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Effect of saline irrigation on physiological traits, fatty acid composition and desaturase genes expression in olive fruit mesocarp

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ABSTRACT

The effect of salinity on physiological traits, fatty acid composition and desaturase genes expression in fruit mesocarp of olive cultivar Leccino was investigated. Significant reduction of shoot elongation (-12%) during salt treatments (80 mM NaCl) was associated with the translocation of Na in the aerial part. After 75 days of treatment, fruits from each plant were subdivided into four maturation groups (MG_0, MG_1, MG_2, MG_3) according to ripening degrees. Na accumulation increased in each MG under salinity, reaching the highest values in MG_1 fruits (2654 mg kg^{-1} DW). Salinity caused an acceleration of the ripening process, increased fruit number and decreased total fatty acids content in MG_3. An increase in oleic acid at MG_1 (53%) was detected, with consequent increase in the oleic/linoleic (41%) and decrease in the polyunsaturated/monounsaturated ratios (30%). Those variations could be explained by the synergic up-regulation of *OeSAD1*, together with the down-regulation of *OeFAD6* transcript levels.

Key words: Fatty acid desaturases, Gene expression, Olive, Olive oil, Salt stress.

Introduction

Olive is an iconic tree of the Mediterranean landscape of agricultural (production of olives and oil) and cultural relevance, even more widely exploited for abiotic stress adaptation studies (Ben Abdallah et al., 2018; Fernández et al., 2014). Olive oil is considered a world's major edible oils rich in oleic acid (55–83%), while linoleic and linolenic acids account for 3.5–21% and less than 1%, respectively (European Commission Regulation, 2003). The relative proportions of these unsaturated fatty acids in olive fruit have a significant effect on several olive oil quality aspects, like nutritional characteristics and technological properties, such as the oxidative stability (Aparicio et al., 1999; Cunnane, 2003; Sofi et al., 2013).

Environmental conditions affect the relative proportions of unsaturated fatty acids in olive fruit (Hernández et al., 2011; 2019). At the end of the 21st century the Mediterranean area will experience very high shortage of water caused by decreased precipitations, increased average annual temperature, increased demand of water for agriculture and civil and industrial uses. To fulfil the demand of water for agriculture, poor quality water (such as saline water) is - and will be even more - utilized for agricultural irrigation. In this context, research on possible use of saline water for olive irrigation is a hot topic (Ben-Ahmed et al., 2009; Ben-Gal et al., 2017; Chartzoulakis, 2005; Weissbein et al., 2008). Since salinity represents a stress factor for olive (Gucci and Tattini, 1997; Tattini, 1994), several studies have been done to understand the impact of saline irrigation on olive oil characteristics (Sebastiani et al., 2016). Experiments with the salt tolerant cultivar Barnea proved

an increase in oleic acid and a decrease in linoleic acid percentages in the extracted oil with increasing salt regimes (Wiesman et al., 2004). Contrastingly, exposure to 100 and 150 mM NaCl in cultivar Koroneiki determines a significant reduction of oleic/linoleic ratio in olive oil (Stefanoudaki et al., 2009). Data reveal that salt stress has some consistent effect on the lipid and phenolic components of olive oil of cultivar Koroneiki mainly in relation to the more salt tolerant cv. Mastoidis (Stefanoudaki et al., 2009).

In addition, Weissbein et al. (2008) screened 12 olive cultivars under saline regimes (1.2 and 4.2 ds m^{-1} of Electrical Conductivity - EC) showing that the oleic/linoleic ratio changes are cultivar-specific but no hypothesis or correlation between the ratio and the genotype tolerance to salinity were done.

While the effect of salinity on olive oil composition has been studied, the impact of saline irrigation on olive fruit fatty acid biosynthetic pathways is still not well documented. Fatty acid biosynthesis in higher plants begins in the plastid, yielding mainly palmitoyl-acyl carrier protein (ACP) and stearoyl-ACP by successive addition of two carbon atoms from acetyl-CoA (Harwood, 2005). Still in the plastid, the soluble stearoyl-ACP desaturase (SAD) desaturates stearoyl-ACP to synthesize oleoyl-ACP, which is the main product of the plastidial fatty acid biosynthesis. The acyl-ACPs are then cleaved by specific thioesterases to free fatty acids, which are then activated to acyl-CoAs and incorporated into the glycerolipid biosynthetic pathway, where can be further desaturated to linoleic and linolenic acids. Two sets of ω6 and ω3 membrane-bound fatty acid desaturases have been

reported, which differ in their cellular localization, lipid substrates, and electron donor system (Shanklin and Cahoon, 1998). The microsomal ω6 and ω3 desaturases (FAD2 and FAD3, respectively) are located in the endoplasmic reticulum, whereas the plastidial ω6 and ω3 desaturases (FAD6 and FAD7/8, respectively) are located in the chloroplast.

In olive, three genes encoding SAD have been reported: *OeSAD1* (Haralampidis et al., 1998), *OeSAD2* and *OeSAD3* (Parvini et al., 2016). With respect to membrane bound desaturases, two genes encoding microsomal oleate desaturases (*OeFAD2-1* and *OeFAD2-2*) have been isolated and characterized by Hernández et al. (2005), while only one *OeFAD6* gene has been reported so far (Banilas et al., 2005; Hernández et al., 2011). In addition, four members of the olive linoleate desaturase gene family have been described, two microsomal (*OeFAD3A*, Banilas et al., 2007; *OeFAD3B*, Hernández et al., 2016) and two plastidial (*OeFAD7-1*, Poghosyan et al., 1999; *OeFAD7-2*, Hernández et al., 2016).

It is generally accepted that plant fatty acid desaturase genes respond to abiotic stress (Upchurch, 2008). Evidence of transcription regulation are reported under water stress (Hernández et al., 2009, 2018; Torres-Franklin et al., 2009), but due to its complexity, fatty acid desaturase genes transcription regulation under salinity stress is poorly studied. Zhang et al. (2009, 2012) showed a key role of *FAD6* and *FAD2* in *A. thaliana* seedlings exposed to 300 mM NaCl. These genes maintain the desaturation level of membrane lipids fatty acid, thus avoiding membrane lipid peroxidation and preserving the ion homeostasis. In olive, transcriptomic

approaches have been recently performed to study the molecular response to saline stress of seedlings from tolerant and susceptible cultivars (Bazakos et al., 2012), and different organs such as leaves and roots (Bazakos et al., 2015). However, no data related to the effect of salt treatment on the fatty acid desaturases gene expression levels in olive fruits have been reported up to date.

With the aim to provide a better explanation of the regulatory mechanism of fatty acid desaturation related to changes in fatty acid composition under salinity stress, a salt sensitive cultivar "Leccino", able to translocate Na from root to aerial part (Gucci and Tattini, 1997; Rossi et al., 2015) and, in particular, into the fruit mesocarp (Moretti et al., 2018) has been used. The effects of salinity on plant physiological traits, fatty acid composition and expression levels of fatty acid desaturase genes were studied in fruit mesocarp at different maturation stages.

