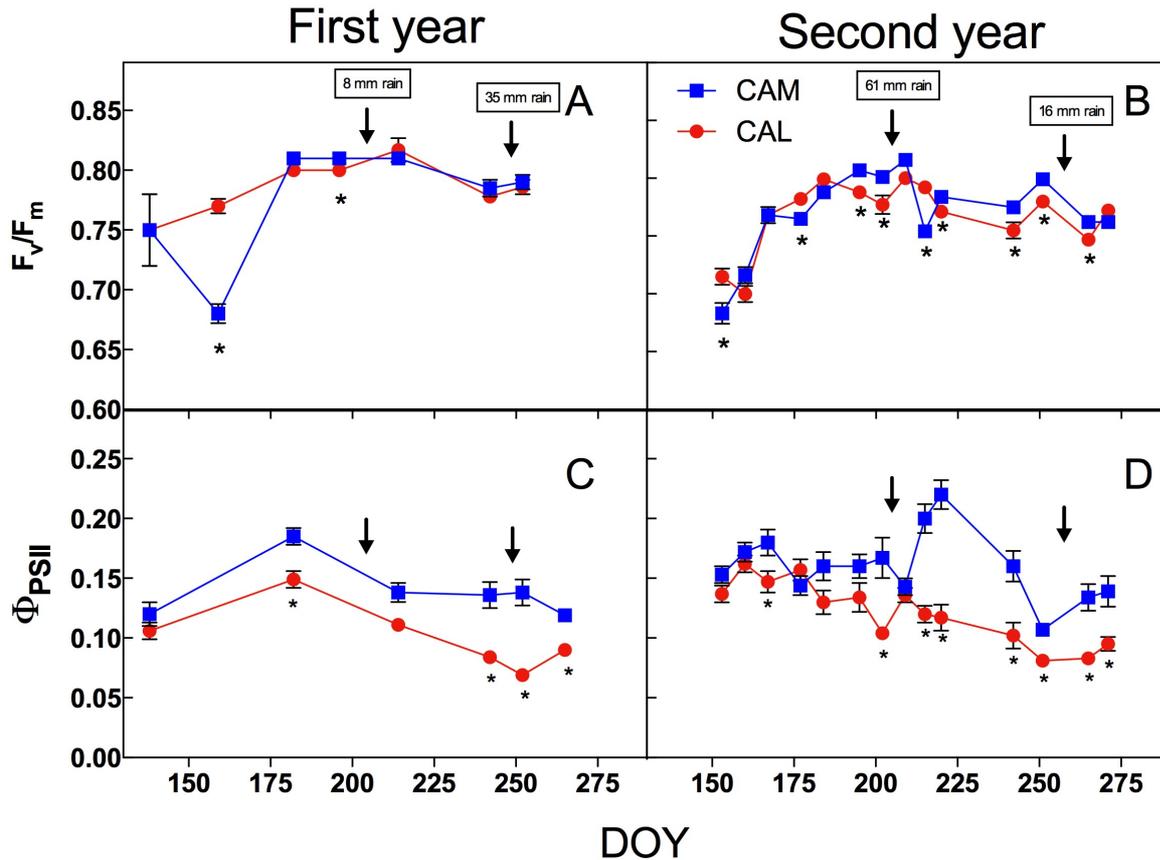


Fig. 3 Maximal quantum efficiency of PSII (F_v/F_m , A) and B), actual quantum efficiency on PSII (Φ_{PSII}) C) and D) in the two theses Calcisol (CAL) and Cambisol (CAM) during first and second year of experiment. Each point is the mean of 50 measurements for F_v/F_m and 15 measurements for Φ_{PSII} . Vertical bars indicate the standard error of the mean. Downward arrows indicate rainfalls.

4.4.5 Chlorophyll content index.

Chlorophyll content was estimated by the optical index CCI. From June to September, this parameter nearly doubled in both years in CAM, and in the second year in CAL years, whereas in the first year CAM showed an increase of just 50% (Fig. 4). After June, as an average, CCI in CAM was higher than CAL leaves by 57% in first year and 25% in the second year.

