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Physiological responses to Megafol® treatments in tomato plants under drought stress: A phenomic and molecular approach

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ABSTRACT

Drought is one of the most significant abiotic stresses that limits the growth and productivity of crop plants. We investigated the physiological and molecular responses of tomato plants treated with Megafol® (Valagro S.p.A), under specific drought conditions. The goal was to evaluate the impact of Megafol®, a biostimulant composed of a complex of vitamins, aminoacids, proteins and betaines, in attenuating the negative physiological responses of drought. Tomato plants were grown in a greenhouse, and physiological parameters were collected using Scanalyzer 3D (LemnaTec, GmbH), a plant phenomics platform. Using this technology it is possible to dynamically study the effects of biostimulants, such as Megafol®, on plant development in terms of early detection of physiological plant stress responses. The results showed that drought-stressed plants treated with Megafol® were healthier in terms of the biomass produced and chlorophyll fluorescence, thus highlighting the higher tolerance to stress of the treated plants. The effects of Megafol® were also studied at a molecular level by analysing the induction of genes typically involved in drought stress responses. Our results demonstrate the efficacy of Megafol® to reduce drought-stress related damage in tomato plants.

1. Introduction

Multi-spectrum analysis (infra-red and visible, ultraviolet light) of reflected or re-emitted light from the plant crown, stem and leaves provides information on the nutritional, hydrological and physio-pathological state of plants, as well as on the plant's ability to intercept light. The relative simplicity and high efficiency of such imaging technology and the subsequent data processing enables it to be successfully integrated in experiments that involve many variables, a high number of sampling intervals, and multiple comparisons. Given that it can extract phenotypic data quickly and efficiently, multi-spectrum analysis has become an interface between molecular genetics and plant phenotyping. In fact, phenotypic analysis of new lines has historically been a bottleneck for fundamental genetic studies in plants (Furbank and Tester, 2011).

With visible light illumination, the reflectance spectrum from leaves is particularly poor. In fact most wavelengths (i.e. colours) are absorbed by the pigments of the photosynthetic apparatus and in particular chlorophyll. Even with this reduced emission spectrum, it is still possible to extract a great deal of phenotypic information, such as the green index, and these plant images are valuable for measuring the many morphometric parameters of plants such as height, width, and biomass (Rajendran et al., 2009). At increasing the wavelengths, the near infra-red (NIR) is important in identifying the chemical functional groups through the resonance frequencies of bond stretching, contracting and bending. In detail, wavelength intervals from 1450 to 1930 nm and around 2500 nm have been already used to study the differences in the water content levels of plants, quantifying water molecules through the OH chemical bonds (Berger et al., 2010). Therefore, this approach allowed to measure the internal water content levels without disturbing the plant repeatedly over time (Petrozza et al., 2009).

Abbreviations: ABA, abscissic acid; DHN, dehydrin; HIS, hue, saturation and intensity; LEA, late embryogenesis abundant; NIR, near infra-red; RGB, red, green and blue.

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Another fundamental parameter largely used is the chlorophyll fluorescence, by which it is possible to see signs of stress in plants much earlier than with traditional phenotypic methods. In recent years, the analysis of chlorophyll fluorescence has become ubiquitous in plant ecophysiology studies, and the principle underlying this technique is relatively straightforward. Light energy absorbed by chlorophyll molecules can (1) be used to drive photosynthesis, or (2) be dissipated as heat or (3) be re-emitted as light – chlorophyll fluorescence (Oxborough, 2004). Under optimal growth conditions much of the energy absorbed from light is directed into photosynthesis and plants emit a basal level of chlorophyll fluorescence. However, when the photosynthesis apparatus is under abiotic stress, the balance is disturbed and more energy must be released through heat and fluorescence (Müller et al., 2001).