2. Materials and methods

2.1. Plant material and salt treatments

Five-years old homogeneous olive trees (*Olea europaea* L.) cultivar Leccino, taken from a commercial certified nursery were grown in pots (33 cm Ø) filled with peat and pumice (60/40; v/v). Pots were accurately covered with a plastic foil in order to avoid rain water leaching effect. The experiment started at fruit pit hardening stage corresponding to 90 days after flowering (DAF) and ended 75 days later (165 DAF). Two treatments (4 l each) were applied on 8 plants per treatment once a week: 0 mM NaCl (control plant, irrigated with Milli-Q water, 18.2 MΩ.cm,) and

80 mM NaCl (saline water – 7.36 g of Na per plant once a week, for a total of 73.6 g of Na per plant).

Plants were fertirrigated at the beginning of the experiment (90 DAF), one month after (120 DAF), and two months after (150 DAF), with 1/3 strength olive medium, pH 5.8 (Rugini, 1984).

2.2. Plant physiological traits and Na accumulation

During 75 days of treatments, the effect of salt stress on efficiency of PSII was monitored. Chlorophyll *a* fluorescence emissions on 30-min dark-adapted leaves were measured, each week, with a portable fluorimeter FMS2 (Hansatech, Germany) at 09:00 - 10:00 in leaves near to the infructescence. The background fluorescence signal (Fo), the maximum fluorescence (Fm), and the potential quantum yield of PS II photochemistry [Fv/Fm=(Fm−Fo)/Fm] were determined. The quantum efficiency of PSII (Φ PSII) was also considerated. Shoot elongation was recorded each 15 days from the beginning of treatments.

At the end of experiment, olives were collected and divided according to Camposeo et al. (2013) into four maturation groups (MG): MG_0 (with green skin), MG_1 (with ≤50% purple skin), MG_2 (with >50% purple skin), MG_3 (with 100% purple skin) and distribution frequency of fruits in each MG was evaluated.

Fresh (FW) and dry weight (DW), flesh/pit ratio and fruit volume were determined on 5 olives per plant (n = 8). Volume was calculated from the longitudinal and

transverse diameters using the formula for a prolate spheroid ($V = 4/3\ \pi\ a2\ b$, where a and b are the transverse and longitudinal radii, respectively).

To determine the Na translocation in aerial part of Leccino plants, olive mesocarp, leaves, stem and rachis, were dried in a forced-circulation oven at 65 °C and grounded with a laboratory mill (IKA-Werke GmbH & Co.KG, Staufen, Germany). Samples (0.2 g) were digested in HNO_3 and used for the Na determination in an atomic absorption spectrometer (model 373; PerkinElmer, Norwalk, CT, USA) equipped with specific lamps. Analytical reference standards of Na were used as a control (WEPAL IPE, Wageningen University).

2.3. Fatty acid analysis

Fatty acid methyl esters of olive mesocarp were produced by acid-catalysed transmethylation (Garcés and Mancha, 1993) and analysed by gas chromatography (Román et al., 2012), using an HP-7890 (Hewlett-Packard, Palo Alto, CA, USA) fitted with a capillary column (30 m length; 0.32 mm inner diameter; 0.2 μm film thickness) of fused silica (Supelco, Bellafonte, PA, USA) and a FID detector. Hydrogen was used as a carrier gas with a linear rate of 1.34 ml min^{-1} and a split ratio of 1/50. The injector and detector temperature was 220 °C, and the oven temperature was 170 °C. Heptadecanoic acid was used as internal standard to calculate the fatty acid content in the samples. Palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids were identified, and quantified as μg mg^{-1} DW of

mesocarp tissue. Ratio between oleic/linoleic acids, and polyunsaturated fatty acid (PUFA)/monounsaturated fatty acid (MUFA) were calculated.

2.4. Total RNA extraction and cDNA synthesis

Total RNA isolation was performed as described by Hernández et al. (2005). Briefly, 1−2 g fresh weight (FW) of frozen olive mesocarp was finely grounded in mortar with liquid nitrogen. 5 ml of extraction buffer (100 mM Tris–HCl, pH 9.0; 100 mM NaCl; 10 mM Na_2EDTA, pH 8.0, and 2% SDS), 50 μl 2-mercaptoethanol and 2.5 ml phenol were added to the homogenized samples. The mixture was supplemented with 2.5 ml chloroform, shaken gently for 5 min, and centrifuged for 10 min at 2500g. Phenol-chloroform extraction was performed twice on the upper phase. Nucleic acids were precipitated adding 0.1 vol of 3 M NaAc, pH 5.2, and 3 vol of absolute ethanol for 30 min at −80 °C. After centrifugation at 2500g for 30 min at 4 °C, the pellet was resuspended in 2.5 ml DEPC–water, and 2.5 ml of 5 M LiCl were added to precipitate the RNA overnight at 4 °C. After centrifugation at 2500g for 30 min at 4 °C, the pellet was resuspended in 1 ml DEPC–water, and RNA was precipitated adding 0.1 vol of 3 M NaAc, pH 5.2, and 3 vol of absolute ethanol for 30 min at −80 °C. Finally, the pellet was washed twice with 70% ethanol and resuspended in 25 μl DEPC–water for analyses.

Quality of RNA was verified by demonstration of intact ribosomal bands in agarose gel electrophoresis, in addition to 1.8-2.0 absorbance ratios ($A_{260/280}$ and $A_{260/230}$, respectively). Contaminating genomic DNA was removed using the

TURBO DNA-free kit, according to manufacturer's instructions (Ambion, USA). First-strand cDNA was synthesized from 5 µg of DNA-free total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with oligo (dT) 20 primer, following the manufacturer's instructions.

2.5. Quantitative Real-Time PCR (qRT-PCR)

Gene expression analysis was performed by qRT-PCR, using a CFX Connect real-time PCR System and iTaq Universal SYBR Green Supermix (BioRad, California, USA). Primers for gene-specific amplification were previously designed using the Primer3 program (http://bioinfo.ut.ee/primer3/) for *OeSAD* genes (Parvini et al., 2016), *OeFAD2* and *OeFAD6* genes (Hernández et al., 2009), and *OeFAD3* and *OeFAD7* genes (Hernández et al., 2016). The primers sequences are reported in Supplementary Table 1. Reaction mix (10 µL per well) contained 1X iTaq-QPCR Master Mix, 100 nM forward and reverse primers, and 2 µL of cDNA of appropriate dilution, which was selected according to the primers amplification efficiency. The thermal cycling conditions consisted of an initial denaturation step of 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 30 s, 60 ºC for 1 min, and 72 ºC for 30 s. The specificity of the PCR amplification and the presence of primer dimers was monitored by melting curve analysis following the final step of the PCR, and beginning at 55 ºC through 95 ºC, at 0.1 ºC s^{-1}. Additionally, PCR products were also checked for purity by agarose gel electrophoresis. PCR efficiencies (*E*) of all primers were calculated using dilution curves with eight

dilution points, two-fold dilution, and the equation $E = [10^{(-1/slope)}] - 1$. The housekeeping olive ubiquitin2 gene (*OeUBQ2*, AF429430) was used as an endogenous reference for normalization. The relative expression level of each gene was calculated using the equation $2^{-\Delta CT}$ where $\Delta CT = (CT_{GOI} - CT_{UBQ2})$ (Livak and Schmittgen, 2001; Pfaffl, 2004). This method gave us an advantage to make comparisons in the level of gene expression across developmental stages and genes. The data are presented as means ± SD of three reactions performed in different 96-well plates, each having two replicates in each plate.

