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ORIGINAL ARTICLE

Olive (*Olea europaea* L.) plants transgenic for tobacco osmotin gene are less sensitive to in vitro-induced drought stress

Cristian Silvestri[1] · Silvia Celletti[1] · Valerio Cristofori[1] · Stefania Astolfi[1] · Bruno Ruggiero[1] · Eddo Rugini[1]

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Abstract Olive is one of the most important tree crops in the Mediterranean region, because of its ability to grow and produce acceptable yields under limited water availability. In this study, the drought tolerance of an olive cultivar Canino was compared to the performance of its derived transgenic line expressing *osmotin* gene from tobacco, obtained by *Agrobacterium*-mediated transformation of Canino cultivar. Shoot cultures of both wild-type (*wt*) and transgenic lines were exposed to drought stress over a 28-day period, and their differential responses to in vitro-drought stress were investigated. After exposure to PEG, most of the shoots from *wt* plants resulted in damage and exhibited decreased levels of chlorophyll, while those of transgenic line did not show injuries and showed a normal growth even when exposed to the highest PEG concentration (4%). After preliminary evaluation we characterized Canino *AT17-1*, by measuring several physiological parameters, including the activities of the antioxidant enzymes (POD and CAT), and the content of malondialdehyde (MDA). Both the activity of catalase and the proline content were higher in the leaves of the transgenic shoots compared to *wt* plants. Consequently, it was observed that the transgenic line accumulated less MDA indicating that the presence of the *osmotin* gene protected the cell membrane from damage by lipid peroxidation. Together, these results could suggest that the transgenic line Canino *AT17-1* was more efficient in the activation of defense responses against oxidative stress with respect to the Canino *wt*. The further finding that the transgenic shoots also showed higher proline accumulation supported the hypothesis that the *osmotin* gene conferred to transgenic shoots increased tolerance to drought stress compared with the *wt*.

Keywords *Olea europaea* L. · Drought-stress tolerance · *Osmotin*-transgenic olive · Proline accumulation · Lipid peroxidation

Introduction

Growth and development of plants mainly depends on the availability of water and nutrients in their habitat (Tilman 1997). Changing climate conditions are particularly concerning for agricultural production as it causes low-resource environments (Seki et al. 2003; Farooq et al. 2011). In particular, rainfall patterns and level of precipitation are projected to change in a manner unfavorable for plant growth, e.g., more wet winters and dryer periods during crop growth.

Drought stress is becoming particularly widespread in many regions resulting in 30% land loss by the year 2021 and more than 50% by 2050 (http://faostat.fao.org/).

Therefore, a better understanding of how plants cope with suboptimal water seems extremely important to develop novel strategies to improve plant water-use efficiency.

Olive (*Olea europaea* L.) plants are naturally grown in arid and semi-arid areas; however, their capability to cope with drought stress is of importance from an economical point of view, since drought stress may affect fruit production and quality (Rejšková et al. 2007).

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✉ Cristian Silvestri
silvestri.c@unitus.it

[1] Department of Agriculture and Forestry Sciences (DAFNE), University of Tuscia, Via San Camillo de Lellis s.n.c., 01100 Viterbo, Italy

Abiotic stresses, such as drought stress, cause serious damage to plants, resulting in reduced growth and huge yield losses (Oerke 2006). Therefore, plants undergo a complex stress response, through signal transduction originating from environmental stimuli, including morphological and physiological adaptations (Pereira et al. 2012).

One of the first consequences of plant exposure to water-deficit conditions is the production of active oxygen species. Therefore, the induction of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activity plays an important role in adaptation of plants to changes in environmental conditions (Candan and Tarhan 2012).

Adaptive response to drought stress is commonly associated with the accumulation of osmotin and osmotin-like proteins (Veronese et al. 2003). Osmotin belongs to the pathogenesis-related (PR) type-5 protein family (Anil Kumar et al. 2015; Van Loon 1997), it not only accumulates in defense responses against pathogens but also in response to abiotic stresses, such as drought and salinity (Singh et al. 1989). High levels of PR-5 were detected in tobacco leaves exposed to salt stress (Van Loon and Kammen 1970; Singh et al. 1989). In vivo transgenic expressing osmotin suggests that osmotin is able to protect chlorophyll and photosynthetic pathways under drought stress, as reported for tobacco (Barthakur et al. 2001) and tomato (Goel et al. 2010).

It has been reported that the *osmotin* over-expression in potato, tobacco, tomato, mulberry and chilli pepper plants enhanced plant tolerance to water deficit and this response could be most likely attributed to increased proline accumulation (Evers et al. 1999; Barthakur et al. 2001; Al-Khayri and Al-Bahrany 2004; Sokhansanj et al. 2006; Goel et al. 2010; Das et al. 2011; Subramanyam et al. 2011). Free proline is accumulated in plants during drought and salt stresses playing the roles of osmotic adjustment, protection of cellular macromolecules and scavenging of hydroxyl radicals (Kishor and Sreenivasulu 2014).

The aim of the paper was to investigate the role of osmotin in drought response and acclimation in olive plants by comparing wt plants and transgenic plants expressing the osmotin gene driven by the 35S promoter.

Drought-stress conditions were achieved in vitro by adding to the proliferation medium exogenous PEG (polyethylene glycol 8000) with concentrations ranging from 0 to 4% (Dami and Hughes 1995). First, preliminary morphological and physiological tests were conducted on the transgenic line Canino AT*17-1* to establish possible differences in the sensitivity degree to drought. In particular, we investigated whether the presence of *osmotin* gene triggered changes in the activities of the antioxidant enzymes (POD and CAT), and the content of malondialdehyde (MDA).

The results of this work shed new light on the efficacy of the transgenic approach for improving drought tolerance in olive, a highly economically valuable plant.

Materials and methods

Selection of transgenic lines expressing osmotin gene

Shoots of the transgenic line Canino AT*17-1*, derived from somatic embryogenesis were proliferated for many years on Olive Medium (Rugini 1984) supplemented with 1 mg/L zeatin trans-isomer and 1 mg/L gibberellic acid (proliferation medium), with monthly subcultures. The somatic embryos were obtained through a "double regeneration technique" (Rugini and Caricato 1995; Rugini and Silvestri 2016; Rugini et al. 2016) and subsequently transformed via *Agrobacterium tumefaciens*, strain LBA 4404, plasmid pK YLX71, carrying the tobacco *osmotin* gene and *NPTII* gene, under control of *35S* promoter, as previously described by Rugini et al. (1999).

Before starting the present experiment, the shoots were tested again for kanamycin resistance, at 150 mg L^{-1} on the proliferation Olive medium, and the presence of the transgenic genes in their DNA were ascertain.

Olive genomic DNA was extracted from young leaves (150 mg of fresh tissue) using a CTAB (cetyl-trimethyl-ammonium bromide)-based method (Doyle and Doyle 1990). DNA concentration and quality were determined by 1% agarose gel electrophoresis and using a Nanodrop Bioanalyzer ND1000 (ThermoScientific). Then, the transformants were screened for the transgene by polymerase chain reaction (PCR) using the osmotin-specific primers Osm-F (5′-CCAACAACCCAACTTGTTAAAA-3′) and Osm-R (5′-CGACAGAATAATTTGACCAAAAG-3′). The PCR conditions were: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 80 s, and final extension at 72 °C for 10 min. The reaction products were separated electrophoretically on 1.5% (w/v) agarose gel, stained with ethidium bromide, and photographed.