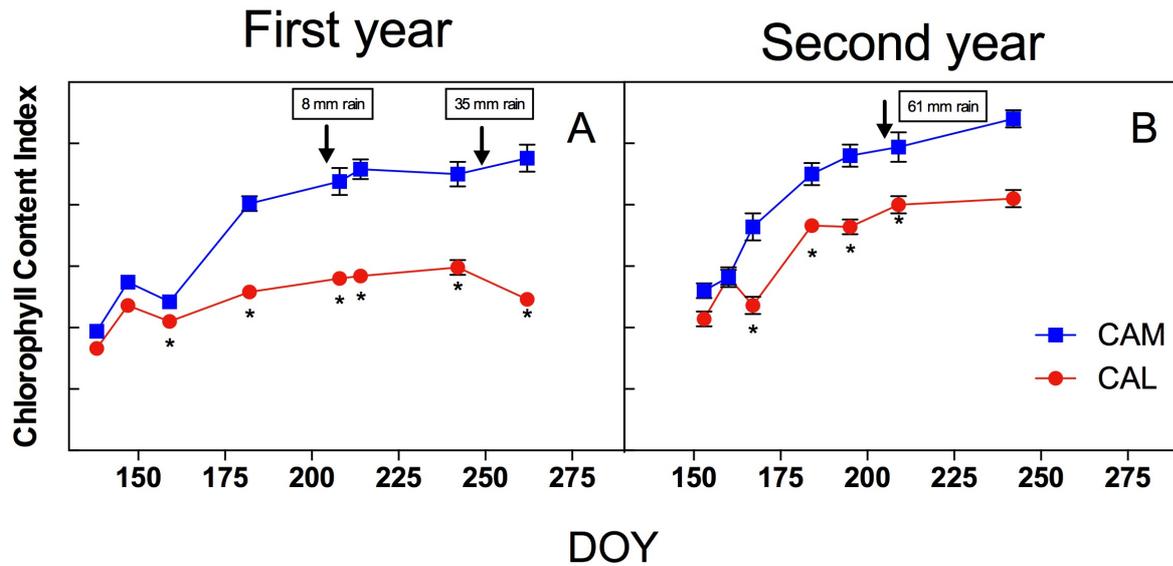


Fig. 4 Optical chlorophyll content index (CCI) in the two theses Calcisol (CAL) and Cambisol (CAM) during first and second year of experiment. Each point is the mean of 60 measurements. Vertical bars indicate the standard error of the mean. Downward arrows indicate rainfalls.

4.4.6 Leaf area index.

Vines of CAM site showed higher leaf area index (LAI, $m^2 m^{-2}$) than CAL in both the two years. These differences occurred essentially in mid and late summer. During this period in the first year the average LAI was 1.23 in CAL and 1.50 $m^2 m^{-2}$ in CAM (Fig. 5, A). Comparable values were found in the second year. However, in mid summer LAI in CAM was 0.98 $m^2 m^{-2}$ higher than CAM (Fig. 5,B)

