PEG induces high expression of the cell cycle checkpoint gene *WEE1* in embryogenic callus of *Medicago truncatula*: potential link between cell cycle checkpoint regulation and osmotic stress

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Abstract

Polyethylene glycol (PEG) can be used to mimic osmotic stress in plant tissue cultures to study mechanisms of tolerance. The aim of this experiment was to investigate the effects of PEG (M.W. 6000) on embryogenic callus of *Medicago truncatula.* Leaf explants were cultured on MS medium with 2 mg L^{-1} NAA and 0.5 mg L^{-1} BAP for 5 months. Then, calli were transferred to the same medium further supplemented with 10% (w/v) 6000 PEG for six months in order to study physiological and putative molecular markers of water stress. There were no significant differences in growth rate of callus or mitotic index ± PEG although embryogenic potential of PEG treated callus was morphologically enhanced. Cells were rounder on PEG medium and cell size, nuclear size and endoreduplication increased in response to the PEG treatment. Significant increases in soluble sugar and proline accumulation occurred under PEG treatment compared with the control. Significantly, high *MtWEE1* and *MtCCS52* expression resulted from 6 months of PEG treatment with no significant differences in *MtSERK1* or *MtP5CS* expression but down regulation of *MtSO*S expression. The results are consistent in showing elevated expression of a cell cycle checkpoint gene, WEE1. It is likely that the cell cycle checkpoint surveillance machinery, that would include *WEE1* expression, is ameliorating the effects of the stress imposed by PEG.

217 words

Keywords: cell cycle, cell division, cell morphology, gene expression, *in vitro*, legumes, *Medicago truncatula*, water stress; *WEE1*

Introduction

Water stress can result in reducing crop yield world-wide (Boyer, 1982; Gonzalez *et al.*, 1995; Smirnoff, 1993) and a recent UN survey has underlined the importance of water deficit in our planet and its effects for the coming generations unless urgent measures are taken. This situation is exacerbated in arid and semiarid ecosystems. Here legumes play a central agroecological role through their ability to use atmospheric nitrogen via the symbiosis with Rhizobia, and thus it reduces the need for fertilizers, improve food security, and generally favour the environment (Araújo *et al.*, 2015; Kohler *et al.*, 2008; Naya *et al.*, 2007; Rubio *et al.*, 2002; Ochatt, 2015). Studying a legume model species is thus timely and *Medicago truncatula* is of particular interest given its rather short life cycle and autogamy. It has a small and almost completely annotated genome (500–550 Mbp) which is publicly available (Goodstein *et al.*, 2012), and it is more drought tolerant than other legume crops such as pea, bean and soybean (Costa França *et al.*, 2000; Galvez *et al.*, 2005;Gonzalez *et al.*, 1998; Motan *et al.*, 1994). In spite of this, previous studies on water stress resistance in *M. truncatula* mostly concerned gene transfer (Alcântara *et al.*, 2015; Araújo *et al.*, 2015; Duque *et al.*, 2016). The assessment of physiological responses (Nunes *et al.*, 2008) and their genetic mechanisms (Badri *et al.*, 2011) is more limited.

Osmotic stress or water deficit can be defined as the absence of adequate moisture necessary for a plant to grow normally and complete its life cycle (Cabuslay *et al.*, 2002). Resistance mechanisms can be grouped into three categories: firstly escape, which enables the plant to complete its life cycle before the most intense period of water shortage, secondly avoidance, which prevents exposure to water stress, and finally tolerance, which enables the plant to withstand stress conditions (Golldack *et al.*, 2014; Levitt, 1972; Zhu, 2002). Some resistance mechanisms are constitutive and active before exposure to water shortage. In other cases, plants exposed to water stress alter their physiology, thereby acclimating themselves to withstand drier conditions. One of the tolerance mechanisms activated under such stress is that of mitigating osmotic stress, via the production of osmolytes such as proline, and soluble sugars, that protect cells against osmotic perturbation (Deinlein *et al.*, 2014; Choudhary *et al.*, 2005; Fulda *et al.*, 2011; Elmaghrabi *et al.*, 2013; Valliyodan and Nguyen, 2006). On the other hand, φw (water potential) is also known to induce a morphological variation in tissues subjected to osmotic stress, notably at the cellular level. Such variation is potentially useful to understand biodiversity by identifying cellular responses to stress that are not necessarily picked up by taxonomic or phylogenetic indices that consider cell shape or size *in vitro* (Ochatt *et al.*, 2008; Ochatt and Moessner, 2010). It is also important for assessing the competence for regeneration

in vitro (Ochatt *et al.*, 2008) following the recovery of tissues with a novel genetic makeup obtained via *in vitro* selection (Elmaghrabi *et al.*, 2013) or gene transfer (Alcântara *et al.*, 2015). Responses to abiotic stress factors involve a reprogramming of the expression of thousands of genes, which in turn result in the modification of a range of cellular and physiological processes (Cushman and Bohnert, 2000; Sreenivasulu *et al.*, 2004; Araújo *et al.*, 2015). One example of tolerance to stress at the molecular level, is the induction of *P5CS* that encodes Δ^1-pyrroline -5-carboxylate synthetase involved in proline biosynthesis (Silva-Ortega *et al.*, 2008). This gene is highly expressed in salt-and drought-tolerant plant species (Choudhary *et al.*, 2005) and it is induced under salt and water stress in many plant species including legumes (Chen *et al.*, 2009). The *P5CS* gene was also up-regulated in *M. truncatula* in response to salt stress (Elmaghrabi *et al.*, 2013). The kinetics of expression of genes involved in the cell cycle in plants exposed to high levels of abiotic stress has been the object of a number of studies (Gill and Tuteja, 2010; Roy, 2016; Zhao *et al.*, 2014). In Arabidopsis, a negative regulator of mitosis, *WEE1*, is strongly expressed in response to abiotic stress (De Schutter *et al.*, 2007). Osmotic stress imposed using PEG also up-regulated oxidative DNA damage and consequently DNA repair enzymes both in imbibed seeds (Balestrazzi *et al.*, 2011) and in plantlets (Macovei *et al.*, 2010). Our recent work with *M. truncatula* also showed an increased expression of *WEE1* and *CCS52* (*CELL CYCLE SWITCH PROTEIN 52*, another gene involved in the cell cycle) in salt-acclimated tissues as well as expression of genes involved in salt tolerance (*SOS1* encoding a Na^+/H^+ antiporter) and embryogenesis *in vitro* (*SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1*, *SERK1*) (Elmaghrabi *et al.*, 2013).

Tissue culture has been used in the selection of water stress tolerant cell lines that have been used to regenerate plants resistant to harsh environmental conditions in a range of crops including *Medicago sativa* L., tomato, soybean and wheat (Sakthivelu *et al.*, 2008; Guóth *et al.*, 2010; Mahmood *et al.*, 2012). Water deficit *in vitro* can be imposed through treatment with PEG 6000 (Ochatt *et al.*, 1998; Guóth *et al.*, 2010; Yang *et al.*, 2012; Rai *et al.*, 2011). The adsorbant property of this inert osmolyte provokes in plant cells and tissues the same or comparable effects to those obtained by drying soil at the same φw and without any other associated detrimental effects (Michel and Kaufmann, 1973). PEG 6000 thus closely mimics soil water stress (Lu *et al.*, 1998) and induces increases in total soluble sugars which serve as an osmoticum, or can be a source of respiratory substrates (Srivastava *et al.*, 1995; Elmaghrabi *et al.*, 2013). Additionally, PEG was shown to stimulate somatic embryogenesis *in vitro* (Attree *et al.*, 1995; Igasaki *et al.*, 2003). PEG 6000 was also used, and at similar concentrations as here

(although osmolarity was expressed in MPa rather than in mOsm/kg as in this work), in studies on PEG-induced DNA damage with *M. truncatula in vitro* plantlets (Macovei *et al.*, 2010) and seeds (Balestrazzi *et al.*, 2011).

As many legumes are grown (or have their center of origin) in regions with an arid to semi-arid climate (Smýkal *et al.*, 2015), a number of studies have identified genes, QTLs, ESTs and SNPs that are responsive to drought stress in several species (Jacob *et al.*, 2016). However, the molecular basis of water stress tolerance is not fully understood in *Medicago truncatula* (Araújo *et al.*, 2015). The aim of the current work was to examine the extent to which treatments with PEG could enhance osmotic stress tolerance potential in callus of *Medicago truncatula*. In addition, the accumulation of osmoprotectants, the effects on cell morphology (shape and size) and division competence, and the expression of *WEE1*, *CCSS52*, *P5CS* and *SOS1* were monitored in the PEG treatments to investigate the mechanism underlying the induced water stress responses as compared to those activated in response to salt stress (Elmaghrabi *et al.*, 2013). The expression of *SERK1* was also analyzed, given its key role on the competence for the subsequent regeneration through somatic embryogenesis of plants that may potentially carry the stress resistance trait acquired.

Materials and Methods

Plant material

Medicago truncatula cv. Jemalong line A17 (2n = 2x = 16, 1C value = 0.48 pg) was used in this study. One hundred leaves were explanted to tissue culture from 4 week old aseptically grown plants onto a medium consisting of MS basal medium (Murashige and Skoog, 1962) with 2 mg L^{-1} NAA (alpha-naphthalene acetic acid; Sigma, Poole, UK) and 0.5 mg L^{-1} BAP (6-benzylaminopurine; Sigma, Poole, UK), hereafter called MANA medium as in Elmaghrabi *et al.* (2013), and dispensed in multi-well dishes as 2 mL aliquots per well. Cultures were incubated at 24/22 ºC (day/night), with a 16/8 h (light/ dark) photoperiod of 90 µmol m^{-2} s^{-1} from warm white fluorescent tubes. After 4 weeks, explants were sub-cultured on the medium above and the frequency of callus initiation assessed.