Growing conditions

Three 250-mL jars each containing ten single-node explants were sub-cultured in 50 mL of proliferation medium as above described and supplemented with three different concentrations of polyethylene glycol 8000 (PEG) (0, 1, 2 and 4%). Concentrations of PEG were selected on the basis of previous experiments to avoid lethal doses (data not shown). Explants were kept under controlled conditions in a growth chamber with a day/night cycle of 16/8 h at 24 ± 1 °C air temperature, 80% relative humidity, and

40 μmol m^{-2} s^{-1} light intensity. Data collection was performed after 28 days in culture.

Plant growth parameters

Fresh weight, shoot length and node number were recorded at the end of the culture period. Dry weight of the shoots was determined by heating the samples at 105 ± 2 °C until constant weight.

The measurements of chlorophyll content per unit area were estimated in attached leaves with a non-destructive portable apparatus, the SPAD chlorophyll meter (Minolta Co., Osaka, Japan), using the youngest fully expanded leaf from the top of each plant and presented as SPAD units.

Proline accumulation

Samples of freshly harvested leaves (100 mg of fresh weight) were collected and proline concentration was determined by spectrophotometer according to Bates and Waklren (1973). Briefly, for colorimetric determination based on proline's reaction with ninhydrin, a 1:1:1 solution of proline, ninhydrin acid and glacial acetic acid was incubated at 100 °C for 1 h. The reaction was arrested in an iced bath and the chromophore was extracted with 4 mL of toluene and the absorbance at 520 nm was determined with a spectrophotometer EVO 60 (Thermo Fischer Scientific Inc.).

Enzymes from whole shoot extracts

Reactive oxygen species-scavenging enzymes were determined in total crude shoot (0.5 g of fresh tissue) extracts obtained according to the method described by Santangelo et al. (2003).

Guaiacol peroxidase (E.C. 1.11.1.7) activity was measured spectrophotometrically at 470 nm using guaiacol as hydrogen donor (Santangelo et al. 2003). Catalase activity (E.C. 1.11.1.6) was evaluated by measuring the decrease in absorbance at 240 nm due to decomposition of H_2O_2, as described in Santangelo et al. (2003). All reported enzyme activities were linear with time and proportional to the amount of extract used. Protein content was estimated according to Bradford (1976), using BSA as standard.

Determination of malondialdehyde content

The level of lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as TBA reactive metabolites according to Astolfi et al. (2005). Briefly, fresh shoot tissues (0.2 g) were homogenized in 10 mL of 0.25% TBA made in 10% TCA. Extract was heated at 95 °C for 30 min and then quickly cooled on ice. After centrifugation at 10,000×*g* for 10 min, the absorbance of the supernatant was measured at 532 nm. Correction of non-specific turbidity was made by subtracting the absorbance value taken at 600 nm. The level of lipid peroxidation was expressed as μmol g^{-1} fresh weight using an extinction coefficient of 155 mM cm^{-1}.

Statistical analysis

Each reported value represents the mean ± SD of measurements carried out from three independent experiments. All the data were statistically analyzed using one-way ANOVA with the GraphPad InStat Program (version 3.06). Significant differences were established by post hoc comparisons (Fisher's LSD) at $P < 0.01$.

Results

Test for transgenic lines expressing osmotin gene

The transgenic line Canino *AT17-1* showed resistance to kanamycin as well as the transgenic somatic embryos from which the shoots originated. On the other hand, Canino *wt* shoots, grown at the same kanamycin concentration, exhibited an inhibition of the axillary bud growth which turned yellow in color (Fig. 1), indicating the absence of the selectable genes. In addition, PCR analyses confirmed the presence of the target gene in shoots of transgenic line Canino *AT17-1* (Fig. 2). Furthermore, the transgenic line Canino *AT17-1* had just been subjected to in situ localization of tobacco osmotin protein, showing the osmotin labeling in the epidermal and sub-epidermal tissues in the transgenic cells (D'Angeli et al. 2001).

Effect of PEG-induced drought stress in in vitro shoot growth

In the control medium (without PEG addition), the shoot height and the node number resulted significantly higher in the Canino *wt* compared to transgenic clone Canino *AT17-1*, while no difference in terms of mean internode length was found (Table 1).

The shoots of *wt* line grown in vitro showed several symptoms of damage, included decreased growth and necrotic leaves following exposure to increasing concentrations of PEG (ranging from 1 to 4%). In contrast, the addition of PEG did not affect most shoot growth parameters of Canino *AT17-1* (Table 1).

In particular, after PEG exposure the shoot length of Canino *wt* was drastically reduced (by 44, 67 and 81% at 1, 2 and 4% PEG, respectively), as the mean node number,

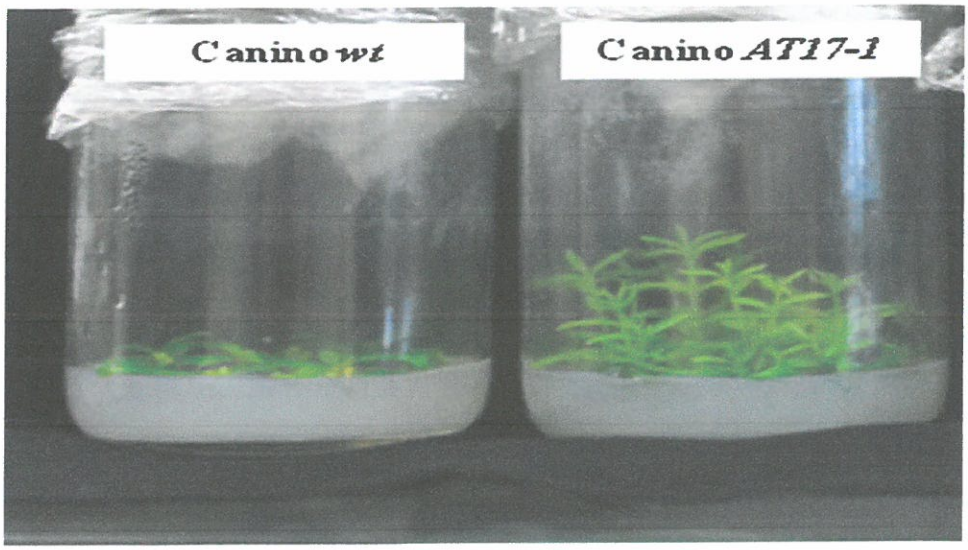

Fig. 1 Canino *wt* (control) and transgenic Canino *AT17-1* growing on selection medium enriched with kanamycin (150 mg L^{-1})

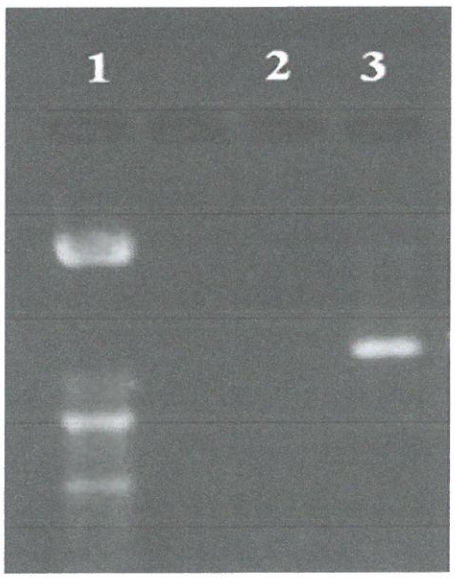

Fig. 2 Analysis of tobacco osmotin gene integration in olive transgenic shoots: (1) molecular weight marker, (2) PCR products of Canino wt (control), (3) PCR products of transgenic clone Canino At 17-1

which was strongly reduced by increasing PEG concentrations (27, 42 and 67% at 1, 2 and 4% PEG, respectively).