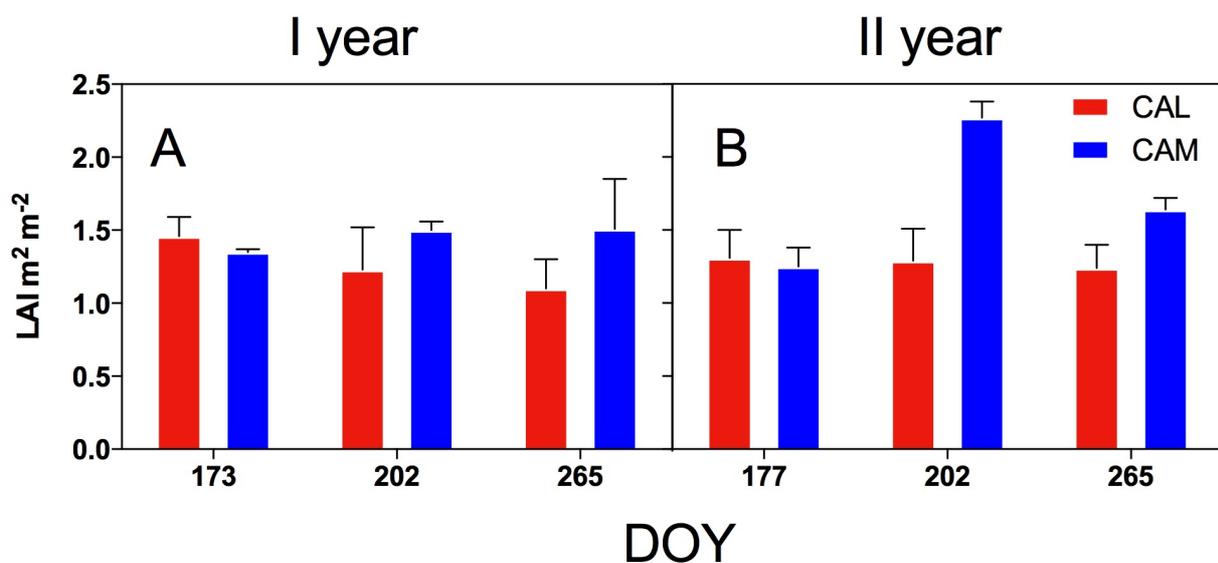


Fig. 5 Leaf area index measured on early, middle and late summer in first (A) and second (B) year in both Calcisol and Cambisol vines. Each point is the mean of 10 measures. Vertical bars indicate the standard error of the mean.

4.5 Discussion

Drought is the most deleterious abiotic stress that plants must cope in their environments (Boyer, 1982). Crop growth and photosynthesis are among the primary processes affected by a reduced availability of water in the soil (Kriedemann and Smart, 1971; Liu et al., 1978; Matthews et al., 1978; Chavez and Rodriguez, 1987; Schultz and Matthews, 1988; Chaves, 1991, Schultz, 1996; Flexas et al., 1999; Maroco et al., 2002; Chaves et al., 2003; Cifre, 2005; Blum 2011), with deleterious effects on yield and grape quality (Kliewer et al., 1983, Bravdo et al., 1985; Matthews and Anderson, 1989), in relation to short-term and long-term water deficit (Winkel and Rambal, 1993; de Souza et al., 2003; dos Santos et al., 2003).

The relationship between soil water content, leaf water status and gas-exchanges can be useful tools to improve water use efficiency in vineyard to optimize yield and grape quality (Cifre et al., 2005; Flexas et al., 2004). Intensity, duration and rate of occurrence of water stress, which strictly depend on how soil water content is depleted during the growing season, are crucial aspects influencing plant responses (Chaves et al., 2009).

The soil water content (θ) and the atmospheric conditions (i.e. the evapotranspirative demand) determine the plant water status during the day, as assessed by the leaf water potential (Williams et al., 1994). In our two-year experiment, an “Aglianico” vineyard was grown in two clay-loam soils, which were characterized by different structure. As a consequence, water availability (the capacity to retain water) was higher in the Eutric Cambisol (CAM) soil than Cambic Calcisol (CAL). Soil water was consumed by the vines through transpiration, and by (the negligible) amount of water, which evaporated directly from the soil surface. θ gradually decreased along the growing seasons but more in CAL than in CAM site, and the occurrence of few significant rainfalls recovered the soil water availability (Fig.1). Leaf water status was strictly affected by soil moisture. This resulted in both a gradual worsening of vine water status, as assessed by Ψ_1 decreasing during the season, more in CAL than

CAM, and by a recovering as an effect of rainfalls (cf. Fig.1 A, B and Fig.1 C,D). Williams et al. (1994) reported in “Thompson Seedless” grapevine that early morning leaf water potential measured in mid-summer was about -1.2 and -0.5 MPa in soil provided with very low or with high soil water content respectively. In our case, we measured in late summer (early morning) Ψ_1 values of -1.67 MPa in the first year and -1.61 MPa in the second year in CAL, and -1.36 and -1.43 MPa in CAM leaves. On the other hand, in late spring of both years, leaf water potential in CAL and CAM vines was as high as about -0.7 MPa (Fig. 1, C, D), similarly to the slightly stressed vines reported by the authors above mentioned. Moreover, our data are quite identical to those reported for two years by Centeno et al. (2010) in a 13-year old “Tempranillo” vineyard grown under rain-fed conditions in Spain. In fact, they found that Ψ_1 gradually decreased from about -0.8 MPa in late spring to about -1.7 MPa in late summer. Therefore, in both the two soil sites vines gradually reached a condition of water stress typical for the stressful summers of the Mediterranean area.