Leaf-derived embryogenic callus was obtained after culture on MANA medium for 5 months. Calli were screened for embryogenesis (i.e. somatic embryos at different developmental stages, identified as spherical glistening nodules when globular, through to elongated greening structures at later stages) or, organogenesis (development of shoots and/ or roots), as reported

elsewhere (Ochatt *et al.*, 1998; Elmaghrabi and Ochatt, 2006; Chen *et al.*, 2011; Ochatt *et al.*, 2013; Ochatt and Revilla, 2016). Only embryogenic calli were transferred onto 25 ml of MANA medium supplemented with or without 10 % w/v (-0.66 MPa) PEG6000 (PEG; Sigma, Poole, UK) for six months in order to acclimate the cultures under conditions that mimic water (osmotic) stress (at least 12 calli per treatment). This PEG concentration was chosen based on previous studies with various species (Biswas *et al.*, 2002, Guóth *et al.*, 2010) and also including *M. truncatula* (Macovei *et al.*, 2010, Balestrazzi *et al.*, 2011). Growth data (g fresh weight, g FW) were recorded and results were statistically analysed ($P \leq 0.05$; Kruskal Wallis followed by a Dunn's test).

Proline and water soluble carbohydrate measurements

Proline content was measured as described in Elmaghrabi *et al.* (2013) and according to Troll and Lindsley (1955) and Boukel and Houassine (1997) from callus tissue (100 mg per sample per treatment) grown on 0 and 10 % (w/v) PEG. All treatments were repeated three times. Optical density was measured using a spectrophotometer (UNICAM; Cambridge, UK) at a wavelength of 528 nm and calibrated using a standard curve of proline solutions (0.1–0.4 mg mL^{-1}; Sigma, Poole, UK).

Determination of soluble sugars was by the anthrone method (Elmaghrabi *et al.*, 2013; Plummer, 1987) using 100 mg callus samples from 0 and 10 % (w/v) PEG treatments (3 replicates). The soluble sugar content was measured spectrophotometrically (UNICAM, Cambridge, UK) at 585 nm and the data were converted to mg L^{-1} using the calibrations established prior to the assay.

Medium and callus osmolarity

For measurements of medium and callus osmolarity a Wescor (model VAPRO 5520, South Logan, USA) vapour pressure micro-osmometer was used and a minimum of three 10 µL samples were measured. For medium osmolarity assessments, 10 mL of the medium were vortexed prior to collecting the 10µL samples to be measured. For callus osmolarity measurements, 1 g fresh weight of tissue was collected in 2 mL of liquid medium and centrifuged (100 g, 10 min, 10°C). The supernatant was carefully removed, the pellet was crushed in an Eppendorf with a pestle and centrifuged (1000g, 10 min, 4°C), and this second supernatant was finally employed for measurements of osmolarity. Results from such measurements, expressed in $mMkg^{-1}$, are the mean $\pm$ S.E. of a minimum of three individual

samples per treatment, and were performed at the time of sub culturing and over at least three consecutive subcultures.

Mitotic index, cell viability and cell morphology

For determinations of C-value stability of calli following *in vitro* selection for several months they were compared to leaf tissues from the original plants. Nuclei were mechanically isolated from about 0.2 g of calli or from a single leaf of *M. truncatula* A17 grown in green house conditions. Tissues were chopped roughly with a sharp razor in 400µl of nuclei extraction buffer and 1.6 mL of staining buffer (Partec®; Canterbury, UK) (Ochatt, 2008). The suspension was filtered through a 20 µm nylon mesh and 4, 6 diamidino-2-phenylindole (DAPI; Sigma, Poole, UK), an A-T binding specific fluorochrome, was added to the filtrate to a final concentration of 1 µg mL^{-1}. The DNA contents of the isolated nuclei suspension were analysed using a Partec PAS-II flow cytometer equipped with an HBO-100 W mercury lamp and a dichroic mirror (TK420). Ten replicated calli for each treatment were analyzed, with a minimum of 3000 to 10000 nuclei per run. The mitotic index was calculated according to the formula: MI = 4 x 4C/ Σ 2C + 4C, where 2C and 4C correspond to the mean integrated value of nuclei in G1 phase and G2, respectively (Ochatt, 2008).

Cell viability was estimated by dual staining with fluorescein diacetate (FDA; Sigma, Poole, UK) and propidium iodide (PI; Sigma, Poole, UK). Cell suspensions (75 µL) from each treatment were mixed with 75 µL of dual staining solution containing FDA (200 µg mL^{-1}; Widholm, 1972) and propidium iodide (PI at 120 µg mL^{-1}) on ice and incubated for 20 min. The FDA molecule is cleaved by the esterases in the cytoplasm into acetate and fluorescein which, being hydrophilic accumulates in the cytoplasm of metabolically active (alive) cells that, upon excitation with the UV light fluoresce yellow-green, while dead cells appear red using a fluorescent microscope. A minimum of 300 cells are counted and results are expressed as the percentage of fluorescing cells referred to the total number of cells in the field.

For the cell morphology characterization, FDA stained slides of the control and PEG-treated cells were observed under the microscope under the UV. The surface area of cells and nuclei was determined at 2, 4 and 6 months of culture, using the image acquisition programmes ArchimedPlus and Histolab (Microvision, France) as reported (Ochatt *et al.*, 2008), and a shape coefficient (Ochatt and Moessner, 2010) was applied at 6 months of culture. Briefly, this shape coefficient (SC) is calculated based on the half length of the cell along its longest (a) and shortest (b) axes, as:

$$SC = \frac{\sqrt{a^2 - b^2}}{a}$$

For each treatment, nucleus and cell size were measured on 10 cells at 2 and 4 months of culture and at least 20 cells at 6 months of culture, and results expressed as the mean ± SE.

The SC distinguishes round from elongated shapes, since SC values close to 1.0 correspond to elongated cells while SC values close to 0.5 correspond to rounder cell shapes.

Real time PCR

RNA was extracted and genomic DNA removed by DNase treatment (Elmaghrabi *et al.*, 2013; Spadafora *et al.*, 2012), and its absence verified using 18S rRNA primers (Spadafora *et al.*, 2011). Retrotranscription was carried out using an Ambion kit (RETROscript Reverse transcription for RT-PCR; Foster City, USA) and 2 µg of RNA. An ABsoluteTM QPCR SYBR Green Mix (Thermo Scientific, Waltham, USA) kit was used for real time PCR. Reactions (in a total volume of 25 µL) consisted of: 5 µL cDNA (1:20 dilution), 12.5 µL ABsoluteTM QPCR SYBR Green Mix, 1.75 µL of each primer (10 µM) and 4 µL H_2O. Reactions were cycled in an MJ Research OPTICON 2 (Quebec, Canada), in triplicate under the following conditions: 95 ˚C for 10 min, 40 cycles of: 95 ˚C for 15 s, 60 ˚C for 30 s and 72 ˚C for 30 s and one cycle of 72 ˚C for 30 s. For testing primer specificity, a melting curve analysis was performed after amplification (from 60 to 98 ˚C with an increasing heat rate of 0.5 ˚C s^{-1}). A relative quantification of gene expression was calculated using the 2-DDCT method (Livak and Schmittgen, 2001). Primers for the target genes: *MtSOS1*, *MtWEE1*, *MtSERK1*, *MtP5CS* and *MtCCS52* are as described in Elmaghrabi *et al.* (2013). Mt18S rRNA primers were used to normalise the results as it was shown previously that 18S rRNA was a reliable reference gene for stress responses in *M. truncatula* (Elmaghrabi *et al.*, 2013), and widely used across a range of different species for developmental and stress-response studies (e.g. Price *et al.*, 2008; Wagstaff *et al.*, 2010).

Statistical Analyses

Unless otherwise stated above, data were analysed using R software (R version 3.3.2, Foundation for Statistical Computing). One or two way (as appropriate) ANOVA tests followed by a Tukey's test, or non-parametric statistical tests (Kruskal Wallis followed by a Dunn's test) were applied to determine differences across multiple samples. Comparisons between pairs of samples were performed using a Student's t-test, or if not normally distributed using a Wilcoxon signed rank test. Regression equation and R^2 value for the growth data were calculated in Excel.

Details of tests applied are provided in the legend to each Figure and all original data are provided as Supplementary materials (S1 to S6).