In the same way, after PEG exposure the shoot length of Canino *AT17-1* was reduced (by 22, 39 and 47% at 1, 2 and 4% PEG, respectively) as the mean node number, which was strongly reduced by increasing PEG concentrations (14, 28 and 24% at 1, 2 and 4% PEG, respectively), but the reduction of growth traits was significantly lower than in the control Canino *wt*.

The transgenic line seems to be less affected by PEG-stress treatments. In particular, at all PEG concentrations tested (1, 2 and 4%) the shoot height and internode number are significantly reduced than the control, but no difference was found among PEG treatments. In terms of mean internode length, the transgenic clones did not show significant differences with respect to the control medium (no PEG addition). All the genotypes tested showed a significant increase in fresh/dry weight ratio when the media were supplemented with PEG at various concentrations.

Proline and chlorophyll content

The proline contents measured in the shoots of Canino *AT17-1* were greater than in those of Canino wt, even in the control condition (absence of PEG) (Fig. 3).

The shoots of Canino *wt* showed a significative proline increase in the presence of PEG 1 and 2%, while the further increase up to 4% resulted in a decrease of proline accumulation (Fig. 3).

In contrast, each increase in PEG concentration in the medium was not followed by an increase of proline content in the shoots of transgenic line subjected to 1 and 2% of PEG, but this value increase markedly at the higher concentration (PEG 4%).

After the 28-day exposure to PEG, Canino *wt* shoots showed typical symptoms of leaf chlorosis. In particular, chlorophyll concentration progressively dropped with increasing PEG concentration in the medium resulting in lower SPAD values compared to control treatment (no PEG addition). In contrast, exogenous PEG addition did not affect chlorophyll content in shoots of transgenic line compared to control, as shown in Fig. 4.

Table 1 Growth parameters—shoot length, node number, internode length, and fresh and dry weight ratio of Canino *wt* and transgenic clone Canino *AT17-1*

Clone	PEG 8000 (% w/v)	Shoot length (cm)	Node number	Internode length (cm)	fv/dw ratio
Canino *wt*	0	20.30 (±2.4)a	20.1 (±1.3)a	0.99 (±0.13)a	0.18 (±0.01)a
Canino *wt*	1	11.44 (±2.3)b	14.7 (±1.6)b	0.81 (±0.12)b	0.24 (±0.02)b
Canino *wt*	2	6.70 (±2.0)c	11.7 (±2.2)b	0.64 (±0.11)c	0.25 (±0.01)b
Canino *wt*	4	3.91 (±0.5)c	6.6 (±1.9)c	0.57 (±0.15)c	0.25 (±0.02)b
Canino *At17-1*	0	11.57 (±1.2)A	14.5 (±0.9)A	0.79 (±0.12)A	0.18 (±0.02)A
Canino *At17-1*	1	9.03 (±1.0)B	12.4 (±0.8)B	0.73 (±0.17)AB	0.22 (±0.02)B
Canino *At17-1*	2	7.05 (±0.8)B	10.5 (±1.1)B	0.67 (±0.10)AB	0.22 (±0.02)B
Canino *At17-1*	4	6.15 (±0.9)B	11.0 (±1.4)B	0.56 (±0.10)B	0.24 (±0.02)B

Data are the means (±SD) from three independent experiments. Different letters (lowercase letters for Canino wt and uppercase letters Canino *AT17-1*) above the data indicate significantly different means as determined by Fisher's LSD at $P < 0.01$

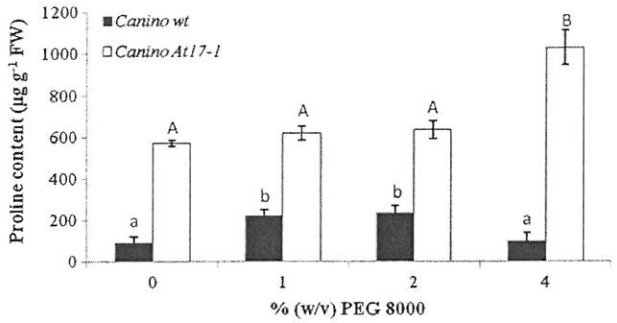

Fig. 3 Proline content in olive genotypes in vitro grown on media supplemented with different concentration of PEG 8000. Data are the means (±SD) from three independent experiments. Different letters (lowercase letters for Canino wt and uppercase letters Canino *AT17-1*) above the data indicate significantly different means as determined by Fisher's LSD at $P < 0.01$

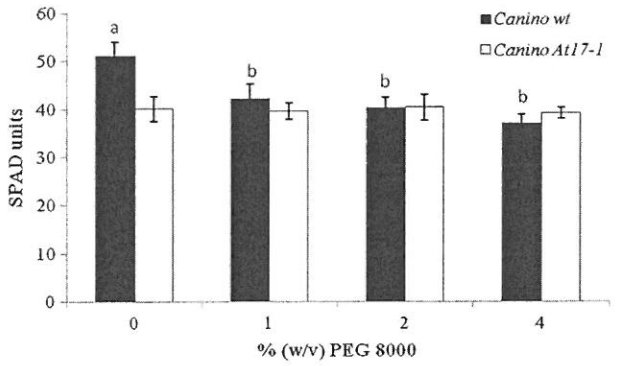

Fig. 4 Chlorophyll content, measured as SPAD units, in olive genotypes in vitro grown on media supplemented with different concentration of PEG 8000. Data are the means (±SD) from three independent experiments. Different letters (lowercase letters for Canino wt and uppercase letters Canino *AT17-1*) above the data indicate significantly different means as determined by Fisher's LSD at $P < 0.01$

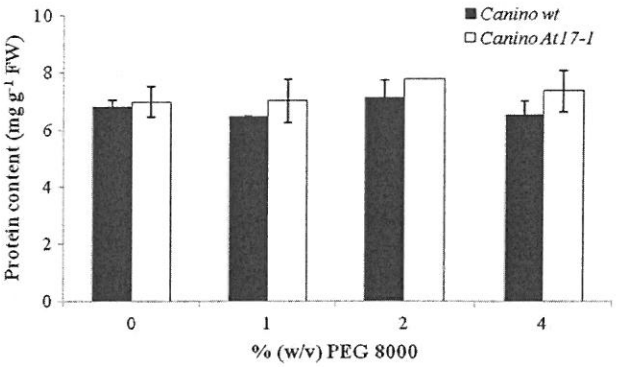

Fig. 5 Protein content (mg g^{-1} FW) in shoots of olive genotypes (Canino wt and Canino *AT17-1*) grown in vitro on media supplemented or not with PEG 8000. Data are the means (±SD) from three independent experiments. Different letters (lowercase letters for Canino wt and uppercase letters Canino *AT17-1*) above the data indicate significantly different means as determined by Fisher's LSD at $P < 0.01$

Total soluble protein content and antioxidant enzyme activity

To investigate the changes induced by PEG treatment, we measured the protein contents and antioxidant enzyme activities in the shoots of *wt* and transgenic line Canino *AT17-1*.