Due to the robustness, relatively low cost, and reasonable analysis time, the use of high throughput plant image analysis could be a very interesting tool for studying plants' physiological responses to different environmental conditions, such as drought stress. Water deficiency is a severe environmental stress, and has a negative impact on crop yields worldwide (Boyer, 1982; Pennisi, 2008). Considering that water resources are decreasing and climate change is expected to increase the amount of dry land, the outlook for agriculture is dramatic (Battisti and Naylor, 2009). It is predicted that in 2050, the global population will increase from the current 6.7 billion to over 9 billion, and over 3 billion will be threatened by water shortages. An increasing population also means a higher demand for food: maintaining a high yield in drought conditions is thus a major priority as is understanding the responses and adaptive mechanisms of crops to water deficit. However, the physiological mechanisms of yield maintenance under drought conditions are poorly understood (Tuberosa and Salvi, 2006), since the mechanisms that plants usually use to maintain growth in low water are complex and still not well characterized. The strategies to cope with water scarcity differ depending on the species and genotypes of the plants, as well as on the type of drought. Generally, plants respond to drought with a series of physiological mechanisms, which include stomatal closure, repression of cell growth and photosynthesis, leading to an overall reduction in plant biomass.

Furthermore, many changes at the cellular and molecular level occur, involving extensive increases in the expression levels of those genes that protect the plant from stress damage (Shinozaki and Yamaguchi-Shinozaki, 2007). These genes can be classified into two major groups, according to their putative functions. The first group includes early response transcriptional activators, including transcription factors and protein kinases. Stress-related transcription factors play an important role in the regulation of drought tolerance and mainly include bZIP, WRKY, MYB, and AP2/EREBP proteins (Abe et al., 1997; Finkelstein and Lynch, 2000; Marè et al., 2004; Song et al., 2005). These transcription factors respond to environmental signals, namely drought or high salinity, also though the involvement of abscisic acid. However, it must be highlighted that both ABA-dependent and independent pathways operate in response to drought (Shinozaki and Yamaguchi-Shinozaki, 2007). Downstream of the activation of the first group of genes operates a second gene group, that includes genes that codify for downstream effectors in the stress response pathway, including structural proteins, osmoregulatory proteins, antioxidant proteins, aquaporins, and late embryogenesis abundant (LEA) proteins (Bailly et al., 2001; Breton et al., 2003; Wang and Ye, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Since they increase markedly in vegetative organs during water deficits, a protective role during water limitation has been suggested (Bies-Etheve et al., 2008; Popelka et al., 2010). The *DHN* (dehydrin) genes also belong to the LEA proteins family, which are up-regulated during drought stress (Zhu et al., 2000) and, for this reason, have been widely studied in higher plants in response to water deficits (Ismail et al., 1999; Nylander et al., 2001). The most important genomic information concerning the responses of different plant species to drought stress, collected so far, has been provided by genome-wide expression profiling under water deficits, including Arabidopsis (Seki et al., 2001; Bray, 2004; Huang et al., 2008), rice (Rabbani et al., 2003; Hazen et al., 2005), barley (Talamé et al., 2007; Guo et al., 2009), maize (Hayano-Kanashiro et al., 2009), sorghum (Pratt et al., 2005), and potato (Vasquez-Robinet et al., 2008).

Despite the agronomic and economic relevance of tomato, this kind of information is lacking, therefore the mechanisms that drive responses to water deficits in tomato have not been well characterized yet, and only a very small number of genes that play a role in drought tolerance have been identified (Atarés et al., 2011; Pineda et al., 2010). Tomato is particularly sensitive to a number of environmental stresses, especially extreme temperature, drought and salinity (Kaloo, 1993).

Improving plant responses to stress conditions might be possible through treatment with specific products, made with natural products and known as biostimulants. The application of biostimulants has a positive impact on plant nutrition and plant growth, and also provides anti-stress effects (Richardson et al., 2004). These important qualities highlight the importance of increasing the knowledge regarding their physiological functions, which are currently unclear.

The aim of this study was to characterize the response to drought stress induced in tomato plants treated with Megafol®, a biostimulant provided by Valagro S.p.A that is known to positively influence crop yield (Parađiković et al., 2011), by collecting information regarding the physiological and molecular changes induced by the stress and the potential positive effects, in terms of tolerance, due to the treatment. The physiological mechanisms were evaluated by image analysis (Scanalyzer 3D System, LemnaTec), using visible light (RGB), florescence, and near infra-red (NIR) chambers.