Results

PEG enhanced callus phenotype and embryogenic competence without reducing its viability

There were no significant differences between *Medicago truncatula* leaf callus cultured on MANA medium or MANA medium supplemented with 10% w/v PEG6000 over the 6 months of culture, and the linear growth rates for control and PEG treatments were 0.155 and 0.168 g $month^{-1}$ respectively (Figure 1). This only represents a 1.08 fold increase in the PEG compared with the control treatment indicating very similar rates of growth in each treatment irrespectively of the presence or absence of PEG in the culture medium. However, these data also suggest that callus tissues were PEG-tolerant already within 1 month of sub-culture and retained such tolerance throughout the experiment. Qualitative observations of callus indicated that those treated with PEG were typically bright green in colour and exhibited clear evidence of embryogenesis as did also the controls; however, the controls were brown in colour and the somatic embryos regenerated looked blocked at an early (globular to heart) developmental stage (Figure 2 A,B). The typical bright green coloration of calli in the PEG treatment would tend to indicate their robustness for both growth and embryogenesis regardless of the length of time in culture, and could also be ascribed to an increased tissue photochemistry linked to the tolerance acquired by onset of a priming process by the long-term culture on PEG. This was confirmed during the cell viability assessments with fluorescein diacetate where calli grown on 10% w/v PEG for 6 months contained 81.00 $\pm$ 1.5 % viable cells compared to 68.00 $\pm$ 5.2 for calli grown only on the MANA medium (Figure 2 C,D).

Mitotic index, cell and nuclear size increase, and cell shape changes in calli cultured on PEG-medium

Flow cytometry (FCM) was used to compare the C value distribution of cells from greenhouse grown *M. truncatula* leaves and callus derived from leaf material cultured for 6 months on 10% w/v PEG6000 (Figure 3). Flow cytometry raw profiles of leaves exhibited two peaks (Figure 3A), corresponding respectively to the nuclei in G1 phase (2C DNA) and those in G2/M (4C DNA), where their analysis after fitting them to Gauss curves resulted in a distribution of nuclei into three subpopulations as follows: G1 77.39%, S 17.32% and G2/M 5.29%, and coupled with a calculated mitotic index of 1.999 (Figure 3C). A very similar profile was obtained from calli cultured on MANA medium alone (not shown) which showed no obvious deviation from the

mother plant tissues from which they originated, while the flow cytometry profiles of calli cultured on PEG was very different (Figure 3B). In the 10% PEG6000 treatment, four peaks were typically detected consistent with 2, 4, 8 and 16 C populations (Figure 3B), and indicative of the occurrence of endoreduplication. The mitotic index was also significantly higher ($P \leq 0.05$) for the calli grown on PEG6000 containing medium (Figure 3C), which is also indicative of the onset of an endoreduplication phenomenon.

Interestingly, cell size also showed a significantly higher value for cells from calli grown on PEG6000 after just 2 months of culture (Figure 4A) and nuclear area was greater on PEG after 4 months (Figure 4B). This is consistent with the occurrence of endoreduplication, and this was coupled with a modified cell shape (Figure 4C), with cells grown on PEG6000 exhibiting a significantly lower SC than control cells. Thus, PEG-grown cells were consistently and significantly ($P < 0.05$) rounder (SC = 0.608 ± 0.117) than control cells which were more elongated (SC = 0.833 ± 0.090) (Figure 4D).

Osmolarity, proline and sugar levels rise following PEG treatment

After six months of 10% PEG 6000 treatment, there was a significant increase in osmolarity of callus in the PEG treatment compared with the control (Figure 5A) while osmolarity of the medium remained more constant. Proline and soluble sugar levels also increased significantly compared with the control (MANA without PEG) (Figure 5B,C). However there were no significant differences in water content between the PEG and control treatments (Figure 5D).

***MtWEE1* expression is highly up-regulated following PEG treatment while *MtSOS1* is down-regulated.**

We chose to examine the expression of five genes as markers of processes related to osmotic (water) stress. These comprised *MtSOS1* (salt stress response), *MtWEE1* (cell cycle checkpoint), *MtSERK1* (embryogenesis) *MtP5CS* (proline metabolism), and *MtCC52* (ploidy marker) in the embryogenic calli treated with PEG. Expression of these genes was measured using quantitative real time PCR after six months of callus culture in PEG6000 (10%) and compared with the control treatment (0 % PEG) and greenhouse grown leaves.

A highly significant reduction in the expression of *MtSOS1* occurred in the 10% PEG compared with the control treatment such that *MtSOS1* transcripts were virtually undetectable, comparable to expression in leaf (Figure 6A). Conversely, *MtWEE1* and *MtCCS52* expression was significantly higher in the PEG treated calli compared with the control treatment which in turn

was higher than expression in leaf (Figure 6B,E). *MtSERK*, and *MtP5CS* were expressed significantly more in the control callus and 10% PEG treated callus but there was no significant difference in expression between the treated and untreated calli. (Figures 6C and D).

Discussion

Osmotic stress, provoked by insufficient ground and/or rain water, is a paramount constraint for plant growth and development. Cultures of callus on media that impose water deficit is a method for generating new, more tolerant, plants. Here we have shown that long term culture of *M. truncatula* calli on medium containing 10% PEG6000 to impose an osmotic stress results in the production of morphologically enhanced calli. An analysis of protective metabolite levels, cellular morphology, cell division and gene expression was undertaken to understand the effects of the imposed stress.

In this work, callus growth was not significantly different plus or minus 10% PEG over a period of six months, suggesting that calli on PEG acquired tolerance to osmotic (water) stress (Figure 1) probably mediated by an early osmotic adjustment which was likely associated to various modifications at the cellular level (Singh *et al.*, 2015). In fact, both cell viability and mitotic index were higher in the PEG treated cells compared to the control indicating a healthy and proliferating culture. It is likely that this sustained viability in the PEG treated cells is due to the activation of defence mechanisms that may include an activation of DNA repair as shown previously (Balestrazzi *et al.*, 2011). The similarity in growth rates following 1 month ± PEG further stresses that tolerance was obtained relatively rapidly. This result differs from those of Biswas *et al.* (2002) who found that in rice, callus proliferation in the presence of PEG was greater than the controls in some genotypes, although this was at a much lower PEG concentration (5-15 gL^{-1}). However in two genotypes of wheat, one drought tolerant, the other drought sensitive, water deficit decreased only slightly in the sensitive compared with the tolerant genotype under water conditions and, it did not change significantly in either the sensitive or tolerant genotype ± 400 mM PEG 6000 (100–400 mOsm; -0.976 MPa; Guóth *et al.*, 2010). Likewise in chili pepper cultures, where there was very good growth after 12 months in 5-10 % PEG8000 (0.57 MPa; Santos-Díaz and Ochoa-Alejo, 1994). Note that after six months of treatment, although osmolarity of the callus increased, osmolarity of the medium did not change since PEG is not metabolised. We decided to analyse both the medium and callus osmolarity as an indirect way of assessing the nutrient consumption from the medium by cells, which impacts their internal salt concentrations, as shown before with various species among which *M. truncatula* (Ochatt *et al.*, 2008). An increased cell osmolarity appeared also to be a

reliable early marker of embryogenic competence (Ochatt *et al.*, 2008; Elmaghrabi *et al.*, 2013). Thus the increased callus osmolarity and embryogenic capacity seen here are in line with previous observations.

In this work (Figure 4) PEG-induced stress resulted in a highly significant increase of the size of both nuclei and cells after 6 months of culture on selection medium. Remarkably, this was also coupled with a consistent and significant modification of the cell shape, reflected by the SC values observed, indicative of an increased elasticity of cell walls under PEG-induced osmotic stress. A similar modification of cell wall elasticity was observed in *M. truncatula* plants subjected to a severe drought stress (Nunes *et al.*, 2008) and in transgenic *M. truncatula* lines expressing the trehalose-6-Phosphate Synthase 1 (*AtTPS1*) from *Arabidopsis thaliana* with altered response to water deficit and recovery (Alcântara *et al.,* 2015). Taken together, these observations suggest a profound elastic modification of the cell walls of water stress tolerant cells, perhaps deriving form a modified ratio among cell wall fractions, and should be the object of future studies.

Similar levels of somatic embryogenesis were observed in the PEG and control calli, however, calli in the PEG treatment were distinctly green in colour compared with the control. This might be consistent with more robust embryogenic callus in the PEG compared to the control treatments (Figures 1 and 2), which is not surprising since MANA medium is not conductive to full maturation of the somatic embryos formed in *M. truncatula* (Ochatt *et al.*, 2013; Ochatt and Revilla, 2016). It may also reflect the fact that *M. truncatula* is adapted to semiarid conditions and even under severe drought stress, pigment content is not affected (Biswal, 1997; Nunes *et al.*, 2008). PEG also improved somatic embryogenesis in other species (Attree *et al.*, 1995; Igasaki *et al.*, 2003). Both control and PEG treatments resulted in similar levels of somatic embryogenesis, which was consistent with the similar expression levels of *MtSERK* in the two treatments. Note that *SERK1* is highly expressed during embryo induction and early somatic embryo development in *M. truncatula* (Nolan *et al.*, 2009) and in *Araucaria angustifolia* (Steiner *et al.*, 2012). The possible stimulation of somatic embryogenesis in response to PEG treatment is consistent with other reports showing stress-induction of this process (Karami and Saidi, 2010), which in *M.truncatula* may be linked to increases in ABA (Nolan and Rose, 1998).