2.6. Statistical analysis and experimental design

The experiment was set up in a completely randomized design (n = 8). Data of shoot elongation (n = 8), photosystem II performance (n = 8), rachis, stem and leaves Na concentration (n = 6), olive fruit traits (n = 8), mesocarp Na concentration (n = 4), and finally, fatty acid composition, ratios and gene expression analysis (n = 3) were subjected to one tailed *t*-test ($P<0.05$). Fruit distribution frequency among maturation groups was analysed by χ^2 test. A two tailed Pearson correlation analysis and linear regression ($P<0.05$) was performed for Na concentration, fatty acids composition and genes expression data in fruit mesocarp. All percentage data were undergone to arcsine square root transformation. Graphs and statistical elaboration were made using Prism5 (GraphPad Software, San Diego, USA).

3. Results

3.1 Physiological effects in plant, and Na accumulation

Leccino treated plants showed a significant reduction of shoot elongation starting to day 60 (-9%) until the end of the experiment (-12%) in comparison with control plants (Fig. 1A).

The reduction was associated with the translocation of Na in the aerial part of Leccino with an average value of 1199, 2493, 4352 mg kg^{-1} in leaves, stem and rachis, respectively (Table 1).

Chl *a* fluorescence in treated plants (80 mM NaCl) indicated a significant decrease in PSII maximum efficiency within dark-adapted leaves (F_v/F_m) starting from the day 45 (–4%) to –8% after 75 days (Fig. 1B). The decrease of Chl *a* fluorescence was observed two weeks before the start of reduction of shoot elongation.

Significant differences in actual quantum yield of PSII (Φ_{PSII}) were also recorded, starting from 30 days of Na exposure, where the difference among control and salt treated plants were 6±4.1% and remaining significantly lower until the end of the experiment (Fig. 1C). Salt treatment (80 mM NaCl) reduce mesocarp FW in MG_0 (-53%) and MG_1 (-25%), as well as the fruit volume in MG_1 (-22%) and the flesh/pit ratio in MG_0 (-75%).

As concern the ripening acceleration of salt-treated fruits, a different MG distribution has been observed in relation to the Na treatments with an increment of

percentage of olives per plant in the MG_3 under salt treatment (31 *vs* 52% in control and 80 mM NaCl treated plants respectively) (Fig. 2A).

Na concentrations in olive mesocarp increase in each MG after salt treatment reaching the range of 2654-1139 mg kg^{-1} DW while in controls plant, the Na background level was in the range of 329-186 mg kg^{-1} DW (Fig. 2 B). The highest difference compared to control was detect in in MG_1 salt treated fruits (329 *vs* 2654 mg kg^{-1}) (Fig. 2 B).

3.3 Olive mesocarp fatty acid composition

The total fatty acid content was analyzed in olive mesocarp showing a significant decrease at the beginning and at the end of fruits ripening in 80 mM NaCl treated plants. In particular, a reduction of -33% and -24% was detected in MG_0 and MG_3 respectively (Fig. 3).

Oleic acid was the main fatty acid found in the mesocarp (Fig. 4) representing around 70% of fatty acids in both salt treatments. The significant decrease of total fatty acid contents observed at the beginning (MG_0) and at the end (MG_3) of fruits ripening in 80 mM NaCl treated plants, was also observed for fatty acid composition at $MG_{0\text{-}3}$. More in details, palmitic acid, oleic acid and linoleic acid decrease of 30, 33 and 35% in MG_0 and of 24, 34 and 42% in MG_3 respectively.

Otherwise a significant increase of 53% in oleic acid and 42% of palmitic acid were observed at MG_1 in salt treated plants compared to control (Fig. 4). Therefore, the oleic/linoleic ratio increased in salt treated plants only in MG_1 and, as a

consequence, the PUFA/MUFA ratio was lower in comparison to control plants (Table 3).

3.4 Fatty acid desaturase genes expression

The relationship among fatty acid composition and the transcription levels of fatty acid desaturase genes at the four MG were investigated. *OeSAD2* gene expression levels were highest in comparison to *OeSAD1* and *OeSAD3* genes under control condition and salt treatment (Fig. 5).

Among the oleate desaturase genes studied, the *OeFAD2-2* gene showed the highest expression levels (Fig. 6), while *OeFAD2-1* gene transcripts were undetectable at the maturation stages studied (data not shown). With respect to linoleate desaturase genes (Fig. 7), we observed higher expression levels of plastidial genes than those of microsomal ones. *OeFAD3A* expression levels were very low throughout fruit development and maturation, whereas *OeFAD3B* transcripts were not detected at the maturation stages studied (data not shown). In contrast, *OeFAD7-1* and *OeFAD7-2* expression levels increased during olive mesocarp ripening (Fig. 7).

Salt stress affected fatty acid desaturases transcript levels at the different MG (Figs. 5-7). In particular, among *SAD* genes, *OeSAD1* and *OeSAD2* showed an increase in their transcript levels compared to control at MG_{0-2} and MG_{2-3}, respectively (Fig. 5 A, B).

Regarding oleate desaturases, *OeFAD6* expression levels decreased in the salt stressed mesocarp (Fig. 6A), while *OeFAD2-2* gene in MG_1 salt stressed mesocarp resulted up-regulated (Fig. 6B). On the other hand, a slight increase in *FAD7-1* expression levels was detected in $MG_{1\text{-}2}$ salt stressed mesocarp, in comparison to control one (Fig. 7A).

4. Discussion

The possibility of using water of low quality, such as saline water, to help the increasing water demand for agricultural development in the olive orchard, is considered from several authors as a promising alternative (Chartzoulakis, 2005) but requires an adequate understanding on its effect on plant physiology and development. Leccino plants, considered sensitive to salt stress (Tattini et al., 1994), give us the opportunity to understand the effect of salinity on fruit. In saline conditions, the shoot elongation of Leccino is reduced with respect to control plants (Koubouris et al., 2015; Moretti et al., 2018; Rossi et al., 2015; Tattini et al., 1992). Under salinity, a decrease in Φ_{PSII} efficiency (Zribi et al., 2009) and a decrease of maximum photosynthetic efficiency (Moretti et al., 2018) proved that Leccino perceive the stress.

As concern fruits, ripening is faster in the stressed plants and Na accumulation in Leccino mesocarp causes an acceleration of maturation process, also observed in "Barnea" trees under two levels of saline irrigation (4.2 and 7.5 dS m^{-1} EC) (Wiesman et al., 2004). Despite olives are non-climacteric fruit, and ethylene

production by ripening olives has been reported to be non-detectable (Rugini et al., 1982), under salt treatment ripening acceleration of salt-treated fruits have been observed. Under salinity, it has been demonstrated that the ethylene production could be quickly stimulated (Tao et al., 2015), and this could be related to the higher number of fruits at MG_3 which was detected in Leccino salt treated plants.

The Na accumulation in mesocarp is reached in each MG, indicating a direct Na translocation in fruit, that also induce a decrease of drupe volume, flesh/pit ratio and mesocarp FW in early maturation stage.