No significant difference in shoot protein concentration was observed in both *wt* and transgenic line upon PEG treatment (Fig. 5). However, several differences were evident between the two genotypes when antioxidant enzymes were considered (Fig. 6).

Antioxidant enzyme activities, such as peroxidase and catalase, were determined in control and PEG-treated shoots from Canino *wt* and transgenic line Canino *AT17-1* plants. Under control conditions, the basal activity of both enzymes was clearly higher in Canino *wt* leaves than in those of Canino *AT17-1* (60% and threefold higher for

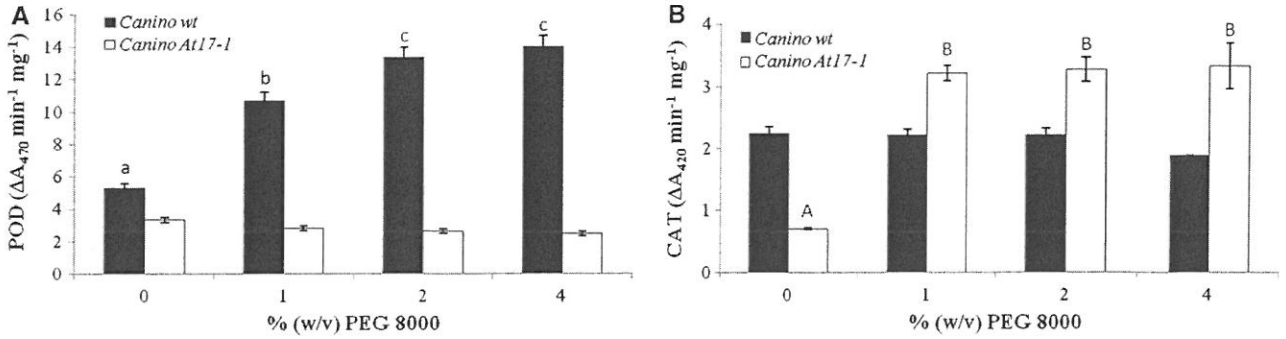

Fig. 6 Changes in guaiacol peroxidase activity (**a**) (ΔA_{470} min^{-1} - mg^{-1} prot) and catalase activity (**b**) (ΔA_{240} min^{-1} mg^{-1} prot) in shoots of olive genotypes (Canino wt and Canino *AT17-1*) grown in vitro on media supplemented or not with PEG 8000. Data are the means (±SD) from three independent experiments. Different letters (lowercase letters for Canino wt and uppercase letters Canino *AT17-1*) above the data indicate significantly different means as determined by Fisher's LSD at $P < 0.01$

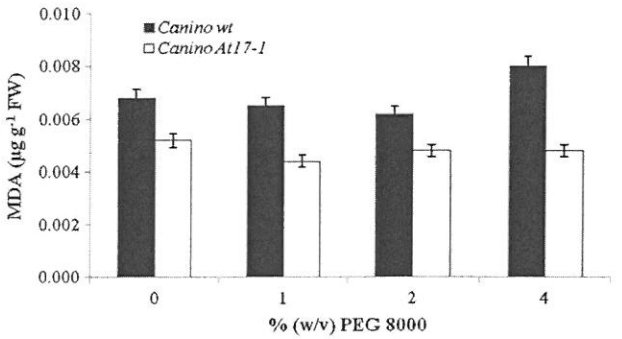

Fig. 7 Changes in MDA content (μg g^{-1}FW) in shoots of olive genotypes (Canino wt and Canino *AT17-1*) grown in vitro on media supplemented or not with PEG 8000. Data are the means (±SD) from three independent experiments. Different letters (lowercase letters for Canino wt and uppercase letters Canino *AT17-1*) above the data indicate significantly different means as determined by Fisher's LSD at $P < 0.01$

peroxidase and catalase, respectively). On the other hand, PEG treatments differently affected both peroxidase and catalase activity.

In Canino *wt* shoots, peroxidase activity of PEG-treated plants was significantly higher than that of the control (without PEG addition) and in particular gradually increased with increasing PEG concentration from 1 to 4%. On the other hand, in Canino *AT17-1* shoots, peroxidase activity remained relatively stable, irrespective of PEG exposure, to values similar to those of control plants. Consequently, we observed that application of the highest PEG concentration (4%) resulted in peroxidase activity in Canino *wt* shoots more than fivefold higher than those found in Canino *AT17-1* leaves.

On the other hand, catalase activity was significantly induced in all PEG exposure experiments, with concentrations ranging from 1 to 4%, in Canino *AT17-1* shoots, while it was almost unaffected by PEG treatment in Canino *wt* shoots.

Lipid peroxidation

As shown in Fig. 7, under control conditions (no PEG addition), MDA content was significantly higher (30%) in Canino *wt* shoots than in those of Canino *AT17-1*. Furthermore, MDA content was increased by 20% following the application of the highest PEG concentration in Canino *wt* shoots, while it did not respond to PEG exposure in leaves of the transgenic line (Fig. 7).

Discussion

Our results provide clear evidence that the presence of tobacco osmotin gene in transgenic olive shoots under stress condition induces an accumulation of osmotically active substances such as proline in the tissues that confer tolerance to osmotic stress. Similar results were obtained in tobacco, cotton and strawberry (Barthakur et al. 2001; Parkhi et al. 2009; Husaini and Abdin 2008) where the constitutive expression of the osmotin gene improved tolerance to water stress and salinity. The significantly higher accumulation of proline in plants exposed to drought stress most likely indicates a reaction to tissue damage and could be considered as an attempt for plants to reduce the oxidative damage, according to Aghaz et al. (2013). Furthermore, Wei et al. (2009) showed that the drought-induced accumulation of proline could be attributed to a down-regulation of proline dehydrogenase.

Under in vitro condition, the transgenic line appeared less affected by the presence of PEG than the *wt*, whereas the leaves and shoots of wild-type counterpart was severely affected by the stress induced by PEG. Irrespective of the presence or absence of PEG, the transgenic line showed similar growth rate and significantly higher than that of untransformed control line, demonstrating that the

transgenic ones are better equipped to tolerate the water stress created by PEG.

In transgenic genotypes, however, the content of chlorophyll was not affected by the presence of the PEG. In transgenic lines of chilli pepper, the content of chlorophylls did not appear to be influenced by water stress induced by PEG, unlike the control, where the increase of the concentration of PEG significantly decreased the amount of chlorophyll in the leaves (Subramanyam et al. 2011). The preservation of considerably higher chlorophyll contents by the transgenic line might be due to stable photosynthetic pigments under water stress conditions and is consistent with previous findings in *Zea mays* (De Souza et al. 2013).

Drought is a major abiotic stress affecting the plant photosynthetic capacity adversely, by decreasing the carboxylation capacity and the related energy dissipation (Wilhelm and Selmar 2011). Under these conditions, a rise in the production of reactive oxygen species (ROS) clearly occurs (Schwanz and Polle 2001; Vranová et al. 2002) and their accumulation is commonly associated with direct or indirect degradation of chlorophyll (del Río et al. 1998; Toivonen and Sweeney 1998) and with promotion of lipid peroxidation which contributes to membrane degradation. Accordingly, the present study showed that Canino *wt* leaves from plants exposed to PEG treatments exhibited decreased levels of chlorophyll and increased MDA content, most likely as a consequence of the generation of ROS, contrary to the transgenic line in which PEG exposure did not affect neither chlorophyll content nor lipid peroxidation.