The physiological, metabolic and gene expression responses of calli to PEG-induced osmotic stress mirrored those found under salt stress treatments (Elmaghrabi *et al.*, 2013) in some respects but not in others, as summarized in Figure 7. In contrast to NaCl treatment, PEG treatment did not result in any increase in water content of the calli compared with the control

although osmolarity did increase. This could be explained by the differential modes of action between NaCl and PEG, as the high MW of PEG exerts a constant osmotic pressure but does not allow its entry across the wall and hence avoids cell plasmolysis which results in different energy costs and different effects on growth (Munns, 2002). However soluble sugars did increase suggesting they are a useful marker of both salt and water (osmotic) stress (Figure 5). PEG also induced a high level of proline accumulation, which was far higher than the largest proline accumulation under stress induced by NaCl (Elmaghrabi *et al.*, 2013), indicating that this might also be a component of osmotic stress tolerance in *M. truncatula* (Figure 5B), as has been found in other species (Deinlein *et al.*, 2014). Validating this hypothesis would require field trials with regenerants from these cultures and goes beyond the scope of this study. However, the expression of *MtP5CS* in callus grown on PEG was similar to the control (Figure 6D) and hence does not correlate with increased levels of proline in the PEG treatment. This was surprising given that this gene encodes an enzyme that is central to proline synthesis and that its expression in *M. truncatula* cultures exposed to salt stress was elevated (Elmaghrabi *et al.*, 2013; Figure 7). It may suggest that this enzyme is not a key regulatory step in proline biosynthesis under these conditions, or that an initial rise in *MtP5CS* expression early in the culture period was sufficient to elevate proline concentrations and that after 6 months culture, increased gene expression was no longer necessary. In other words, whether modifying proline metabolism and the expression of genes involved in it, such as *P5CS*, may or not be used for engineering drought tolerance, and which approach should be adopted for such modification to be done remains uncertain (Bhaskara *et al.*, 2015).

More predictably, PEG did not induce *MtSOS1* expression which was down-regulated compared with the control. This gene is highly expressed in salt stress conditions as it encodes a protein that functions as a membrane-bound Na^+ antiporter and contributes to Na^+ depletion in the cytoplasm (Feki *et al.*, 2011; Smith *et al.*, 2010). Therefore the PEG data indicate a different (non ionic) pathway leading to osmotic stress tolerance compared with NaCl (ionic) tolerance (Figure 7) as reported by Elmaghrabi *et al.* (2013).

The expression of *MtCCS52* was upregulated by the PEG treatment (Figure 6E). In Arabidopsis this gene is a regulator of ploidy level and its expression is positively correlated to endoreduplication. In *M. truncatula* cultures exposed to long-term NaCl treatments this gene was up-regulated (Figure 7), alongside and increase in endoreduplication (Elmaghrabi *et al.*, 2013). Given the clear evidence for endoreduplication in the osmotic stress-resistant cultures here (Figure 3), the upregulation of *MtCCS52*is consistent with its role in Arabidopsis (Vanstraelen *et al.*, 2009).

That *MtWEE1* expression was more highly expressed in the PEG treatment suggests that this gene may have a role in maintaining normal growth in a treatment that mimics osmotic stress conditions. *WEE1* kinase might be necessary to regulate normal cell size in the face of ion toxicity and osmotic (non-ionic) water stress although this could only be resolved by exposing calli from *M. truncatula wee1* knockouts to these treatments. Alternatively, as a gene that is expressed in the DNA damage and DNA replication checkpoints, it may be induced in response to either single strand or double strand DNA breaks as it is in Arabidopsis (De Schutter *et al.*, 2007). However, Gonzalez *et al.* (2004) observed high expression of *LeWEE1* in tomato (*Lycopersicon esculentum* Mill.) which was correlated with endoreduplication during fruit development. Our results do not seem to indicate that there has been irreversible DNA damage due to the osmotic stress imposed on callus, and it would therefore be legitimate to link this to *WEE1* expression and its role in replication checkpoint and DNA damage and the possibility that the PEG concentration used and the long-term culture on it resulted in priming (Singh *et al.*, 2015). Other genes could also been involved in the process though, and, in this respect, in order to protect their gene integrity from DNA damage plants are capable of activating a specific response system that regulates the cell cycle, but also DNA repair and programmed cell death where genes such as *Suppressor Of Gamma response 1* (*SOG1*) (Yoshiyama *et al.*, 2014) and *Breast Cancer 1* (*BRCA1*) (Block-Schmidt *et al.*, 2011) are known to play a central role in DNA repair, chromosome segregation and chromatin remodeling So the increase in *MtWEE1* seen here may be both linked to the increase in endoreduplication and required to protect the cells from DNA damage induced by the PEG-induced osmotic stress treatment.

Conclusion

The data reported in this study of responses to 10% PEG compared with no PEG controls indicates that at this level of osmotic stress it is possible to induce a high level of embryogenesis with no penalty on growth rate. This appears to be achieved by up-regulating protective mechanisms such as the production of osmoprotectant solutes and switching on the expression of *MtWEE1*. The increase in *MtWEE1* and *MtCCS52* expression may cause an increase in endoreduplication while protecting the cells against the potentially damaging effects of the osmotic stress on DNA integrity.

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Figure Legends

Figure 1. Mean (± S.D.) callus growth over 6 months on MANA medium with 10% w/v PEG compared with control (MANA medium). Different letter combinations indicate significant differences ($P \leq 0.05$; Kruskal Wallis followed by a Dunn's test) (n > 8). See Supplementary File S1 for data and statistical analyses.

Figure 2. Callus phenotype and viability after 6 months on 10% w/v PEG and control media: **(A)** control callus; **(B)** PEG-selected callus, arrows indicate somatic embryos; viability of control callus after FDA staining observed under transmission **(C)** or UV **(D)** light; viability of PEG-selected callus after FDA staining observed under transmission **(E)** or UV **(F)** light. Scale bars are A = 2.13 mm, B = 3.34 mm; and C through to F = 200 µm.

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745
746 **Supplementary material: Data and statistical analyses**
747 **S1:** Data for callus growth of control and PEG treatments shown in Figure 1
748 **S2:** Data for Mitotic Index of control and PEG treatments shown in Figure 3(C)
749 **S3** - Data for cell and nuclear are of control and PEG treatments shown in Figure 4(A) and 4(B)
750 **S4** - Data for Shape Coefficient calculations of control and PEG treatments shown in Figure 4(C)
751 **S5** - Data for control and PEG treatments shown in Figure 5: (A) osmolarity of media and callus; (B)
752 proline accumulation; (C) soluble sugars; (D) water content
753 **S6** -Data for Realtime PCR of control and PEG treatments shown in Figure 6
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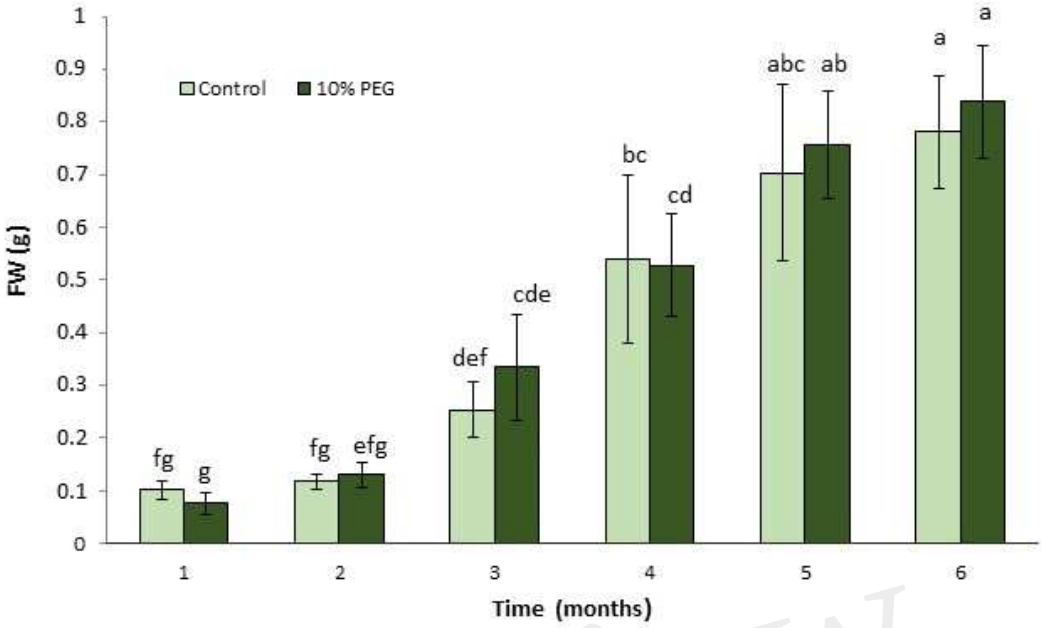
755