As reviewed by Chaves (2010), most grapevine genotypes can be classified as “drought avoiding”, because under soil water deficit conditions – or in stressful atmosphere – a strong control is exerted on stomatal closure in order to limit water loss through transpiration. In other words, these genotypes show a “pessimistic” comportment as regard the expectance for future rainfalls and soil water availability and therefore show a (near) isohydric behavior because their responses to water deficit results in leaf water potential tending to remain high. On the other hand, other cultivars have been reported to have an “optimistic” behavior as they exert less control on stomatal conductance, which tends to remain high, whereas leaf water potentially drops more, resulting in an anisohydric condition. Giorio et al. (2007) reported for the cv “Falanghina” grown in two soils with different water availability in the “Telesina” Valley (BN, Italy) a near isohydric behavior. In fact, midday leaf water potential during the dry and hot stressful summer conditions (July and August), ranged from about -1.4 to -1.5 MPa (in both the two soil sites) while stomatal conductance decreased from about 0.6 to a value as low as 0.1 mol m⁻² s⁻¹. Similarly,

Schultz (2003) reported for vineyards grown in France, near isohydric behavior for the cv “Grenache” and anisohydric behavior for the cv “Syrah”. *Grenache* showed midday leaf water potential decreasing during spring and summer with no differences between the irrigated control and the (rain-fed) water stressed treatment, whereas stomatal conductance was significantly lower in the stressed than control vines. Conversely, *Syrah* maintained significant differences between the two treatments for both Ψ_1 and g_s . It is worth to note that both the general trends and the minima reached in late summer reported in Shultz (2003) are pretty comparable with our measurements of Ψ_1 and g_s . In our case, *Aglianico* showed large differences between the two soil sites as regard stomatal conductance, indicating water saving (pessimistic) behavior. However, the moderate differences in leaf water potential between CAL and CAM, indicating a certain degree of isohydric behavior, were more evident in the second as compared to the first year (cf. Fig.1 C and Fig.2A, cf Fig. 1 D and Fig. 2 B).

The relative importance of stomatal and non stomatal limitations to photosynthesis of plant under water stress have been widely studied, and debated as regard the occurrence of the numerous components in relation to intensity of water deficit (Flexas et al., 2004). Physiological responses of grapevine to water stress and the effects on the photosynthetic performances have been detailed discussed as a means for irrigation scheduling, aimed to improve vineyards water use efficiency (Cifre et al., 2005). The approach of these authors was to adopt light saturated maximal stomatal conductance as an integrative parameter for assessing intensity of water deficit in order to predict and improve vineyards water use efficiency. The authors indicated specifically for grapevines, three phases of water stress on the basis of three g_s ranges: from 0.6 to 0.15, from 0.15 to 0.05 and lower than 0.05 mol m⁻²s⁻² for mild, moderate and severe water stress, respectively. The hyperbolic relationship between A and g_s (Farquhar and Sharkey, 1982) imposes the relationship of intrinsic water use efficiency (A/g_s) vs. g_s . Such a model implies that in the first phase A/g_s slightly increases (as A decreases less than g_s) and C_i decreases because stomatal closure