2. Materials and methods

2.1. Plant material and growing conditions

The experiments were performed in April 2012 at the Plant Phenomics laboratory at the ALSIA Research Center Metapontum Agrobios, in Southern Italy. Tomato plants (cv Ikram, Singenta AG) were grown in a greenhouse under natural ambient light conditions. Four weeks after seeding, after the third true leaf was fully expanded, plants were transplanted into white vases (16 cm diameter, 20 cm height) for the actual experiment. The vases, volume max. 4 L, contained 3.5 L of soil consisting of a 50:50 mixture of peat moss and river sand.

At the moment of transplanting into the pots, 20 units of nitrogen (N_2), 40 units of anhydrous phosphite (P_2O_5), and 20 units of potassium oxide (K_2O) were added to the soil mixture. Drought stress was induced after the third or fourth leaf, non-cotyledon, had fully formed and continued until the end of the experiment.

Plant samples and plant images were taken starting three days prior to the beginning of the drought stress (Day 0), between 7 am and 9 am. The control plants were irrigated with 200 mL every day both before and during the drought stress experiment. This quantity of water was previously determined to be optimal for plant growth in Southern Italy in April, when the experiment was conducted. Drought stress was imposed by withholding irrigation. For those plants subjected to drought stress, irrigation was completely suspended until the first symptoms of withering (at day 5 of drought stress) when 50 mL of water was administered. Verification of drought stress was conducted with a plant leaf porometer (Decagon Devices SC-1). Confirmation of plant stress was obtained when, on the fourth day of the drought stress experiment, stressed

plants revealed a 5 fold lower level of water transpiration rate when compared to regularly irrigated plants.

Megafol® treatment was performed on Day 0, by spraying homogenously a 2 mL per liter solution over the leaves. Megafol® is a mixture of amino acids (proline and tryptophan), glycosides, polysaccharides, organic nitrogen and organic carbon (Parađiković et al., 2011). The apparatus used to spray the plants was a 6 L agricultural pressure sprayer (VOLPI).

Five days after the cessation of irrigation (indicated as S1, and representing the drought stress), the plants lost all turgidity and an emergency irrigation of 50 mL water per plant was administered (S2). A further five days after the emergency irrigation, a period of recovery was established with a watering regime of 200 mL water per plant per day (*R*). The experimental scheme made use of 13 replica plants for each test and was completely randomized.

For the molecular analysis, leaves were harvested and subsequently transferred into liquid nitrogen. Three leaves (central position) were harvested from each of the three different plants. The three leaves were pooled and represent a single sample out of the three biological replicates. Plants used for sampling leaves were excluded from subsequent imaging or additional leaf sampling. Leaf samples were taken on the same days as imaging. The pre-drought samples were used to establish a baseline of gene expression of the plants. All samples were taken at the same time of day (9 am), to minimize climatic variation (i.e. cloudy or sunny days), since at 9 am the impact of the presence/absence of clouds is minimal for the greenhouse microenvironment, being the time for warming-up of the greenhouse due to high sunlight limited to a few hours after sunset. In this manner the hydration state of the plants was predominantly due to the availability of water in the vase and not due to possible transient increases in transpiration at peak day temperatures.

2.2. Meteorological data acquisition

Both the minimum, maximum, and average daily temperatures and the maximum daily solar radiation were measured throughout the experimental period. The same measurements were also collected during the imaging and sampling processes, from 7 am to 9 am. Meteorological data was measured every 30 min with a datalogger (Watchdog Model 450, Spectrum Technologies, Inc.)

2.3. Non-destructive measurements

Phenotypic measurements of the plants were carried out non-destructively by plant imaging. Images of plants were taken over the course of the experiment with a plant image capturing system (Scanalyzer 3D LemnaTec GmbH). Three imaging chambers with different levels of illumination, near infra-red (NIR), white light (RGB), and fluorescence (UV), were used. For each chamber three images were taken, one from above the plant and two laterally at an orthogonal angle. The images collected in the NIR chamber were used to evaluate the plant water content. Images from the RGB chamber were used to evaluate the state of health of the plant via colour classification (i.e. green healthy tissue, yellow chlorotic tissues, and brown necrotic tissue), as well as for morphometric measurements, such as digital biovolume and height. Finally, images from the fluorescence chamber were used to evaluate the state of the photosynthetic apparatus.