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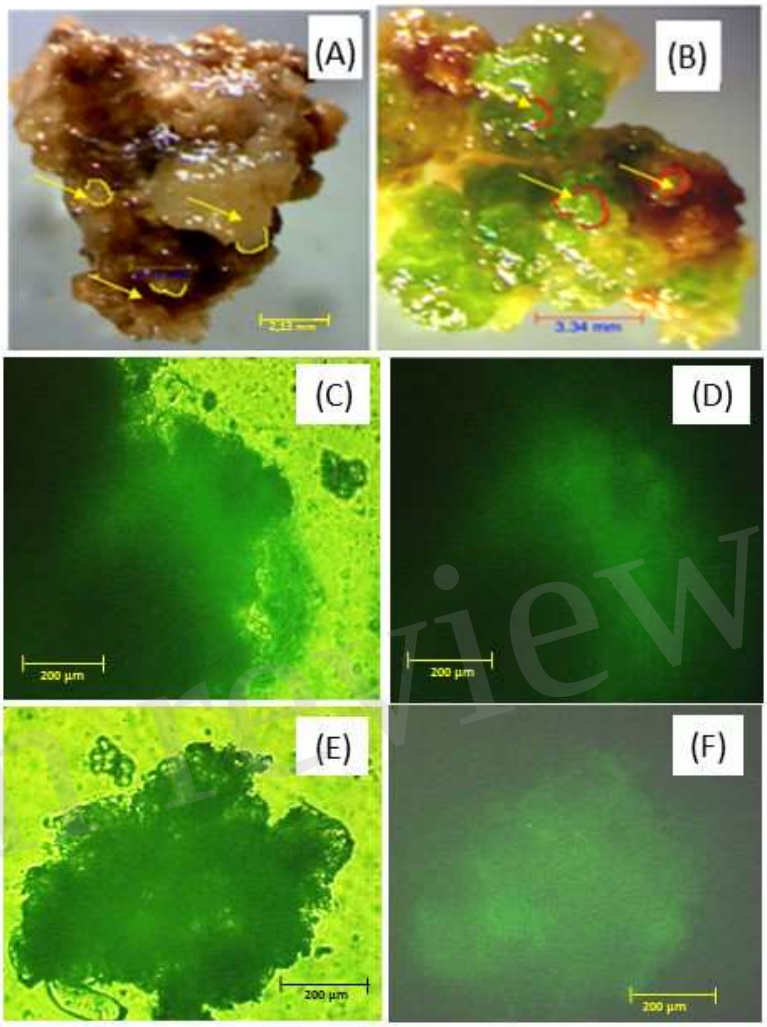

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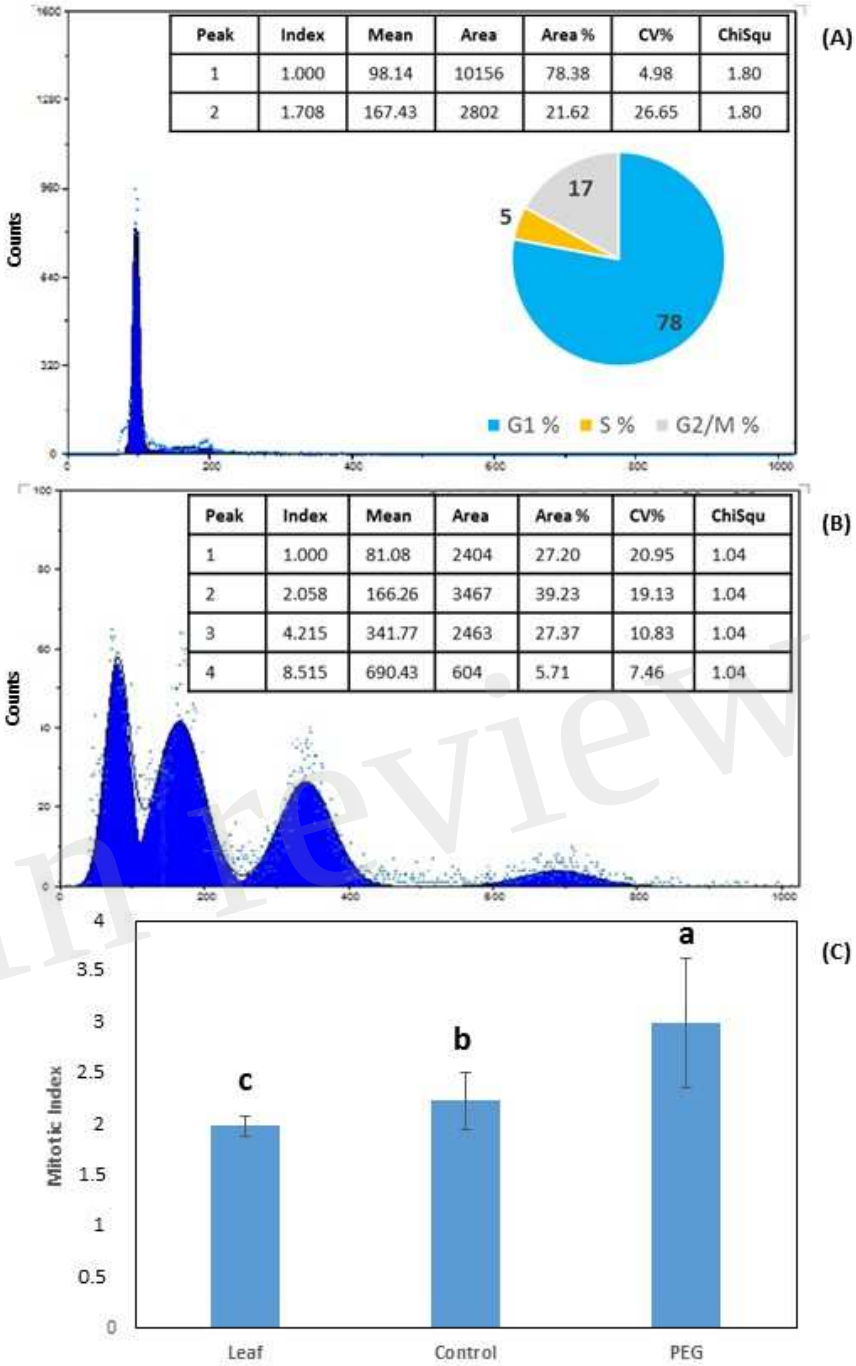

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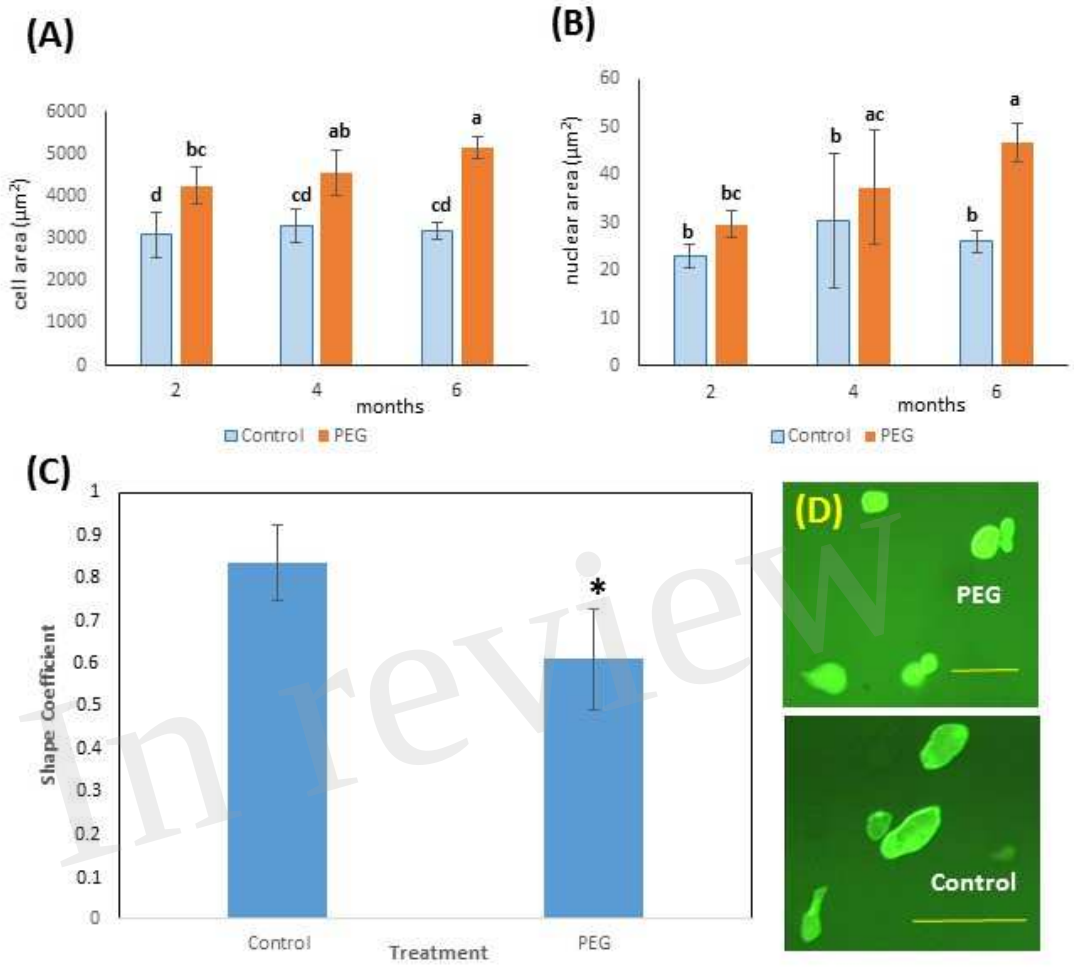