induces CO₂ shortage in the mesophyll where no effects are yet occurring on the photosynthetic machinery. In the second phase A/g_s increases more steeply, meanwhile Ci still decreases. Electronic transport rate (ETR) is still sustained by photorespiration, despite decreased CO₂ fixation, while increased thermal dissipation of excitation energy (non photochemical quenching, NPQ) is observed. Moreover, also the mesophyll conductance can be affected under moderate water stress. During the third phase, when water stress is severe (g_s < 0.05 mol m⁻² s⁻¹), A/g_s starts a steep decrease, as A is being reduced much more than g_s. The severe water deficit conditions also induce a further decrease in NPQ and a reduction of ETR (both A and photorespiration are impaired). At metabolic level, reductions in both CO₂ carboxylation of Rubisco and RuBP regeneration are observed, the latter due to impairments of photochemistry efficiency. Under these conditions a steep increase in Ci is observed. In fact, the reduced diffusion of atmospheric CO₂ through stomata as well as the mesophyll diffusion are overridden by the impairments of photosynthetic machinery, with increased ratio between photorespiration and carboxylation, resulting in an increased intracellular CO₂ concentration. Under these conditions, photosynthesis may not quickly recover upon irrigation or rainfalls. Therefore, under mild water stress, limitations to photosynthesis are due to the regulation of stomatal closure. Conversely, non-stomatal limitations are dominant under severe stress conditions. However it is also known that under field conditions characterized normally by long-term gradual imposition of water stress the probability of non-stomatal limitations is not much high (Chaves 2012).

As regard the environmental conditions experienced by our *Aglianico* vines in the first year, the variations in stomatal conductance during the season were reflected by a similar behavior of assimilation (Fig. 2 A, B, C, D). It is worth to note the amelioration of stomatal conductance and assimilation during spring. One reason of this could be the ontogenetic effect on gas exchange during leaf development (Gucci, 1997; Xien and Luo, 2003). However, since DOY 182 (starting of July) g_s in CAL vines showed a reduction higher than A, leading to an increase of intrinsic water use

efficiency until end of August (Fig. 2 E). With the occurrence of a rainfall, g_s recovered more than A, and as a consequence A/g_s steeply decreased. Conversely, CAM vines showed a much stable pattern of A/g_s along the season with a moderate increase quite late in the season (DOY 252, early September). However, this increase was caused by a substantial recovering for A, and not for g_s , in response to a rainfall. As regard the second year, a rainfall of 61 mm occurred in July, and it was heavy enough to induce the recovering of soil moisture as well as of plant water status in both CAL and CAM. In fact, θ and Ψ_1 rose to same values in both CAL and CAM sites (cf. Fig. 1 B and D). The plant response to water deficit followed what expected by the Cifre model but with some differences. Until late July (around DOY 200), both A/g_s and C_i remained almost stable in the two theses as like the previous year (Cf. Fig. 2 F and Fig. 2 E, Cf Fig. 2 G and Fig. 2 H). Interestingly, during this period for both years, Ψ_1 never dropped below -1.5 MPa (Fig. 1 D), therefore inducing similar responses of gas exchange (Fig. B). Afterwards, conversely to the first year, mitigation of soil water deficit occurred in mid-summer due to the heavy rainfall (DOY 205, Fig. 1 B). During next 10 days, in contrast to the previous year, high stressful atmospheric conditions (highly evaporative-demanding atmosphere) were imposed to vines when soil water deficit was strongly recovered (Fig. 1, B). Therefore, the rate of g_s reduction was quite enhanced (Fig. 2 B) resembling the effect of a short-time intense water stress experiment. Those conditions may have partially modified stomatal response to drought in the second year as compared with this first year. Actually, in the second year the differences in both A/g_s and C_i between the two soil-site vines resulted mitigated (from late July to late August, from DOY 205 to 242, Fig. 2, F) as compared with the previous year. The relationships of both assimilation and water use efficiency *versus* g_s for the two years (Fig. 6, A, B) summarize the dynamic of gas exchange response with the occurrence of water stress, which is indicated by variation of the integrative parameter of stomatal conductance (Cifre, 2005). It is quite interesting to note that, during first year, vines of both CAL and CAM soils did not get into the above-described third phase, because

there were no drops in A/g_s at low g_s values. Moreover, the more stressed CAL vines, showed higher A/g_s at low g_s than CAM, therefore approaching the end of the second phase more than CAM vines (Fig. 6 A). As regard the second year (Fig. 6 B), vines in both sites reached lower g_s at pretty high A/g_s . However, CAL vines were the sole to enter the third phase characterized by severe water deficit, as showed by A/g_s decreasing to low values at low g_s around DOY 251 (Fig. 6 B, Fig. 2 F).