Ben-Ahmed et al. (2009) reported that 'Chemlali' olives in an orchard irrigated with high saline (EC = 7.5 dS m^{-1}) for two years had lower fruit weight and size, compared with good quality (EC = 1.2 dS m^{-1}) water.

The decrease of palmitic acid, oleic acid and linoleic acid at the beginning (MG_0) and at the end (MG_3) of fruits ripening in salt treated plants, was a consequence of the decrease of total fatty acid contents. Those findings are in agreement with Stefanoudaki et al. (2009), who observed an oil % content reduction together with increasing concentration of NaCl in irrigation water, with more pronounced effects on "Koroneiki", the cultivar less salt-tolerant among those studied. Na accumulation did not change oleic/linoleic ratio in mesocarp of MG_0 and MG_{2-3}, indicating that future olive oil will keep stability during storage maintaining its health properties (Rotondi et al., 2004). On the contrary, oleic/linoleic ratio rather increase at MG_1 such as palmitic acid as previously detected in Koroneiki olives irrigated with 100 and 150 mM NaCl, and in Chemlali olives irrigated with

moderately saline water (4.7 dS m^{-1} of EC) (Bedbabis et al., 2010; Stefanoudaki et al., 2009).

The fatty acid desaturases transcript levels found in the control plants, confirmed that *OeSAD2, OeFAD2-2* and both *OeFAD7* genes are the main contributors in olive mesocarp to the oleic, linoleic and linolenic acid synthesis, respectively, as reported previously in Picual and Arbequina (Hernández et al., 2009; 2016; Parvini et al., 2016). The participation of fatty acid desaturases in the response to saline stress was demonstrated by Im et al. (2002), who reported that the antisense expression of Arabidopsis *FAD7* gene in transgenic tobacco plants reduced salt tolerance. More recently, it has been described that salt stress supressed the growth of transgenic tomato plants overexpressing tomato *FAD3* antisense sequence (Wang et al., 2014).

Regarding olive *SAD* genes, results showed that Na accumulation in mesocarp brought a general trend of increase of *OeSAD1* expression level, and about an increase of *OeSAD2* expression in MG_{2-3} in contrast to *SAD* gene down-regulation in leaves of *Phaseolus lunatus* under salt stress (Zhang et al., 2011).

OeSAD1 gene up-regulation together with *OeFAD6* gene down-regulation, could explain the increase of oleic acid (and so the oleic/linoleic ratio) in MG_1 salt treated fruit mesocarp.

FAD6 gene was demonstrated to be very important in salt tolerance of *Arabidopsis thaliana* 8-day-old seedlings under 300 mM NaCl treatment (Zhang et al., 2009)., showing an increase in expression levels after 24h of salt treatment. Moreover, in

the Arabidopsis *fad6* mutant, Na accumulation reached higher level in comparison to wild type, suggesting that FAD6 protein was involved in ions homeostasis. In fact, even though the mechanism remains to be clarified, it has been hypothesized that disruption of FAD6 function impaired the integrity of cell membranes at high salinity condition, mostly for the decrement of polyunsaturation level of thylakoidal fatty acid membrane (Zhang et al., 2009). Anyway, although the decrease of expression levels of *FAD6* has been observed at MG_0, MG_1 and MG_3, a clear decrease of polyunsaturation level, expressed by lower PUFA/MUFA ratio, has been observed only at MG_1, because the increment of the monounsaturated oleic acid. The decrease of PUFAs level was reported also by Bebdabis et al. (2010) in "Chemlali" olive oil under moderate saline water irrigation (EC=4.7 ds/m).

OeFAD2-2 gene increased their transcript levels in MG_1 fruits mesocarp and similar results were obtained in *Phaseolus lunatus* leaves, where *FAD2* gene was induced by 0.15 mM NaCl (Zhang et al., 2011). Moreover, treatment with 300 mM NaCl in *Arabidopsis thaliana* seedlings showed an up regulation in *FAD2* starting after 6h of the treatment (Zhang et al., 2012). Once more, this is because lipid composition and high level of polyunsaturated fatty acids (PUFAs) in plants membrane largely affect tolerance to abiotic stress such as salt stress, mostly for the essential role in the biophysical characteristics and proper function of membrane-attached proteins (Berberich et al., 1998; Chalbi et al., 2015; Cooke and Burden, 1990; Deuticke and Haest, 1987; Mikami and Murata, 2003; Upchurch,

2008; Wu et al., 2005). In fact, in Arabidopsis *fad2* mutant, vacuolar and plasma membrane polyunsaturation was lower, and the Na^+/H^+ antiporters exchange activity was reduced. As a consequence, *fad2* accumulated more Na^+ in the cytoplasm of roots and was more sensitive to salt stress during early seedling growth. This suggests that FAD2 mediates vacuolar and plasma membrane fatty acid desaturation, essential for the proper function of membrane attached Na^+/H^+ exchangers, and to maintain a low cytosolic Na^+ concentration for salt tolerance its relative gene expression is increased (Zhang et al., 2012). A good polyunsaturation level in plants membranes and the up regulation of *OeFAD2-2* observed in our experiment could be and effort by plant to maintain this membrane integrity, in response to the down-regulation of *OeFAD6*, which tend to decrease the PUFAs level.

In relation to olive *FAD7* genes, the slight increase observed in *OeFAD7-1* transcript levels in $MG_{1\text{-}2}$ salt stressed mesocarp was in agreement with the induction of *FAD7* gene observed in maize roots treated with 0.4 M NaCl (Berberich et al., 1998).However, except in the case of *OeFAD6, OeSAD1* and *OeSAD2* genes, we could not observe a correlation between changes in fatty acid composition and those detected in fatty acid desaturase genes expression levels in developing mesocarp under saline irrigation conditions. One possible explanation could be that fatty acid composition of total lipids determined in this work reflects mainly fatty acids esterified to triacylglycerols. It could be possible that other minor lipids, like phospholipids or galactolipids, undergo changes in their fatty acid

composition. In addition, the existence of post-transcriptional regulatory mechanisms of olive fatty acid desaturase genes cannot be discarded.

Data reported represent a step forward in understanding the molecular regulation of fatty acid desaturation pathway under salinity, one of the most important environmental problems in the Mediterranean area. Considering that 80 mM NaCl treatment in Leccino fruit accelerate ripening and that the fatty acid content at the end of the ripening period decrease, a decrement of the oil yield could occur in the final product. Therefore, the use of salt water irrigation in Leccino plants from pit hardening to veraison has to be investigated more, in terms of timing of salt treatments and Na application in order to elucidate the complexity of salinity stress and fatty acid pathway interaction. Moreover, the possible application for direct agronomical practice, like irrigation (in mature olive orchards) alternating high quality water with different level of saline water will be necessary.

Table 1. Na concentration (mg kg^{-1} DW) in leaves, stem and rachis, of *Olea europaea* L. cultivar Leccino after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Values are means ± standard deviation (n = 6). Data were analyzed by one tailed *t*-test. ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$, ns = not significant.

	NaCl (mM)		*t*-test
	0	80	
Leaves	130±53.9	1200±618.6	***
Stem	190±94.7	2494±911.2	***
Rachis	323±178.9	4352±1562.9	***

Table 2. Leccino drupe parameters from MG_0, MG_1, MG_2 and MG_3 after 75 days from the beginning of the experiment. For flesh/pit ratio, Volume (mm^3), fresh weight (FW), dry weight (DW), each value is the mean ± standard deviation of (n=8). Results were analyzed by *t*-test.