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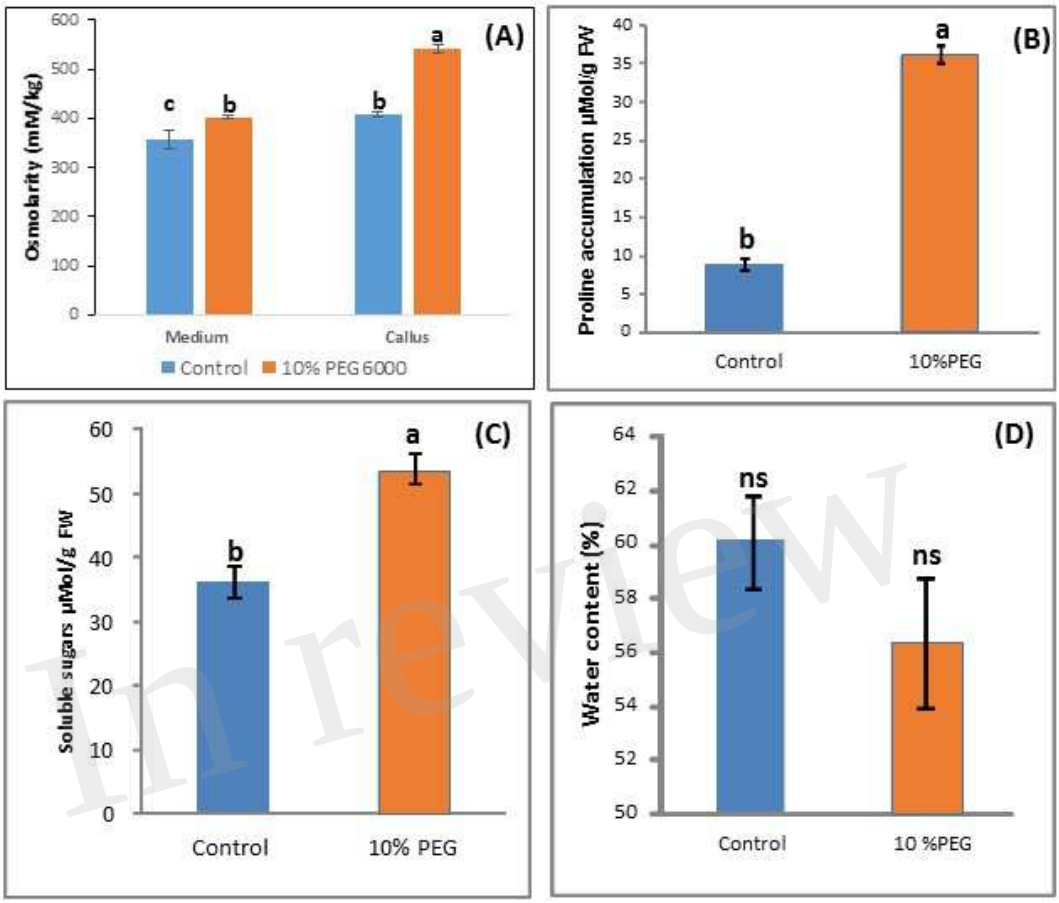

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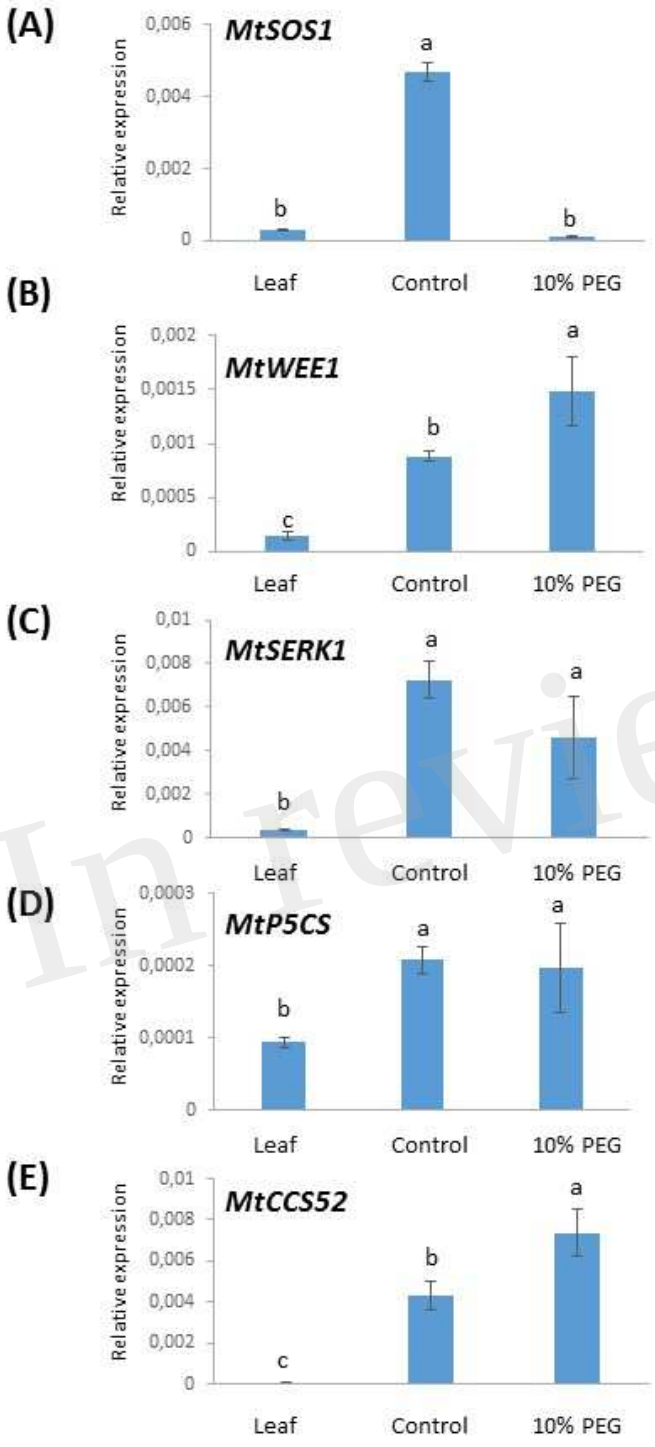

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Growth (fresh weight)			
Cell Viability (%)			
Embryogenic competence			
Chlorophyll (green tissue)			
Endoreduplication			
Mitotic index			
Medium osmolarity			
Callus osmolarity			
Water content			
Proline accumulation			
Soluble sugars			
Relative expression of *MtSERK1*			
Relative expression of *MtWEE1*			
Relative expression of *MtCCS52*			
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Relative expression of *MtSOS1*			

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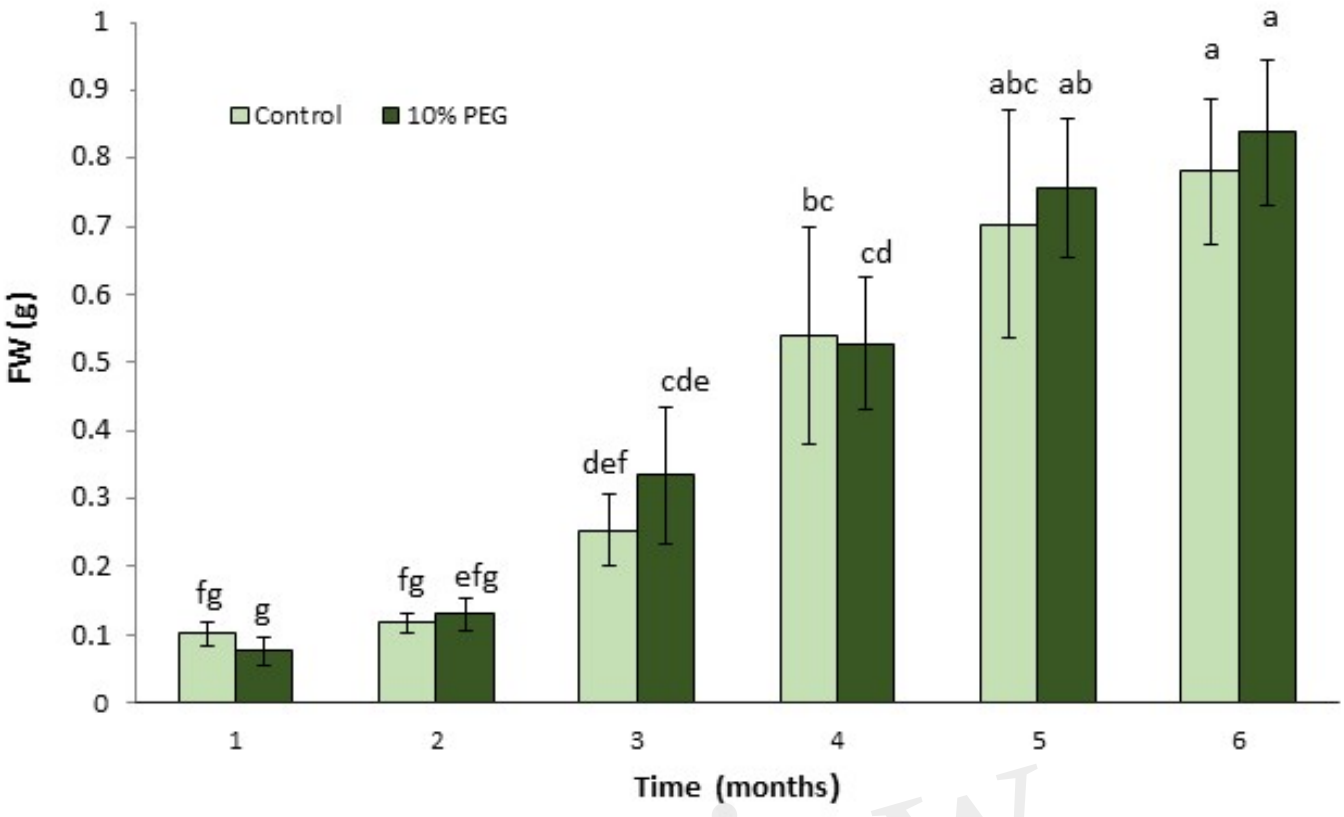