2.4. Image analysis

In order to extract phenotypic measurements from the images, the plant had to be separated from the background. This separation was carried out either by converting the image colour space to HSI (hue, saturation and intensity) or using the intensity channel, if the image contained a sufficient contrast between the plant and the background. Otherwise a mathematical formula, involving the three channels of the RGB colour space, was used to calculate a new grey-scale image with sufficient contrast. This grey-scale image was then subjected to thresholding to create a binary mask to extract the plant from the image. The one exception was for the NIR top view images, where the mask from the RGB top view plant image was resized and transposed to the NIR image. The measurement of digital biomass, correlated with fresh weight, was carried out as described by Eberius and Lima-Guerra (2009). Specifically, the areas of the plant in the three orthogonal images from the RGB chamber were applied in the following formula:

$$\sum \text{pixel sideview } 0^\circ + \sum \text{pixel sideview } 90^\circ + \log\left(\sum \text{pixel} \frac{\text{topview}}{3}\right)$$

To measure a stress index histogram of the hue channel, pixels (from the HSI colour space) from the images generated in the fluorescence chamber were used to determine the hue value with the greatest value for non-stressed and then for drought stressed plants. Applying the formula

$$\frac{C_{\text{control}} - C_{\text{stress}}}{C_{\text{control}} + C_{\text{stress}}}$$

an index value from −1 to +1 was calculated. This value is indicative of the shift in the modal value of the histogram of the fluorescence image pixels, where lower values represent a shift to the right and higher values a shift to the left. This shift is significant in that the hue channel represents the colour or wavelength of the light, the scale running from 0 to 255 or red to violet. An index shift to lower values is representative of a shift in the histogram toward higher energy or violet light and indicates lower photosynthetic efficiency, which is interpreted as greater stress. Histograms from the grey-scale images generated in the NIR chamber were divided into 10 groups, representing a decrease in water content as the colour value increased from 0 to 255. For the analysis only the category with the lowest value, or the highest water content, was considered. In all cases, comparisons of the colour class data for the pixels in RGB, fluorescence, and NIR were made with the untreated control.

2.5. Statistical analysis of data

Phenotyping results were analyzed using one-way analysis of variance (ANOVA), and the means were compared using the Duncan's New Multiple Range Test (MRT; $p < 0.05$). Data were reported in Table 1.

2.6. Molecular analysis

Total RNA was extracted from each sample using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St Louis, MO, USA), according to the manufacturer's instructions. Electrophoresis using a 1% agarose gel was performed for all the RNA

Table 1
Phenotyping results of the experimentally treated plants for the digital biomass and high water content index at the end of the experiment, and for the stress index two days after treatment.

Tesis	Digital biomass	High water content index	Stress index
Untreated control	383617a	0.292a	0.541a
Megafol control	400213a	0.292a	0.495a
Untreated drought stress	279845c	0.258c	0.128c
Megafol drought stress	309353b	0.278b	0.314b

For each column median values with significant differences, as calculated by the Duncan's New Multiple Range Test with $p < 0.05$, indicated by different letter grouping.

samples to check for RNA integrity, followed by spectrophotometric quantification. Contaminating DNA was removed using a TURBO DNA-free kit (Ambion, www.ambion.com). RNA was then reverse-transcribed using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA; www.bio-rad.com). Expression analysis of tomato drought stress marker genes (*Solyc02g084840* and *Solyc03g025810*) was performed by real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems). These genes were selected because they are orthologous of Arabidopsis *RAB18* and *RD29B* genes, respectively (Lang and Palva, 1992; Cutler et al., 2010). Both *RAB18* and *RD29B* are strongly upregulated by drought (Ding et al., 2011). Quantitative PCR was performed using 40 ng cDNA and iQ™ SYBR® Green Supermix (Bio-rad Laboratories, Hercules, CA; www.bio-rad.com), according to the manufacturer's instructions. *SlEF1A* (*S. lycopersicum elongation factor 1-alpha*) and *SlUBQ* (*S. lycopersicum ubiquitin*) were used as reference genes. The relative quantization of each individual gene expression was performed using the geometric averaging method (geNorm; http://medgen.ugent.be/~jvdesomp/genorm/).