Parameters	Maturation Group (MG)	NaCl (mM)		*t*-test
		0	80	
Flesh/Pit ratio	MG_0	0.8±0.35	0.2±0.04	*
	MG_1	1.5±1.23	0.6±0.13	ns
	MG_2	1.5±1.56	0.7±0.2	ns
	MG_3	0.8±0.24	1.0±0.13	ns
Volume (cm^3)	MG_0	2.3±0.65	1.9±0.54	ns
	MG_1	2.3±0.31	1.8±0.48	*
	MG_2	2.3±0.40	2.1±0.37	ns
	MG_3	2.3±0.31	2.3±0.11	ns
FW Flesh (g)	MG_0	2.1±0.02	1.0±0.16	***
	MG_1	2±0.33	1.5±0.27	*
	MG_2	2.1±0.44	1.8±0.15	ns
	MG_3	2.4±0.49	2.1±0.24	ns
DW Flesh (g)	MG_0	0.5±0.23	0.4±0.10	ns
	MG_1	0.9±0.65	0.4±0.11	ns
	MG_2	1.0±0.89	0.5±0.08	ns
	MG_3	0.6±0.18	0.06±0.09	ns

Table 3. Oleic/linoleic ratio and PUFA/MUFA in each maturation group of Leccino mesocarp after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Data (n=3) are means ± standard deviation. Data were analyzed with one tailed *t*-test. *, $P<0.05$, ns = not significant.

Parameters	Maturation group	NaCl (mM)		*t*-test
		0	80	
Oleic/linoleic	MG_0	8.71±1.1	9.20±1.2	ns
	MG_1	8.13±1.4	11.47±0.9	*
	MG_2	13.47±3.3	10.39±0.6	ns
	MG_3	9.06±0.7	10.71±2.4	ns
PUFA/MUFA	MG_0	0.13±0.01	0.12±0.01	ns
	MG_1	0.13±0.02	0.09±0.01	*
	MG_2	0.08±0.02	0.10±0.01	ns
	MG_3	0.12±0.01	0.10±0.02	ns

Figure legends

Fig. 1. Time course of (A) Shoot length, (B) maximum photosynthetic efficiency (Fv/Fm), (C) Φ_{PSII} (actual quantum yield of PSII - arbitrary units) every 15 days in leaves near to infructescence of (*Olea europaea* L.) cultivar Leccino during 75 days of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Values are the means ± standard deviation (n = 8). Results for each timing day were analyzed by one tailed t-test. Data significantly different are indicate with *, P<0.05. **, P<0.01; ***, P<0.001. ns= not significant.

Fig. 2. (A) Distribution of olives per plants (%) in different Maturation Group (MG) after 75 days of 80 mM NaCl treatment in comparison to control (0 mM NaCl). (B) Na concentration (n = 4) in mesocarp of *Olea europaea* L. cultivar Leccino in different MG. For each MG data were analyzed by one tailed t-test. ***, P<0.001; *, P<0.05, χ2-test was performed and significant difference are reported in table.

Fig. 3. Total fatty acid content (µg mg^{-1} DW) in each MG of *Olea europaea* L. cultivar Leccino mesocarp after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Values are means + standard deviation (n = 3). For each MG data were analyzed by one tailed *t*-test. *, *P*<0.05; ns= not significant.

Fig. 4. Fatty acid composition in maturation groups (MG0, MG1, MG2, MG3) of Leccino mesocarp after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Palmitic acid (16:0); palmitoleic acid (16:1); stearic acid (18:0); oleic acid (18:1); linoleic acid (18:2); linolenic acid (18:3). Data (n = 3) are means + standard deviation and were analyzed with one tailed t-test. **, P<0.01; *, P<0.05, ns = not significant.

Fig. 5. *OeSAD1* (A), *OeSAD2* (B), *OeSAD3* (C) relative transcript abundance in each maturation group (MG) of Leccino mesocarp after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Bars represent mean + standard deviation. Data were analyzed with one tailed t-test (n=3). ns= not significant; *= P<0.05; **= P<0.01; ***=P<0.001.

Fig. 6. *OeFAD6* (A), *OeFAD2-2* (B) relative transcript abundance in each maturation group (MG) of Leccino mesocarp after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Bars represent mean + standard deviation. Data were analyzed with one tailed t-test (n=3). ns= not significant; *= P<0.05; **= P<0.01; ***=P<0.001.

Fig. 7. *OeFAD7-1* (A), *OeFAD7-2* (B), *OeFAD3A* (C) relative transcript abundance in each maturation group (MG) of Leccino mesocarp after 75 days (165 DAF) of 80 mM NaCl treatment in comparison to control (0 mM NaCl). Bars represent mean + standard deviation. Data were analyzed with one tailed t-test (n=3). *= P<0.05; **= P<0.01; ***=P<0.001; ns= not significant.

Conflict of interest

The authors have no conflicts of interest to declare.

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Contributions

SM he has contributed to, data collection and elaboration (molecular and physiological analyse, statistical elaboration of data), manuscript draft and its final approval.

AF she has contributed to the planning of the experiment, data collection and elaboration (sampling olive fruits, Na concentration, chlorophyll fluorescence analyse, statistical elaboration of data), manuscript draft and its final approval.

MLH she has contributed to data collection and elaboration (fatty acid analysis and desaturase genes expression) statistical analyses of data, manuscript draft and its final approval.

JMMR he has contributed to fatty acid analysis and desaturase genes expression data elaboration, manuscript draft and its final approval.

LS he has contributed to the planning of the experiment, statistical analyses of data, manuscript draft and its final approval.