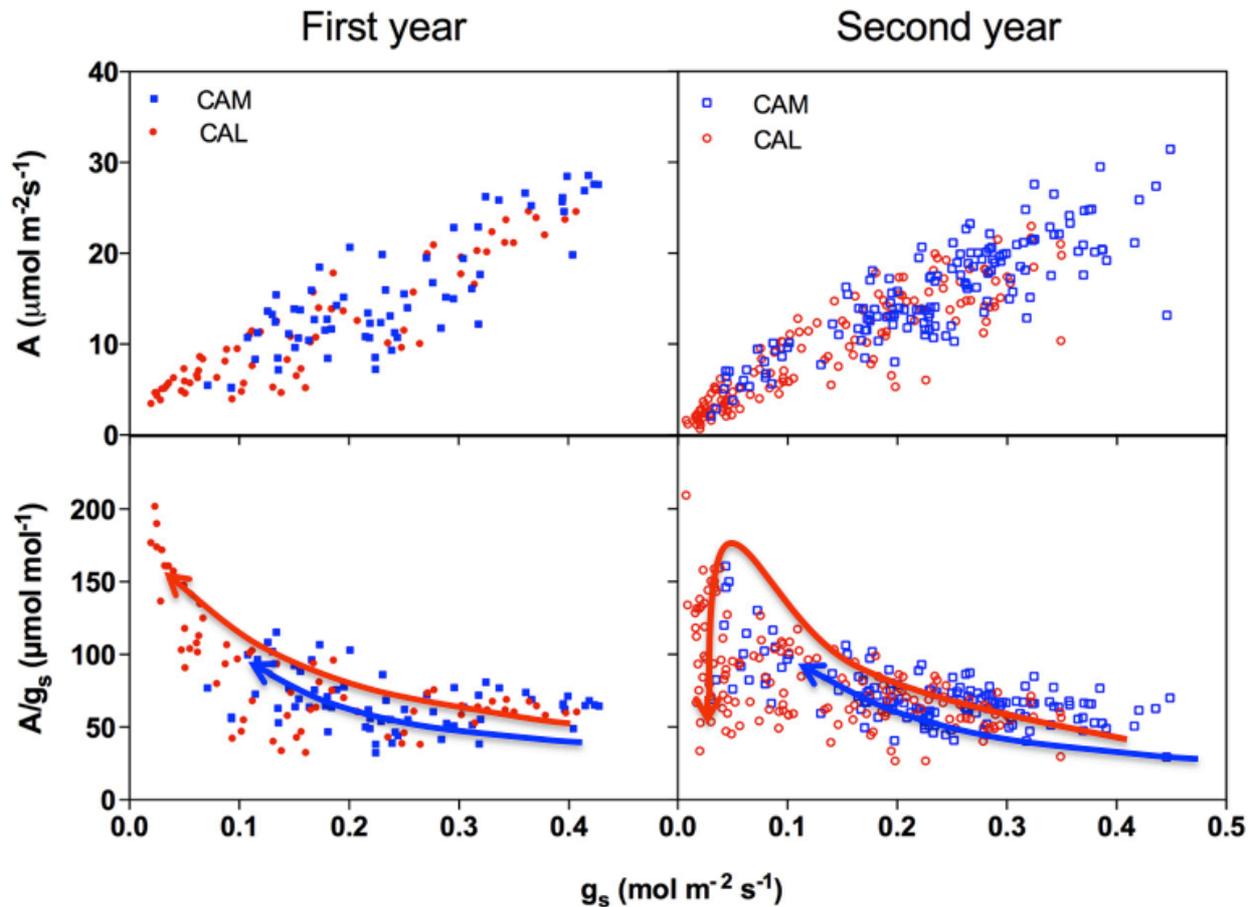


Fig. 6 Relationships of intrinsic water-use efficiency (A/g_s) and assimilation (A) with stomatal conductance (g_s) in grapevines (*V. vinifera* L.) in first and second year of trials. All data are single values, taken from 10 to 13 h at saturating light intensity. Arrows indicate the dynamics of temporal occurrence during the experiment.