The following specific primers were used: *Solyc02g084840*, 5′-ATGGAACTCAGGCTGGACAC-3′ (forward), 5′-TCTTCCTTCTACCGCCATGT-3′ (reverse); *Solyc03g025810*, 5′-ACACGAGCAGCATTCACAAG-3′ (forward), 5′-GCCCATTGACACAACTTCCT-3′ (reverse); *SlEF1a*, 5′-GCTGCTGTAACAAGATGGATGC-3′ (forward), 5′-GGGGATTTTGTCAGGGTTGTAA-3′ (reverse); *SlUBQ*, 5′-CACCAAGCCAAAGAAGATCA-3′ (forward), 5′-TCAGCATTAGGGCACTCCTT-3′ (reverse).

3. Results

3.1. Environmental conditions during treatments

The minimum, maximum, and average daily temperatures measured throughout the experiment are shown in Fig. 1A, while Fig. 1B reports the maximum daily solar radiation. Two days after Megafol® treatment (Day 2, S1), the first full day of drought stress, the climatic conditions were particularly warm, accompanied by high solar radiation (500 W m^{-2}), creating a particularly strong combination of heat and drought stress. Other high temperature days and solar radiation occurred during the recovery phase (*R*), so the symptoms of stress were less pronounced. In addition, the second day after treatment also experienced the greatest daily change in temperature. The sixth day after the Megafol® treatment was particularly cloudy and humid, the change in temperature during the day was insignificant and the daily solar radiation was low (100 W m^{-2}). The relative meteorological data during imaging and sampling of the plants are reported in Fig. 2.

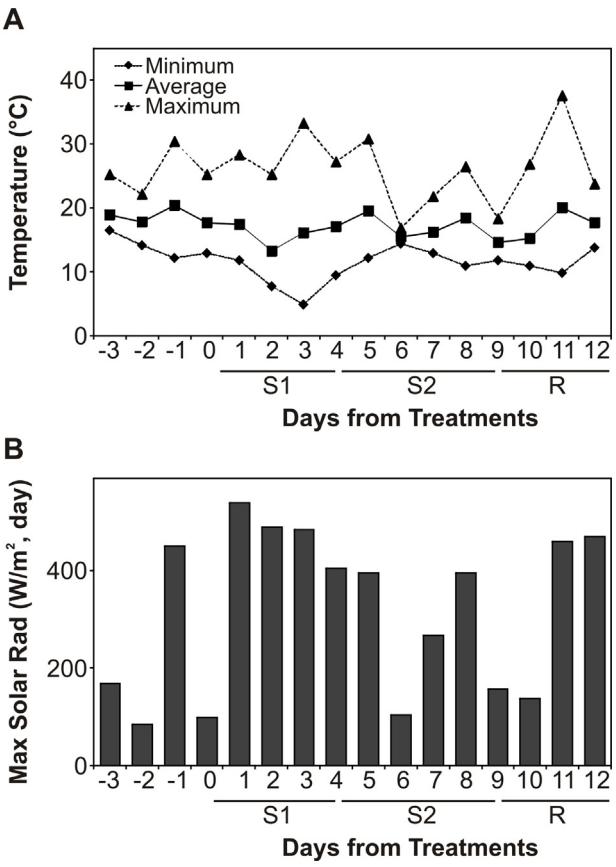

Fig. 1. Meteorological data during the experiment. Minimum, maximum, and average daily temperatures (A), and the maximum daily solar radiation (B) were measured throughout the experimental time frame.

3.2. Stress Index

The calculated stress index for all the experimental plant groups showed statistically insignificant differences up to the day of treatment and drought stress (Day 0, Fig. 3). Plants that were treated with Megafol® did not show a drought-dependent decrease in the stress index when compared to the controls one day after irrigation was stopped. On the second day of drought, the stress index of Megafol® treated plants was lower than that of controls, but higher than the one recorded for untreated, drought stressed plants. The Megafol® treated plants kept a higher stress index along the duration of the S1 phase (Fig. 3) until Day 4 of drought stress, when the stress index of Megafol® treated plants was the same as the one of the "untreated drought" plants (Fig. 3). At this point it was decided to administer 50 mL water to the severely dehydrated plants (S2). With this emergency irrigation the Megafol® treated plants responded better than the untreated ones for the following two days, after which differences became statistically insignificant. The normally irrigated plants (*R*), whether Megafol® treated or not, did not show statistical differences in their stress index. Daily variations in the stress index were due to variations in the daily solar radiation. This is to be expected as the calculated stress index was obtained from chlorophyll fluorescence, and variations in the available light would naturally affect the photosynthetic apparatus of the plants.