In particular, the MDA concentration in plant tissues is considered a marker of the degree of membrane damage resulting from ROS (Wang et al. 2009), so this finding could suggest that the transgenic line Canino *AT17-1* was more efficient in the activation of defense responses against oxidative stress with respect to the Canino *wt*.

The development of protective mechanisms is a crucial step to cope with oxidative stress. One of these mechanisms is the induction of antioxidant machinery, which involves the functioning of a number of enzymes such as superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, which allow to eliminate excessive ROS accumulation (Shah et al. 2001).

In this study, we evaluated the general antioxidant status of the leaves from Canino *wt* and transgenic line Canino *AT17-1* plants by measuring changes in peroxidase and catalase activity profiles following PEG treatments and demonstrated significant difference in the response of different genotypes to drought, for both examined parameters.

In particular, regarding the PEG effect, the MDA production inhibition and the lack of effective chlorophyll degradation in the transgenic line Canino *AT17-1* plants might be related to the stronger scavenging activity, namely the higher catalase activity, with respect to the wild type. In contrast to catalase activity, we found that MDA contents were not correlated with the pattern of peroxidase activity, remaining relatively stable in the transgenic line. Somewhat surprisingly, peroxidase activity increased gradually with increasing magnitude of water stress (PEG concentration) in the wild type, in which we also found a higher MDA level, supporting the occurrence of oxidative stress.

The discrepancy in catalase and peroxidase activity profiles could suggest the minimal involvement of peroxidase enzyme in regulation of drought stress tolerance. However, it cannot be ruled out that the different activity patterns may result from differences in enzyme functions. Catalase is one of the most potent enzymatic antioxidant in plants, catalyzing the conversion of H_2O_2 to water and oxygen. Peroxidase is also capable to remove H_2O_2 from chloroplasts and from the cytosol, but needs to accept electrons from various donor compounds (e.g., phenols, lignin precursors, auxin and secondary metabolites) (Mehlhorn et al. 1996). Furthermore, peroxidases also play a role in modulation of physiological processes in plants, including biosynthesis of cell walls (Bacon et al. 1997). Thus, this observation could indicate that changes of peroxidase activity due to PEG exposure in wild type likely reflect modifications of cell wall properties, as a part of the adaptive response to water stress. Furthermore, our results suggest that peroxidases could have a different role in the metabolic response mechanisms induced to cope with drought stress in olive genotypes with different tolerance. Compared to wild type, the transgenic line appeared to be able to alleviate damage induced by PEG treatment by increasing catalase activity (Lima et al. 2002). On the other hand, the fact that wild type showed lower catalase activity under stress, together with higher MDA content and lower chlorophyll content, could explain its worse response to drought stress (Feierabend et al. 1992; Streb et al.1993).

The results of this work shed new light on the efficacy of the transgenic approach for improving drought tolerance in olive, a highly economically valuable plant. Furthermore, our results could also have implications for the use of osmotin as a potential targeted therapeutic drug for humans (Anil Kumar et al. 2015). The functional and structural homology between osmotin and adiponectin, an adipocyte-specific protein, which has been suggested to play a role in the development of insulin resistance and atherosclerosis (Narasimhan et al. 2005; Miele et al. 2011; Naseer et al. 2014), must be carefully considered as well as plant use for the production of drugs.

Author contribution statement CS was responsible for conception and design of experiments, data analysis, drafting of the manuscript and edited the paper. SC

conducted the enzyme analysis and data analysis. VC took care of study conception and design and drafting the manuscript. SA was responsible for conception and design of experiments, data analysis, drafting of the manuscript and edited the paper. BR conducted molecular analysis. ER took care of study conception and design and edited the manuscript. All authors read and approved the manuscript.

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Cristian Silvestri


Stefania Astolfi


Silvia Celletti


Valerio Cristofori


Antonella Minandri


Giampiero Ubertini


Bruno Ruggiero


Eddo Rugini

STRATEGIES FOR THE IMPROVEMENT OF WATER STRESS TOLERANCE OF ACTINIDIA (ACTINIDIA DELICIOSA A. CHEV) AND BIOCHEMICAL MARKER RESEARCH FOR EARLY SELECTION.

Actinidia is one of the species cultivated in the Mediterranean area known for a prominent susceptibility to water deficiency. Irrigation in the most critical phases of the annual cycle plays a fundamentally important role not only in assuring adequate growth of the fruits and, therefore, a good crop yield, but it also influences the plant's productivity in the successive year by preconditioning mineral nutrition and flower induction. A weakening of the plant caused by a water deficiency can also result in it becoming more susceptible to biotic stresses.
Numerous studies have been conducted on the irrigation of actinidia orchards and on the physiology of the species aimed at verifying the crops' behaviour under water stress, which have allowed for the acquisition of useful information for crop improvement.
Diverse species belonging to the Actinidia genus are found in nature, and numerous are the hybrids as well. Natural mutations or spontaneous crosses have given origin to a great variety of different characteristics and, thus, access to a vast genetic base utilisable for the selection of new varieties (*Testolin et al., 2016*). Nonetheless, many interesting agronomical traits, such as cold climate adaptation, disease resistance, including drought tolerance, are not present in the most cultivated variates.

The principal causes which have stood in the way of fully harnessing the genetic potential and have rendered traditional genetic improvement lengthy and costly, have been the dioecious nature of the plant, the fact that the male individuals' genetic background is unknown, in addition to a relatively long juvenile phase.
Furthermore, the different levels of ploidity amongst actinidia species represent another obstacle for the interspecies hybridisation; numerous attempts at producing plantlets through interspecies hybridisation have failed (Pringle 1986*;* Mc Nelage and Considine 1989; Mu *et al* 1990; Ke *et al* 1991).
The genetic transformation techniques represent an extremely advantageous opportunity for the genetic improvement of

woody fruit-bearing plants, including actinidia, which usually are characterised by a lengthy youth period, a high level of heterozygosity and by a low genetic variability. In addition, unlike other technologies applied to date, this technique allows for the targeted "improvement" of certain traits of widely used commercial varieties in a brief amount of time, performing a sort of "gene therapy," without carrying out a profound modification to the plant's DNA, thus maintaining the commercial varieties' peculiar traits. The first transgenic woody plants, with potentially agronomically useful genes, have been obtained precisely in actinidia (*Actinidia deliciosa* A. Chev.) on a late-blooming pollinator, GTH (Giallo Tardivo Hayward) which was transformed with *A. rhizogenes rolABC* genes (Rugini 1989; Rugini *et al.,* 1991). These pioneering works were followed by others, which have lead to the constitution of female actinidia plants of the cv. Hayward some transgenic for *rol* genes and others for *osmotin* (Rugini *et al.* 2000) which were the object of in-field experimentations up to the year 2012 hosted by the Research University of Tuscia (Rugini, 2012).