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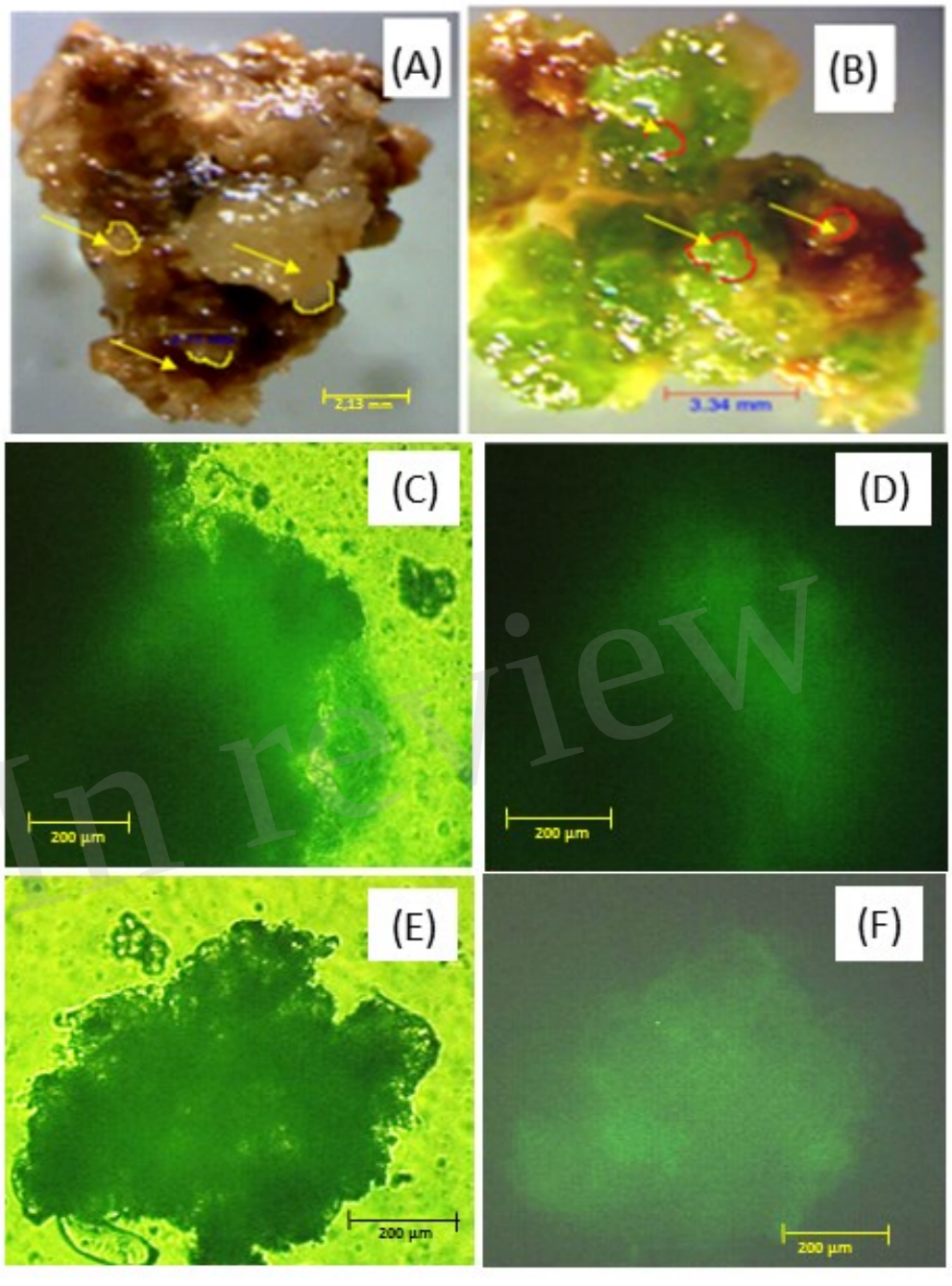

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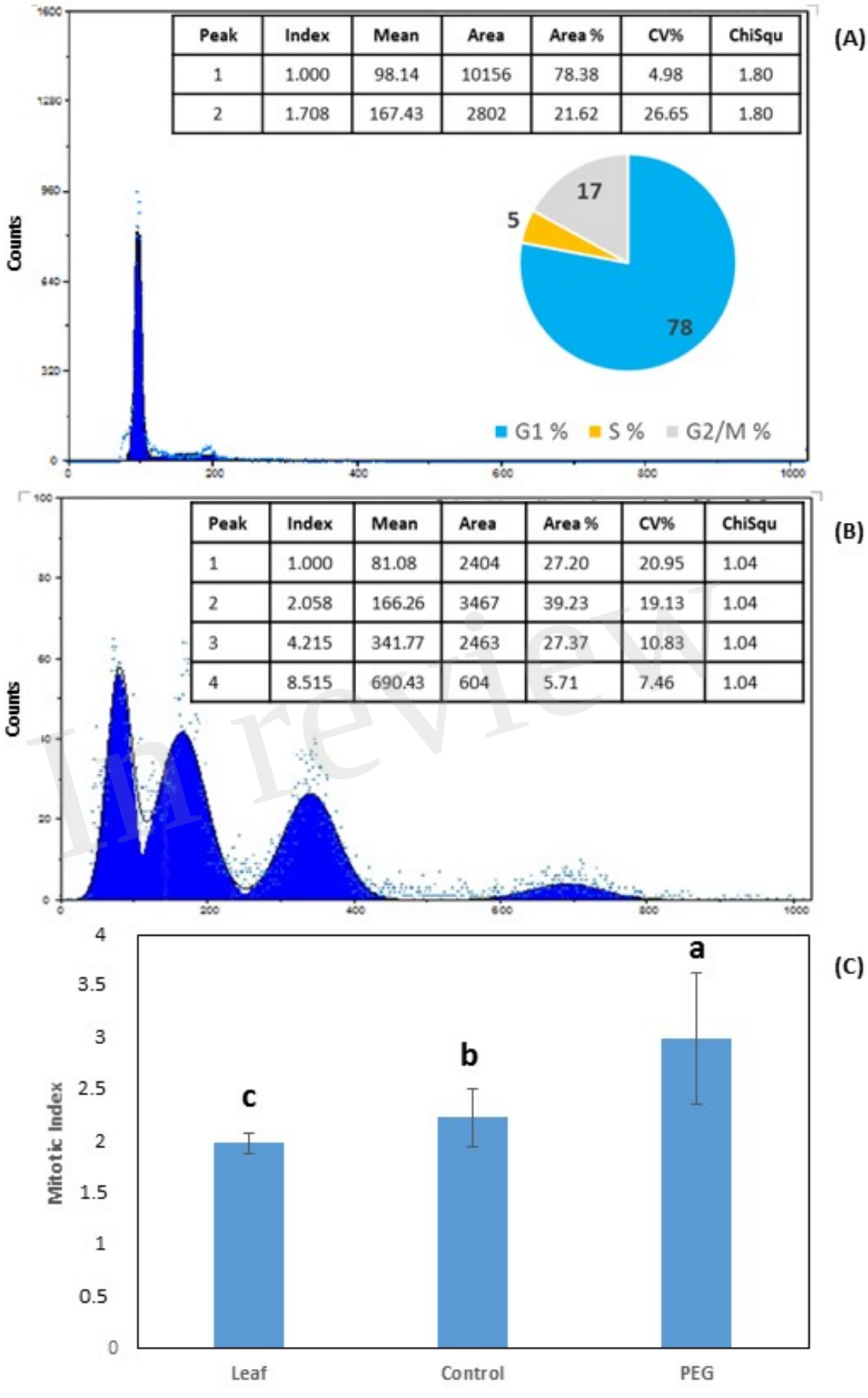