3.3. Biomass changes in response to drought stress and Megafol® treatment

The calculated digital biomass of the plants is shown in Fig. 4A. Clearly, the plants treated with Megafol® showed a delayed response to the drought stress (Day 2 after treatment) in comparison with the untreated plants. This response manifested itself as a reduction in the calculated digital biomass. Not only was the response delayed, but the Megafol® treated plants maintained a greater digital biomass throughout the rest of the experiment. This indicates that the Megafol® treated plants were more resistant to drought stress. No differences between the Megafol® and control plants were noted for the unstressed plants. The digital biomass can be correlated with the weight of the plants, but is strongly influenced by the projected area in the two dimensional images. These two dimensional images were used to calculate a volume: when plants are subjected to drought stress and loose turgor pressure, a reduced digital biomass results. This sensitivity to plant volume can be seen when the fresh weight results (Fig. 4B) are compared to the calculated digital biomass (Fig. 4A). Plants that were not drought stressed did not show any significant differences

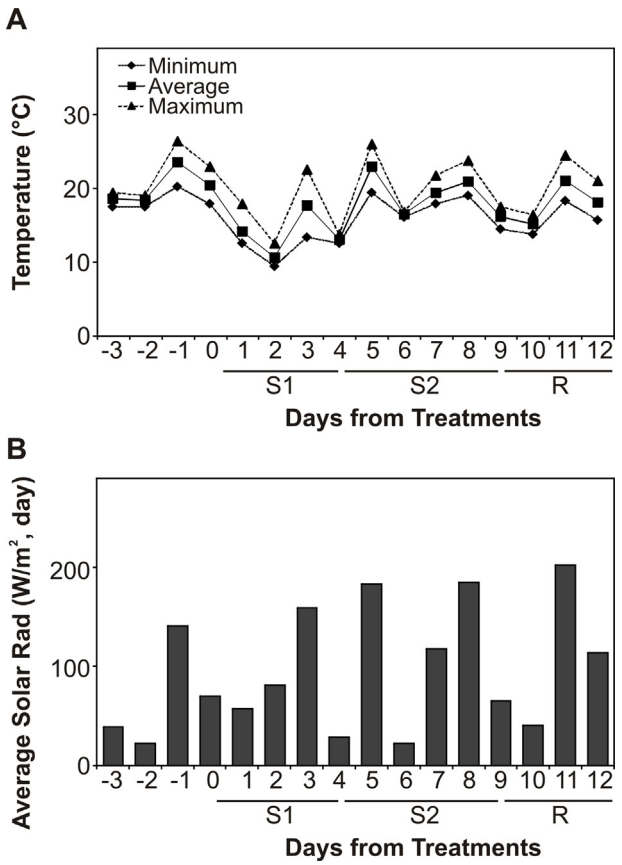

Fig. 2. Meteorological data measured during the period of imaging and sampling, from 7 am to 9 am. Minimum, maximum, and average daily temperatures (A), and the average solar radiation (B).