Materials and Methods

The in-field observations, on 11 years old plants, were focused on foliage water loss in the following genotypes:

1) male plants GTH (Giallo Tardivo Hayward) modified with the *rol ABC* from *A. rhizogenes* using the bacteria's natural promoter;
2) Cv Hayward variety plants modified with *rolABC*, *rolB, rolC* genes through the bacteria's natural promoter;
3) plants grafted with reciprocal grafts between GTH *rolABC* and GTH wt;
4) Cv Hayward variety plants modified with the tobacco *osmotin* gene under the control of the constitutive promoter *CaMV35S* (Rugini, 2012; Rugini 2015);
5) Control plants (wt) of both GTH and Haward;
6) Hayward plants regenerated from callus (regenerated Hayward wt).

Furthermore, analyses were performed relative to certain physical parameters of the terrain on soil samples collected in the experimental area at 40 cm of depth, immediately following those performed on the foliage water loss, namely: granulometric evaluation (sand, lime and clay according to the dimensional limits of the I.S.S.S., with the wet sieving and sedimentation method or pipette method) and the water retention curve including the calculation of the hydrological constants of the field water capacity (-33kPa) and the point of permanent wilting (-1500 kPa) using the method of the Richards' pressure plate apparatus -).

The *in vitro* and biochemical analyses were performed upon two transgenic genotypes of actinidia: Hay *osm1* and Hay *osm166*, derived from two different transformation events and compared with the respective controls: Hayward *wt* and a genotype of Hayward *wt* regenerated from callus which had stood out in field for its tolerance to drought. The shoots were multiplied on a solid substrate for multiplication composed of salts and MS vitamins (Murashige and Skoog, 1962), with added BAP (0,7 mgL^{-1}), NAA (0,01 mgL^{-1}) and Plant Agar 0,6%, pH 5.8, enriched with various concentrations (0, 1, 2, 3%) of polyethylene glycol (PEG), before sterilising in autoclave at 121˚C for 20 minutes. The tests were conducted in pots with 250 mL capacity with 50 mL of substrate. 3 pots containing 10 shoots each were used for each of the various PEG concentrations.

The evaluations, conducted after 4 weeks of growth in a grow room at 23˚C, evaluated the count of newly formed shoots and the relative number of nodes per shoot, besides the weight of the basal callus. A portion of the plant material (shoot without basal callus) was preserved at -80˚C for biochemical analyses that, in particular, focused on the calculation of proline concentration, total protein, thiolic compounds, and of malondialdehyde (MDA) and the variation of the activity of certain stress-related enzymes related to water stress (guaiacol peroxidase and catalase), utilising techniques previously described by Bradford (1976), Santangelo *et al.* (2003) and Astolfi *et al.* (2005). The principal scope was the identification of enzymatic and non-enzymatic markers capable of precocious selection among a multitude of genotypes of the ones most tolerant to water stresses. Specifically, the objective was to check for possible differences in the water stress response mechanisms activated in plants transformed with osmotin and in control plants regenerated from callus (regenerated Hayward wt).

Results and discussions

Field tests

The granulometric evaluation of the soil upon which the plants subject of the work persisted allowed for its classification as "loamy" according to the relative triangle of the I.S.S.S. texture. The knowledge of the water retention curve, specifically for the soil involved in the

experimental tests was useful for a comparative valuation including the gravimetric humidity content found in the soil at the moment of sampling. In the experimental area the average humidity, in the period of sampling, dropped below the point of permanent wilting, as can be deduced in comparing it with the water retention curve (*Table 1*). The plants transformed with the *rol* genes exhibited the notable phenotypic traits conferred by these genes and already described in previous works (Rugini *et al.,* 1997 Gutiérrez-Pesce and Rugini 2008; Rugini 2012 and 2015) in addition to a better tolerance to water stress, due probably to the improved efficacy of the foliar apparatus. This affirmation finds a confirmation in the fact that, among the grafted plants, only transgenic (genes *rolABC* and *rolB*) grafts resulted tolerant to drought, while the reciprocal ones did not (*Table 2*). Hayward *rolB* plants, which, beyond their tolerance to water stress, also presented fruits of analogous dimensions to those of the mother plant (Rugini 2012 and 2015) are of particular interest from an agronomic and commercial point of view. The plants transgenic for the *osmotin* exhibited a regular flowering and fruit production analogous to the control, without showing apparent morphological differences, including the sizing and shape (Rugini *et al.,* 1998; Rugini, 2012); the fruits exhibited a clear tolerance to *Botritis cinerea* (botrytis) and to *Cadophora luteo-olivacea* (cadophora) as reported in previous works (Rugini *et al.,* 2011). Similarly, the leaves presented the same property, verified with artificial infections with botrytis mycelium in agar disks (*Figure 1*). Likewise to the *rolABC* and *rolB* plants, they showed a tolerance to water stress, deducible from the state of wilting observed in comparison to other control plants in soil humidity conditions nearing the point of wilting (*Table 2*). Among the non-transgenic plants a somaclone of particular interest (regenerated Hayward wt), produced from the callus of Haward wt, which showed a nearly similar tolerance in field to the one of transgenic plants for osmotin and *rol* genes.

Table 1 Granulometric composition and hydrological components of the soil (±DS) at the moment of sampling for the hydric state of the leaves of 11 year old Actinidia plants in the summer period (end of June 2012).

Granulometry (I.S.S.S.)	% clay 50,53	%lime 23,78	%sand 25,69
Field capacity (-0.33 kPa) (Humidity % in weight)		38,08. ± 0,77	
Point of wilting (-1500 kPa) (Humidity % in weight)		24,94 ± 0,18	
Gravimetric humidity % (Humidity % in weight)		23,63 ± 1,95	

Table 2
Assessment of the state of foliage wilting of the different self-rooted or grafted genotypes present in the field in absence of irrigation. (A = Wilted; VA = early wilting; V = Not wilted), at the moment of explantation (end of June, 2012, starting point of wilting irreversible).

THESIS	STATE OF FOLIAGE WILTING
HAY wt	A
Regenerated HAY wt	V
HAY Osmotin (various clones)	V-VA
GTH wt	A
GTH rolABC	V
HAY rol ABC	V
HAY rol B	V
GTH rol ABC / GTH wt	V
GTH wt / GTH wt	VA
GTH wt / GTH rol ABC	VA
HAY wt / HAY wt	A
HAY wt / GTH rol ABC	A

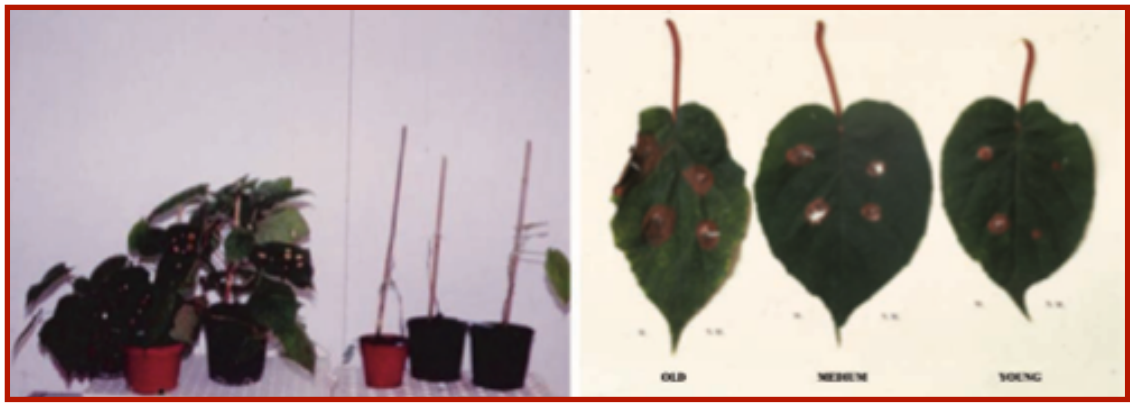


Figure 1 Artificial infections with *Botritis cinerea* on leaves and fruits with an agar tile containing mycelium of the fungus, performed on all the leaves (old, mature, young). Note how the control plants have lost all their leaves (right) following treatment with the fungus, while the transgenic ones (left) have limited the sickness to the surface occupied by the tile.