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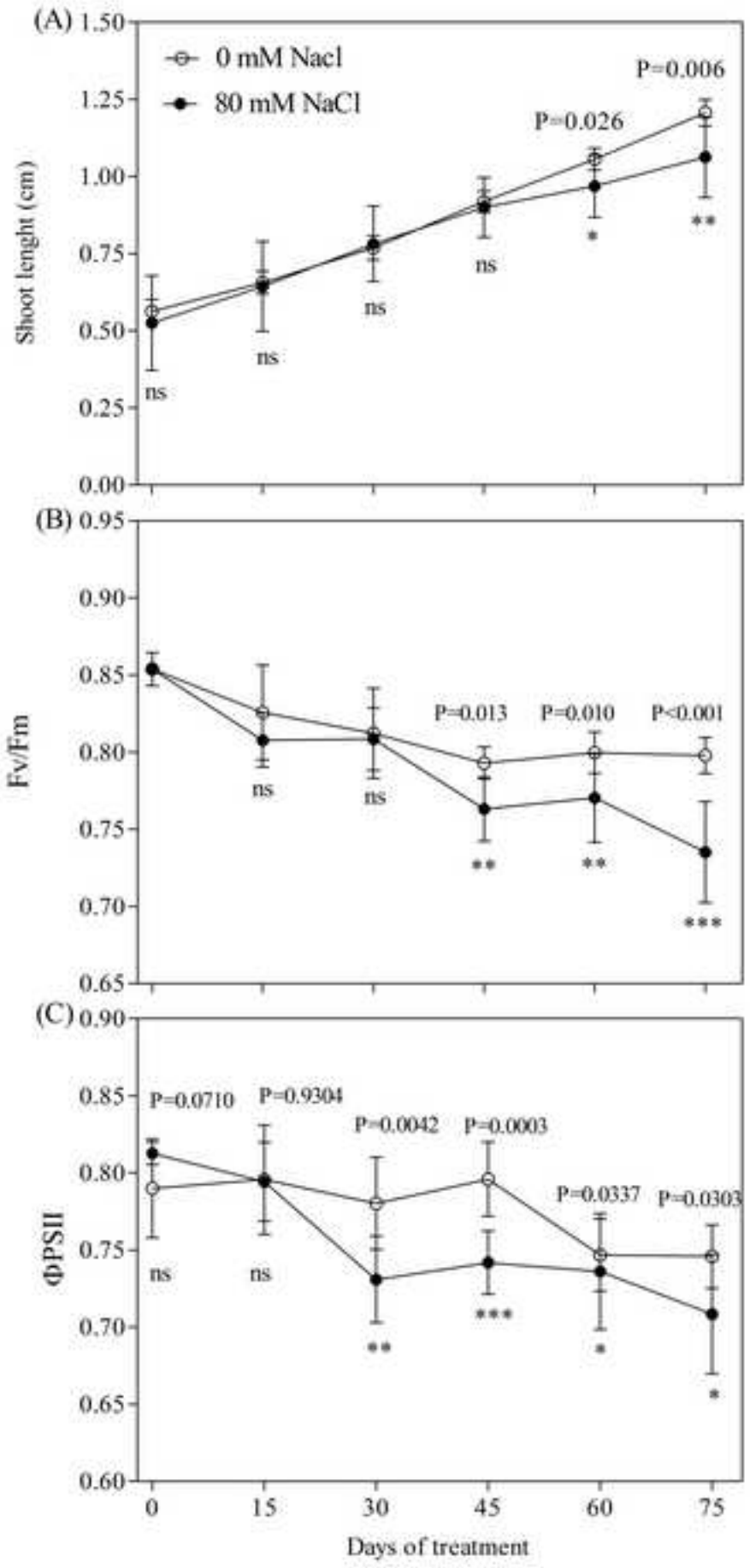

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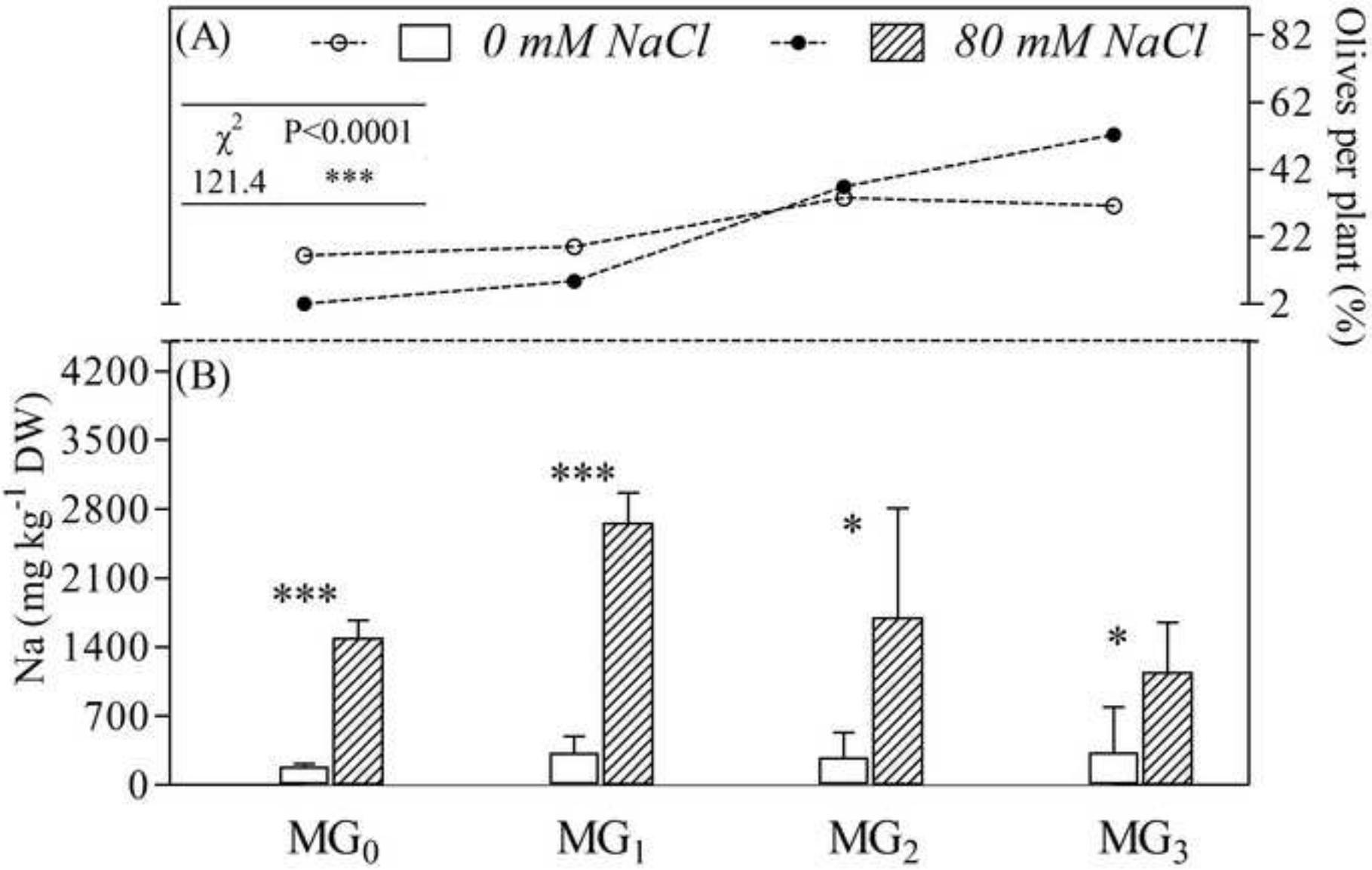