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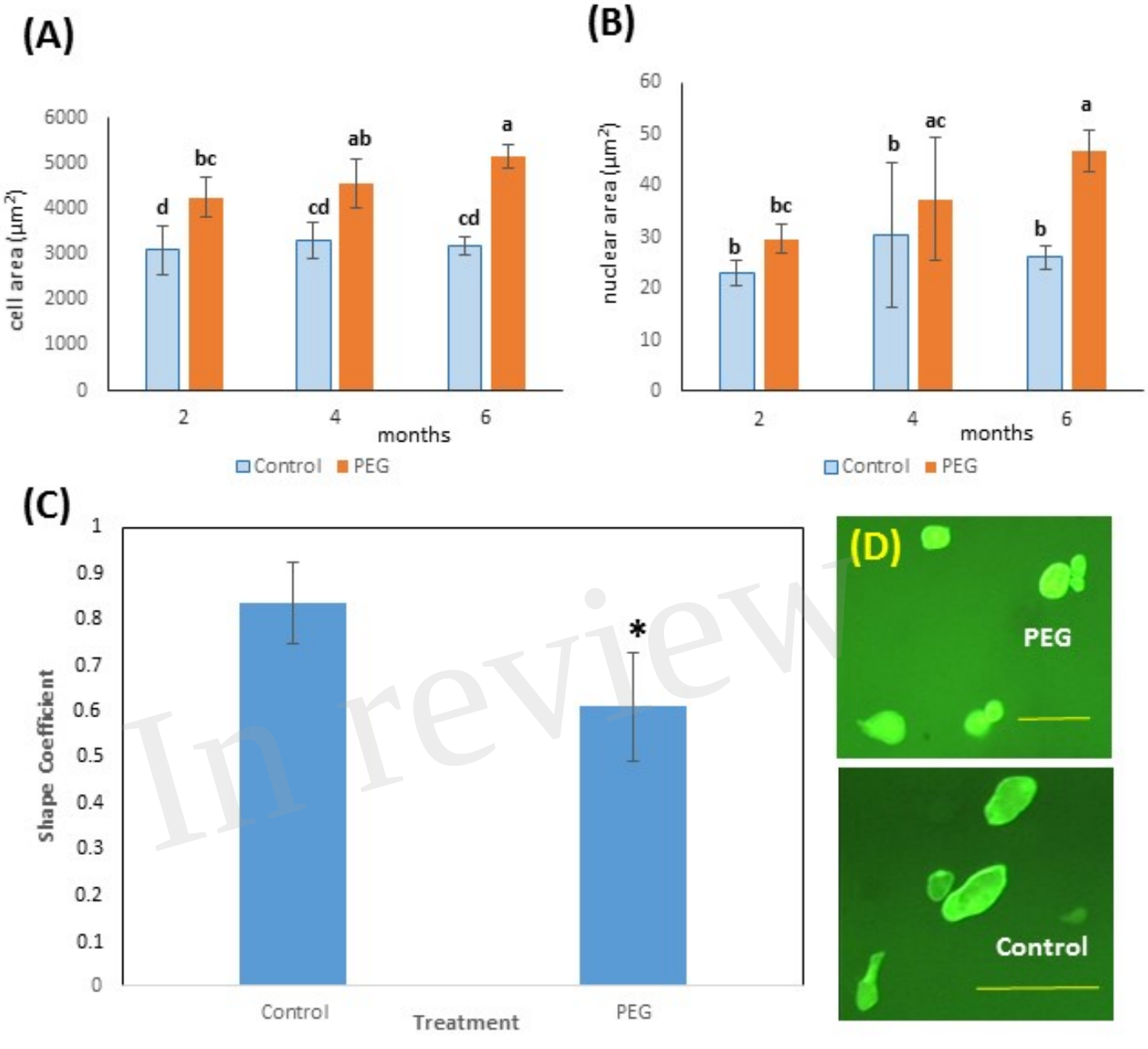

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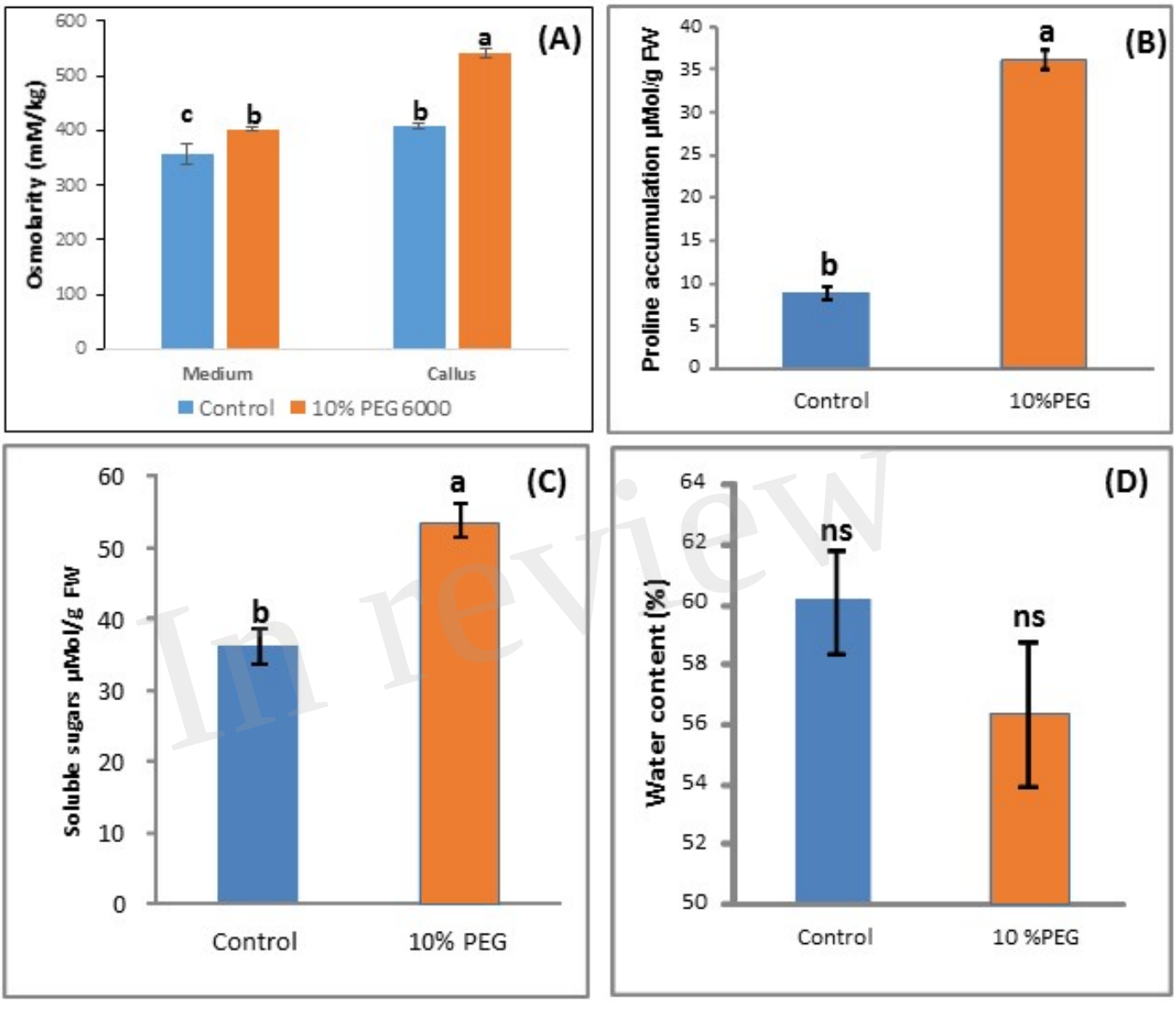

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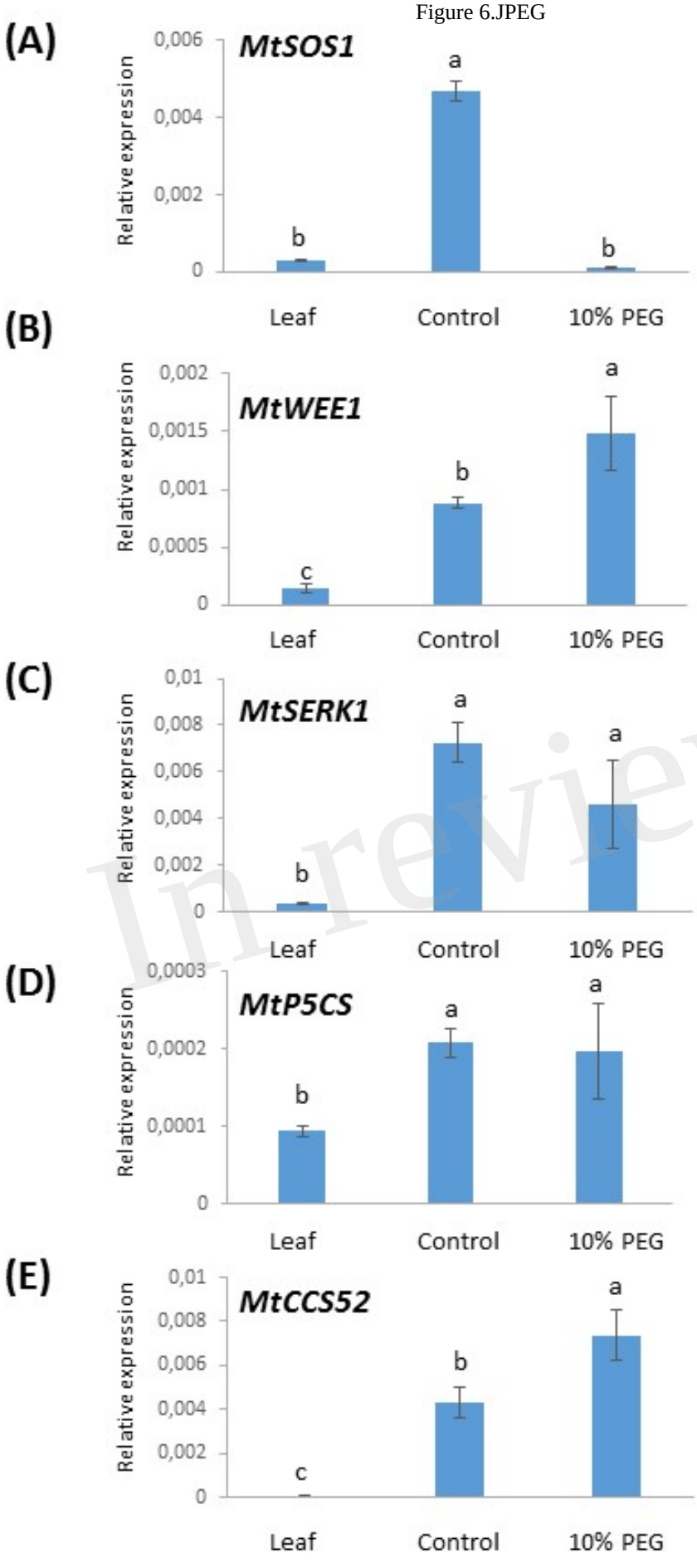

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