Laboratory tests

The tests conducted in the laboratory evaluated the response *in vitro* to water stress induced in shoots of two transgenic osmotin kiwi genotypes, derived from two distinct transformation events in contrast to the Hayward wt and regenerated Hayward wt controls. The transformed shoots showed a superior tolerance to PEG-induced water stress compared to the Hayward wt control and, quite similarly to the new stress-tolerant genotype, regenerated Hayward wt (*Table 3*).

Table 3 Growth parameters (Average number of shoots, number of nodes, weight of the basal callus) of the Hayward wt clones (control), regenerated Hayward wt, Hayward osm1 and Hayward osm166. The averages, which are indicated with a different letter for each group of genotypes, show the significant differences over p≤0,05 (Tukey Test).

Clone	PEG 8000 (% w/v)	Shoot Number	Number of Nodes	Basal Callus
Hayward *wt*	0	1,67 a	5, 11 a	0,25 a
Hayward *wt*	1	1,56 a	3, 67 b	0,14 b
Hayward *wt*	2	1,68 a	3,88 b	0,10 b
Hayward *wt*	3	1,75 a	3,77 b	0,16 b
regenerated Hayward *wt*	0	2,22 a	7,11 a	0,52 a
regenerated Hayward *wt*	1	1,89 a	6,01 ab	0,25 b
regenerated Hayward *wt*	2	2,01 a	5,01 b	0,29 b
regenerated Hayward *wt*	3	1,61 a	5,55 b	0,20 b
Hayward *osm 1*	0	1,68 a	5,22 a	0,46 a
Hayward *osm 1*	1	1,70 a	5,00 a	0,29 b
Hayward *osm 1*	2	1,77 a	5,03 a	0,16 b
Hayward *osm 1*	3	1,65 a	4,89 a	0,16 b
Hayward *osm166*	0	1,84 a	6,33 a	0,36 a
Hayward *osm166*	1	1,81 a	6,70 a	0,23 ab
Hayward *osm166*	2	1,72 a	5,44 a	0,12 b
Hayward *osm166*	3	1,76 a	5,67 a	0,14ab

The growth of the cultivated shoots showed the same parameters even at the highest concentration of PEG (3%) and similarity to those placed in the control substrate lacking PEG. To the contrary, the growth of shoots of the cv Hayward wt was progressively reduced with the increase of PEG concentration and the leaves were visibly damaged. The regenerated Hayward wt genotype showed a superior growth performance compared to cv Hayward wt, both in terms of number of shoots and nodes as well as basal callus (statistical calculation not shown in table 2). This genotype possessed a major vigour *in vitro* and in field (data not shown). Considering this is a case of regeneration, and, therefore, of a plant produced from competent cells, possibly with different epigenetic traits, it may have acquired a reinvigoration, similarly, but to a minor extent, observed in many genotypes derived from crosses. A tentative hypothesis may be advanced that this phenomenon grows more evident the more the cultivar ages, in other words the number of times agamic multiplication is performed on it increases, unless additional modifications take place due to somaclonal variability. This behaviour has also been observed in the first olive plants of the cv Canino regenerated through somatic embryogenesis from adult maternal tissues. These plants, in fact, exhibited a major youthfulness and vigour in respect to those propagated by stimulation of the axil buds or by cutting (Rugini personal communication).

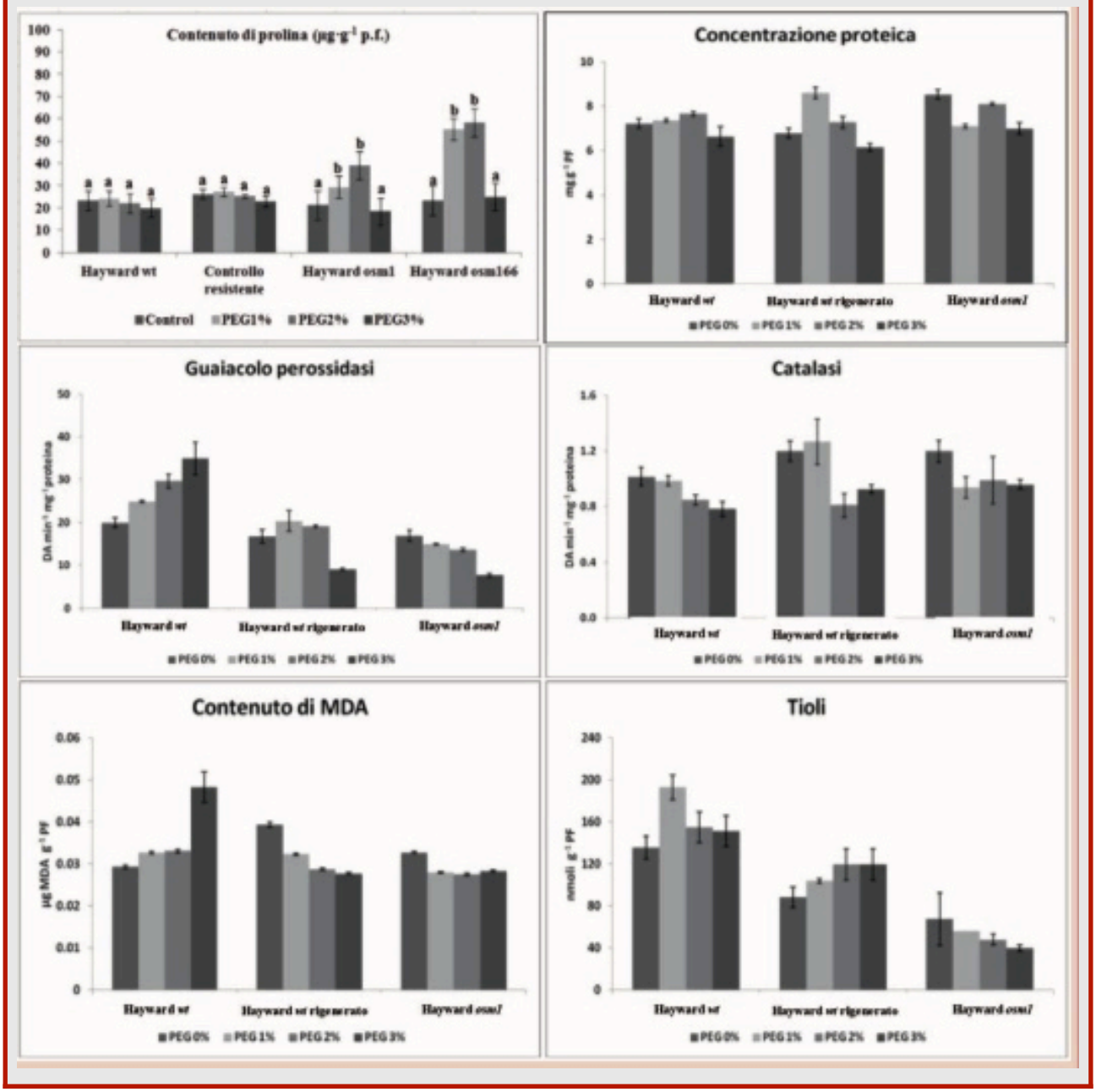
The analysis of the measured biochemical parameters confirmed that the tolerance to induced stress is accompanied by changes at the level of enzymatic activity and of the accumulation of metabolites involved in stress mechanisms (*Figure 3*).