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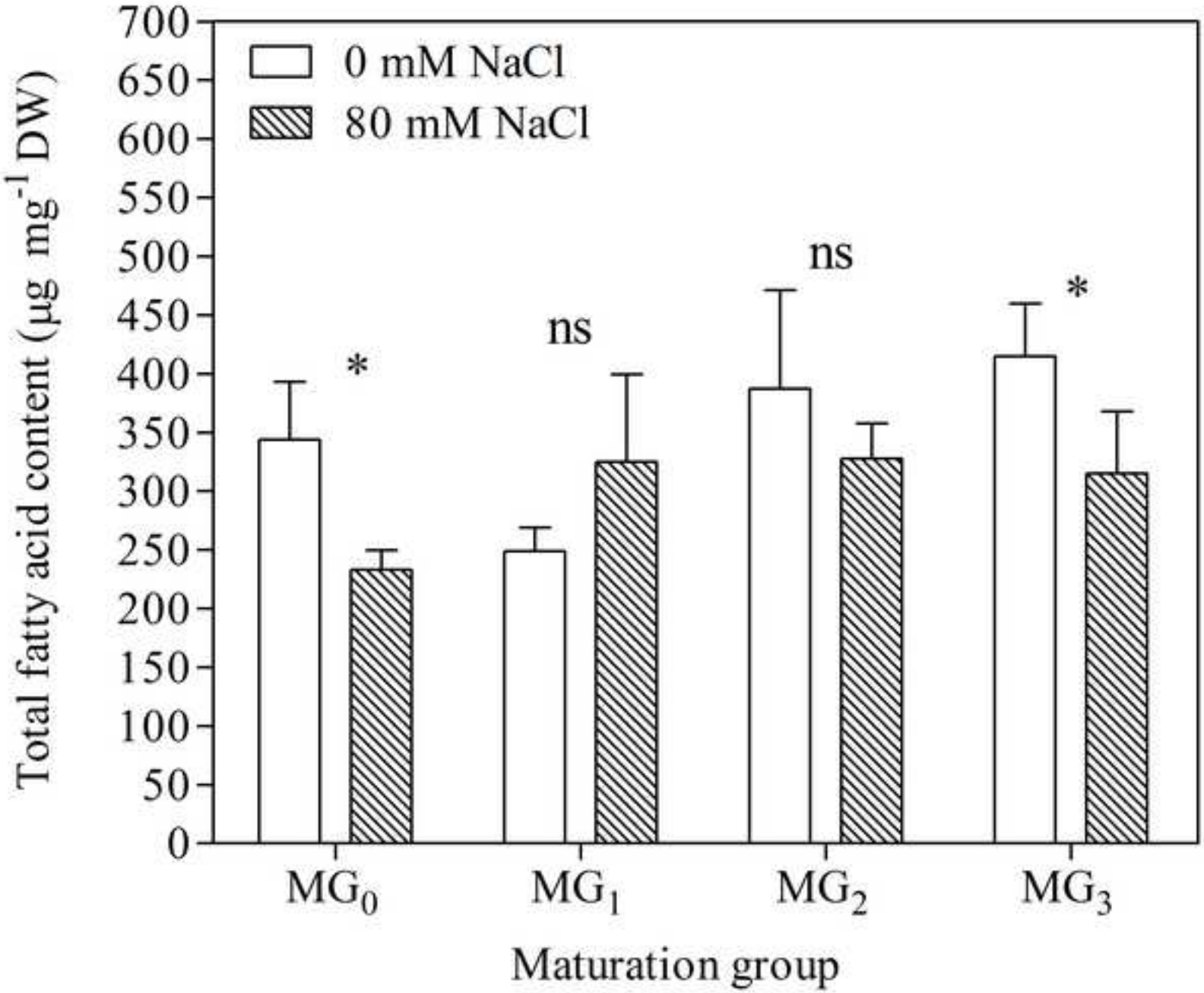

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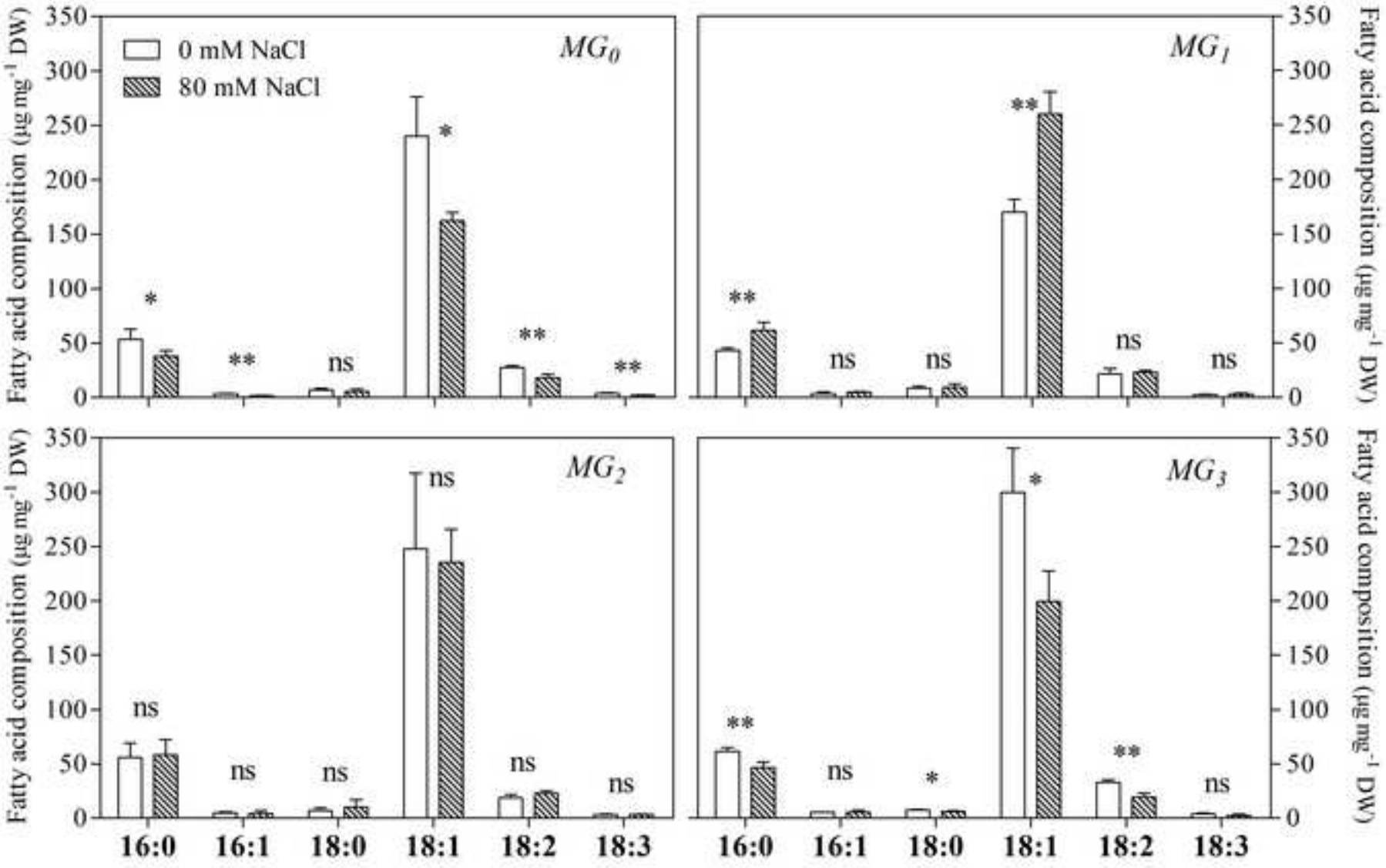