Zsófi et al. (2009) on the basis of the Cifre approach analyzed the response of the grape variety “Kèfrankos” in two sites with higher and lower soil water availability in the continental environment in Hungary. Their grapevines also showed a clear separation between the two theses, but never entered the third water stress phase. However, the authors indicated the thresholds of stomatal conductance of 0.23 and 0.06 $\text{mol m}^{-2} \text{s}^{-1}$ for the passage into the second and third phase, respectively. In our case, it was quite difficult to clearly select the threshold for entering in the second

phase, without additional information. However, CAL vines entered the third phase in the second year when stomatal conductance was about $0.04 \text{ mol m}^{-2} \text{ s}^{-1}$.

Our data of photochemistry efficiency confirmed that CAL vines in late summer of second year were submitted to severe water stress implying the occurrence of non-stomatal limitations to photosynthesis. In fact, in that period we observed a clear difference in both F_v/F_m and Φ_{PSII} (Fig. 3 B, D) at g_s in CAL of about $0.04 \text{ mol m}^{-2} \text{ s}^{-1}$. Flexas et al. (2004) actually reviewed that F_v/F_m is decreased at stomatal conductance below a value of about $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$, fairly similar to what occurred in our case. Fanizza et al. (1991) submitted several grapevine cultivars to one month water stress, resulting in Ψ_1 of about -1.55 MPa , compared with -0.8 MPa of the Control. These authors found that total chlorophyll content of about $45 \mu\text{g cm}^{-2}$ in the control decreased to about $35 \mu\text{g cm}^{-2}$ in the stress vines. They also found (unusually) a linear relationship between the optical chlorophyll index and the actual leaf Chl content. Therefore, their data are comparable with our results (Fig. 4 A, B), which confirm the damaging effects on the photosystems, affecting the photosynthetic performances (e.g. Scotti et al. 2015).

The capacity of vineyards to accumulate dry matter depends on the whole-canopy net photosynthesis, which in turns is determined by the canopy leaf area and its spatial distribution, which affects light interception, and on the ability to use it and to assimilate CO_2 through leaf photosynthetic performances (Kliewer and Dokoozlian, 2005). The *Aglianico* vineyards here studied were submitted to both green pruning and fruit thinning in order to achieve the best equilibrium between vegetative and reproductive growth, aiming to obtain grape and then wine of best quality. In fact, LAI measured in spring, mid- and late-summer was quite stable in both the two sites and in both the two years, with the exception of CAM vines in mid summer of second year (Fig. 5 A, B). These data confirm the strong control exerted by the grower on the vegetative growth, and the same occurred for grape load left on the vines (data not shown). The grape yield (data not shown) and quality of grape and wines were better

in vines of CAL than CAM site. This occurred to several parameters of grape and wine quality as reported in Tab. 1 (data from A. Gambuti, unpublished data). It is worth to note that such difference were stronger in the first as compared with second year, in which CAL vines encountered severe water stress during mid summer.

	First year		Second year	
	CAL	CAM	CAL	CAM
Must soluble solids (°Brix)	23.5	21.6	23.6	22.2
Titrateable acidity (g/L)	7.3	6.8	5.4	6.9
Total anthocyanins (mg/kg)	626.4	455.4	695.8	522.4
Total phenolics (mg/kg)	2347.5	1485.0	1723.5	1747.5

Table 1 Results of the experimental must and wine analyses from the vineyards in each year.