In plants subjected to water stress, a reduction of the photosynthetic activity is generally confirmed, which causes a lower CO2 assimilation rate and the accumulation of reducing agent potency. The latter event determines the increase of reactive oxygen species (ROS) and of free radicals (Schwanz and Polle, 2001; Vranovà et al., 2002); such stress conditions require, therefore, a increase in antioxidant enzyme activity. The plants contain, additionally, numerous non-enzymatic antioxidant compounds, which can detoxify the ROS and, thus, diminish the negative effect of the stress.

In this work, we evaluated the variation in the activity of two antioxidant enzymes - catalase and peroxidase, which are involved in the elimination of H_2O_2 from the chloroplasts, and the concentration of the non-proteic thiols as a non-enzymatic marker, was also analysed. On the other hand, we analysed the content of MDA as a indicator of the oxidative stress damage, which allows for the valuation of cellular membrane lipid peroxidation levels. The results seem to indicate that the transgenic genotype plants could be capable of confronting water stress and suffering fewer damages, as indicated by the lower levels of MDA compared to the *wt.* However, in this type of response, there appears to be no implication of either peroxidase or catalase, which do not show relevant variations in the same genotype, following subjection to stress. A different discussion may be had over the regenerated drought-tolerant genotype, which shows, like the transgenic one, lower levels of MDA, but, unlike the transgenic one, shows perceivable variations of the two measured anti-oxidative enzymes, which could explain these plants' better capacity of response to water stress generated by exposure to PEG. After all, the control genotype is subjected to oxidative stress damage (increase of MDA levels following PEG treatment) to which it responds with an increase of antioxidant enzyme activity, principally of peroxidase. The fact that the peroxidase activity increases in Hayward genotype wt tissues during the progressive exposure to water stress, while remaining constant and even decreasing in the transgenic genotype, can be in part explained considering that the peroxidases participate in the modulation of the cell wall properties during plant growth (Bacon *et al., 1997*) and, therefore, a increment in the activity of these enzymes may reflect variations of the mechanical properties of the cell wall, which, in turn, could be correlated to an adaptation to water stress, which would be necessary in the Hayward wt genotype but not in the transgenic genotype (inasmuch as it appears not to suffer damages). Therefore, even in the *wt* genotype, the peroxidase enzyme would not have only a antioxidant role. In order to explain the better performance of the transgenic genotype in terms of water stress, we evaluated the concentration of thiol compounds in the leaves of these plants to verify whether the lack of damage was due to a greater production of these compounds for the neutralisation of the ROS.

The thiols, similarly to cysteine and glutathione, are organic sulphur traces abundantly available in plant tissues and, in normal conditions, the glutathione is the predominant trace.

Figure 3 Average content of proline (express in µg•g^{-1} of fresh weight), proteic concentration (mg g^{-1} f.w.), guaiacol peroxidase (∂A min^{-1} mg^{-1} proteins), catalase (∂A min^{-1} mg^{-1} proteins), MDA content (µg g^{-1}f.w.), of thiols (mmol g^{-1} f.w.) of Hayward wt (control), regenerated Hayward wt, Hayward *osm1* subject to various concentrations of PEG used. Different letters on the graphic are used to indicate significant differences per P<0.01 (Tukey test).



Still, against all expectations, the thiol concentration was augmented in the *wt* genotypes and in the resistant, but not in the transgenic.

The significance of such results and, overall, the interpretation of the behaviour of the transgenic genotype, remain an open question and further elucidations will certainly be drawn from the study of the activity of other antioxidant enzymes, such as superoxidedismutase (SOD) and ascorbic peroxidase (APX), which are key enzymes in the anti-oxidative system of not only chloroplasts, but also other cellular compartments. Furthermore, the measuring of GSH content variations (which could be masked on the inside of the total thiol fraction) could explain the defence mechanism through which the plant operates to reduce the effects of water stress.

In conclusion, from the preliminary results obtained in this work of comparing osmotin transgenic plants to the somaclone — both of which drought tolerant — it may be affirmed that certain physiological parameters, identified in plants transgenic for the osmotin gene, could be employed as possible markers

for the precocious selection of drought-tolerant plants derived through other techniques of genetic improvement. Furthermore, the plants over-expressing osmotin, in addition to representing an opportunity for sustainable agriculture, through the manifestation of greater resistance in terms of biotic and abiotic stresses, could also have important implications for the protection of human health. It is known, in fact, that osmotin belongs to the PR (Pathogen Related) protein family, of the 5th type (Anil Kumar *et al.,* 2015) and, in nature, it is only produced by plants in response to pathogen attack or abiotic stresses of various kinds. It has been observed that osmotin is capable of protecting the chlorophylls and the photosynthesis related metabolic pathways (Barthakur *et al.* 2001; Goes *et al.,* 2010), thus determining the increased proline accumulation, which performs a re-equilibration of osmotic balance and the protection of the cell structure (Subramanyam *et al*., 2011). In terms of human health, of particular importance is the role that osmotin plays in the veterinary and human field of applications, inasmuch as this plant protein is capable of mimicking the activity of the human hormone Adiponectin (Kadowaki *et al.,* 2005).

Adiponectin is a hormone produced by the adipose tissues and is involved in various metabolic processes, such as glucose regulation and the catabolism of fatty acids both in the body and the brain. The scarcity of adiponectin results in diseases such as diabetes, liver conditions and cardiovascular problems (Kadowaki and Yamauchi, 2005; Tang *et al.,* 2005; Holland and Scherer, 2013). For these reasons, today there is a growing interest in the research on osmotin, seen as a protein with a therapeutic potential targeted to humans. Recent publications have demonstrated that osmotin is capable of recovering damages caused by senile dementia, Alzheimer's, diabetes, colitis and Crohn's disease, thanks to its capacity to activate the Adipor1/Sirt1 receptors (Shah *et al*., 2016) and, therefore, to activate the cellular enzymatic cascade improving the functionality of mitochondria and, thus, potentiating the cell's vital activity, including the control of neoplastic growth. A project is underway at the Research University of Tuscia, in Viterbo for the extraction and purification of recombinant osmotin from the transgenic genotype actinidia plants, which, unlike control plants, express the recombinant gene for osmotin from tobacco *Wisonsin 38* with a 35S viral promoter, and, therefore, produce the protein at a factor which could vary between 4% and 16% of the total protein content. These genotypes are, *de facto*, perfect bioreactors capable of supplying osmotin, of great therapeutic worth, in good quantities and in a context of being ready for human and animal consumption, making the commercialisation of this substance economically advantageous for the pharmaceutical industry and plant suppliers alike.

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Dipartimento di Scienze Agrarie e Forestali (DAFNE) Università degli Studi della Tuscia, Via S. Camillo de Lellis, 01100 Viterbo