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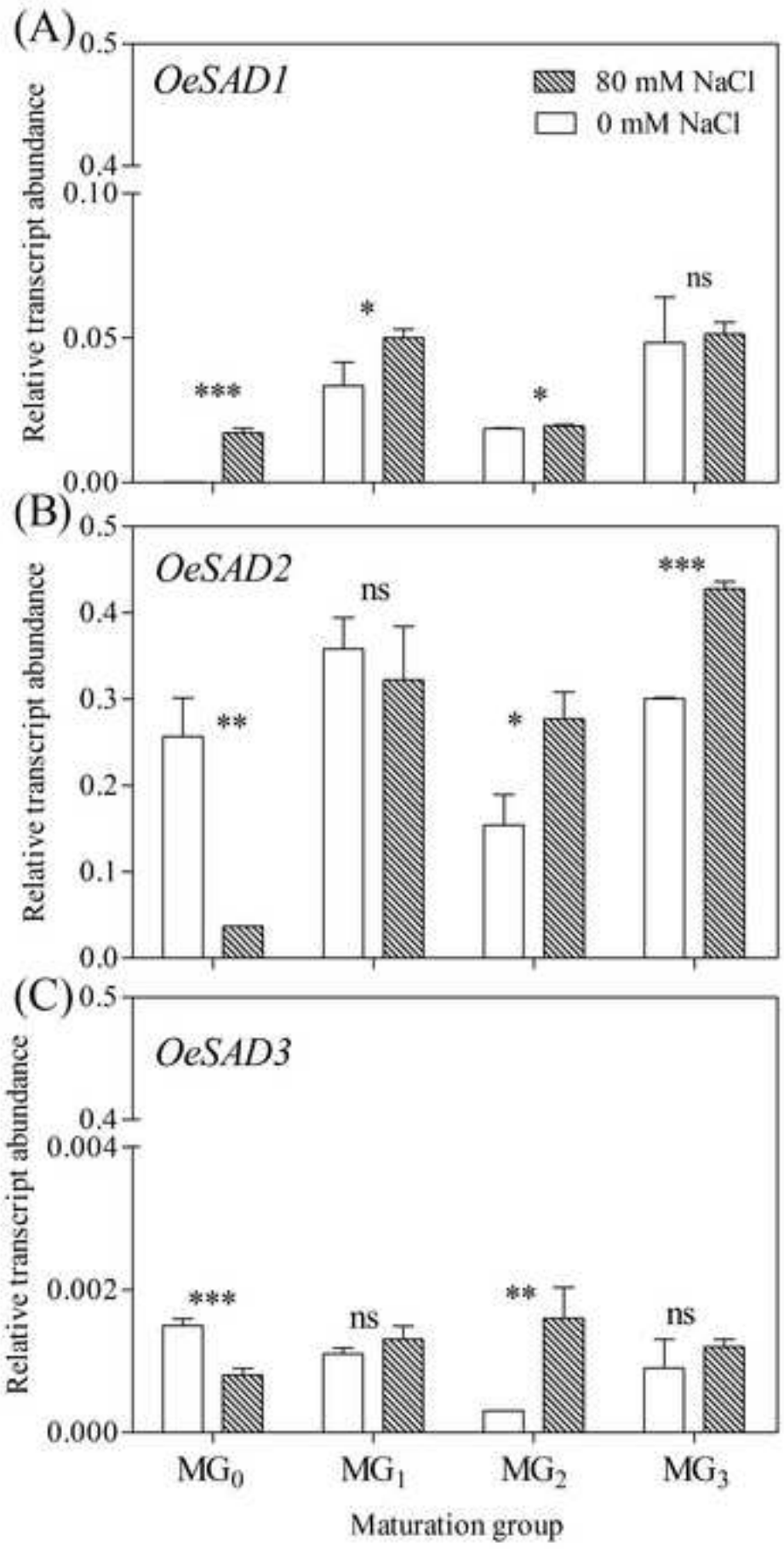

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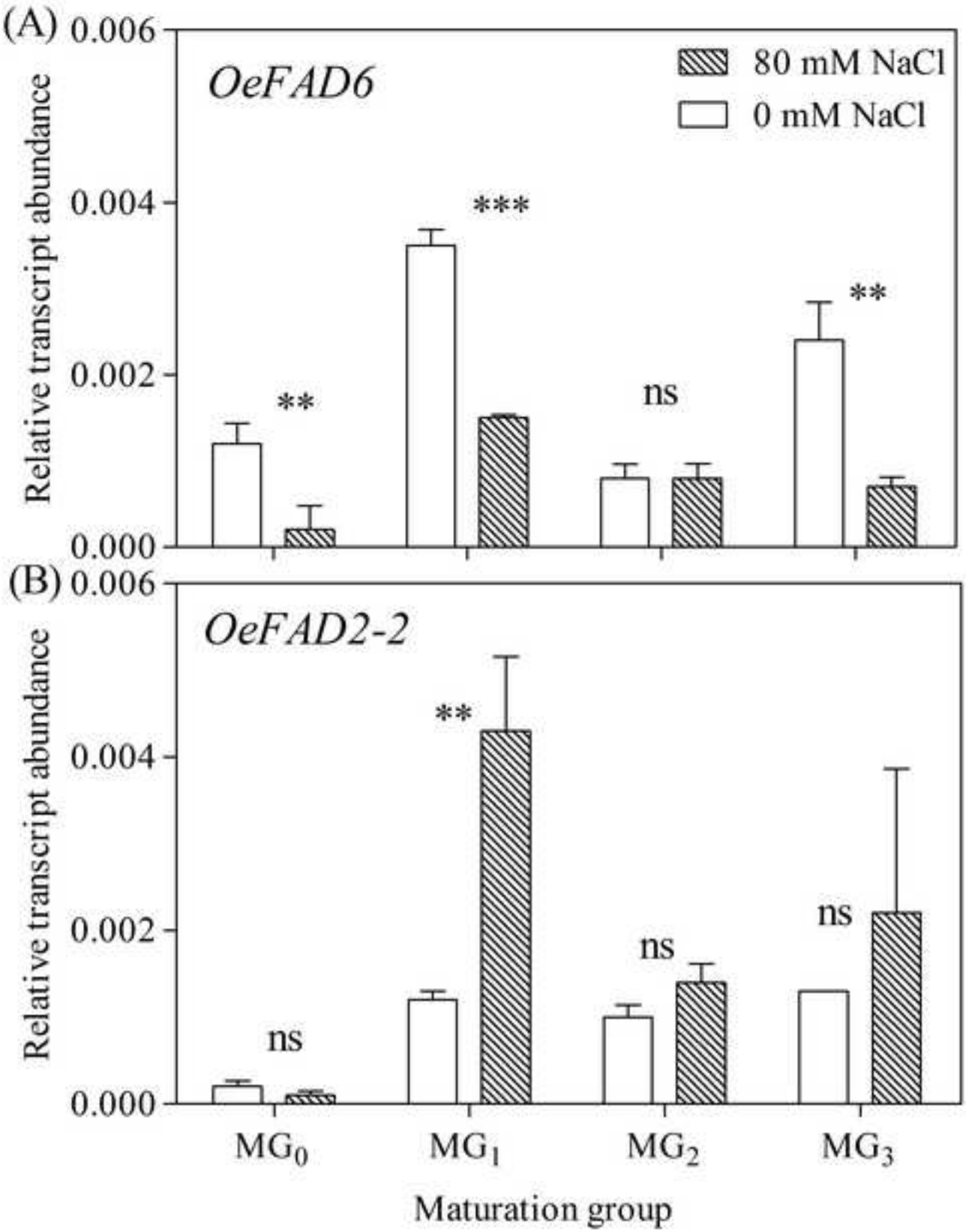

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