4.6 Conclusions

The different hydrological properties of the two soils significantly affected the dynamics of the soil moisture available to the vines from spring to late summer in a two-year experiment. A gradual water stress was imposed to vines as soil moisture was consumed through evapotranspiration by the vineyards. However, vine water status was more affected in CAL site, as showed by the more sustained decrease in leaf water potential as compared with CAM vines. Recovering in both soil water content and leaf water status occurred as an effect of rainfalls. Stomatal conductance and photosynthetic CO₂ assimilation followed the trends showed by leaf water status. Analyses of the dynamic of intracellular CO₂ concentration, and the relationship between intrinsic water use efficiency (A/g_s) and g_s , the latter being an integrative parameter of water stress, reveal information leading to relevant conclusions. The long-term gradual imposition of soil water deficit under field condition resulted in a mild to moderate intensity of water stress imposed to the vines. Grapevines succeeded to bear water stress conditions by controlling water loss through stomatal closure. This significantly reduced photosynthetic assimilation, yet without significant impairments of photosynthetic machinery. Nevertheless, in the second year a severe water stress was imposed to CAL vines in a short-term period. In this

case, A/g_s vs. g_s and the dynamic of C_i indicate the occurrence of non-stomatal limitations to photosynthesis. Moderate water deficit reduced yield but improved berries quality especially in the first year.

General Conclusions

Plant responses to water and salt stress were investigated in five experiments along three years. Plants were grown in pots on soil (tomato) or on a soilless system (pepper) in glasshouse, or under open-field conditions (grapevine).

Two Italian long-term storage tomato landraces, *Locale di Salina* and *Pizzutello di Sciacca* were submitted to rapid, intense water stress by withdrawal of irrigation and recovering by re-watering. Two genotypes of sweet pepper, *Cazzone Giallo* and *Quadrato d'Asti*, were submitted to long-term stress at several level of salinity. *Aglianico* grapevines experienced long-term water deficit in soils with high or low capability of water retention.

Morphological, physiological and biochemical responses to those different types, intensities and dynamics of abiotic stress were analysed to investigate the underlying plant response mechanisms.

The two tomato landraces both showed a clear response to the imposed intense soil water deficit by regulation of stomatal closure and photosynthetic activity, and recovering upon re-watering. Physiological responses were strictly linked to biochemical mechanisms of response to water stress as both ABA and proline increased concomitantly with severe stress conditions. The activity of PARP was influenced by both stress imposition and ABA response. Although future confirmation is needed at the field level, the data revealed that the two landraces showed a wide physiological and biochemical plasticity to deal with water stress.

Pepper genotypes under long-term salt stress showed great impairment of water status, and accumulated significant amount of both Na^+ and Cl^- in stems and leaves. Photosynthetic assimilation and photochemistry at high levels of salinity were dramatically impaired throughout two distinct phases. Stomatal closure dominated

photosynthetic limitations during first 30-40 days of salt stress imposition. Conversely, afterwards non-stomatal limitations had a relevant role with the probable significant contribution of specific ion toxic effects. Moderate salinity did not affect physiological parameters in both genotypes. In several physiological aspects and in marketable yield response the genotype *Cazzone Giallo* resulted more salt sensitive than *Quadrato d'Asti*.

The different hydrological properties of the two soils significantly affected the dynamics of the soil moisture available to the vines of *Aglianico* from spring to late summer. A gradual water stress was imposed to vines as soil moisture was consumed through evapotranspiration by the vineyards. However, vine water status was more affected in the soil site with lower capability of water retention, as showed by the more sustained decrease in leaf water potential as compared with the vines grown in the soil site with high water retention. Recovering in both soil water content and leaf water status occurred as an effect of rainfalls. Stomatal conductance and photosynthetic CO₂ assimilation followed the trends showed by leaf water status. Analyses of the dynamic of intracellular CO₂ concentration, and the relationship between intrinsic water use efficiency (A/g_s) and g_s , the latter being an integrative parameter of water stress, reveal information leading to relevant conclusions. The long-term gradual imposition of soil water deficit under field condition resulted in a mild to moderate intensity of water stress imposed to the vines. Grapevines succeeded to bear water stress conditions by controlling water loss through stomatal closure. This significantly reduced photosynthetic assimilation, yet without significant impairments of photosynthetic machinery. Nevertheless, when a severe water stress was imposed in a short-term period to vines grown on soil with low water retention, A/g_s vs. g_s and the dynamic of C_i indicated the occurrence of non-stomatal limitations to photosynthesis. Moderate water deficit reduced yield but improved berries quality especially in the first year.

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