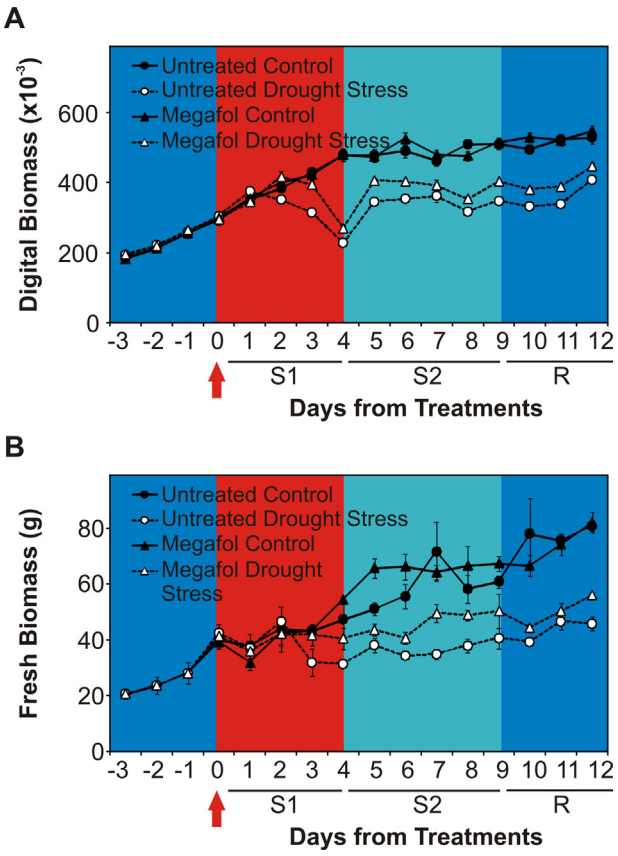

Fig. 4. Digital (A) and fresh biomass (B) throughout the experimental time frame. Circles represent the untreated (*filled*) and drought stressed (*empty*) samples, whereas triangles represent the Megafol® controls (*filled*) and stress-treated plants (*empty*).

in their digital biomass (whether or not treated with Megafol®). The drought-stressed plants showed, instead, a much lower digital biomass (Fig. 4A) with concomitant fresh weight losses, when compared to their controls. For the drought stressed plants, the higher digital biomass detected in the Megafol® treated plants was maintained throughout the experiment, even after irrigation of the plants was reinstated for a recovery phase.

3.4. *Megafol® influences the high water content class index*

The fraction of plant area with the highest water content class is reported in Fig. 5. As expected, the irrigated plants showed much higher levels during the drought stress period which was already evident on Day 1 in S1. The drought stressed plants showed small but significant differences with or without the Megafol® treatment, although with the application of 50 mL water (emergency irrigation, S2) a much greater difference in high water content class levels was observed. Interestingly, the Megafol® treated plants

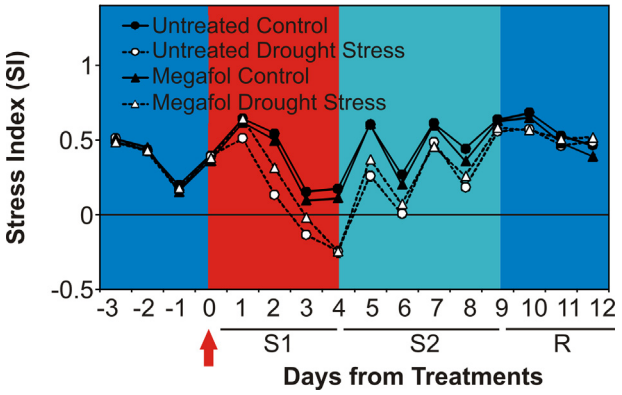

Fig. 3. Stress index during the experiment. The stress index (SI) is a measure of the state of health of the photosynthetic apparatus of the plants and is calculated with the formula $(C_{control} - C_{stress})/(C_{control} + C_{stress})$ which results in a value from −1 to +1.

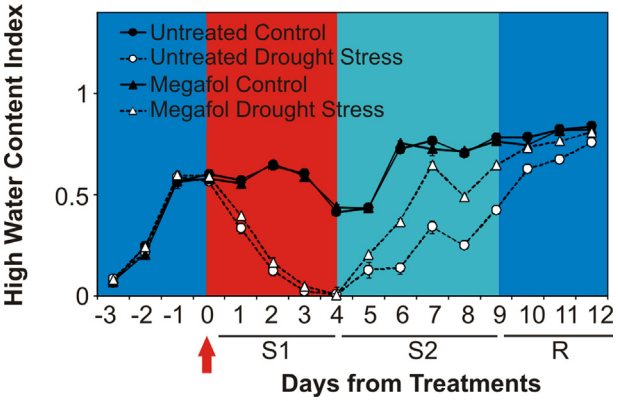

Fig. 5. High water content index in tomato plants throughout the experimental time frame. The water content is reported as a high water content index. The pixels from the grey-scale images generated in the NIR chamber were divided into equidistant classes according to their intensity. The groups levels indicate the absorbance of NIR light, which is directly related to the quantity of water in the plant tissue. For this experiment only values from 105 to 255 were considered and divided into 10 classes. The high water content index was calculated by adding the relative area for the first three classes, those of the highest water content, and is reported as a fraction of the total area.

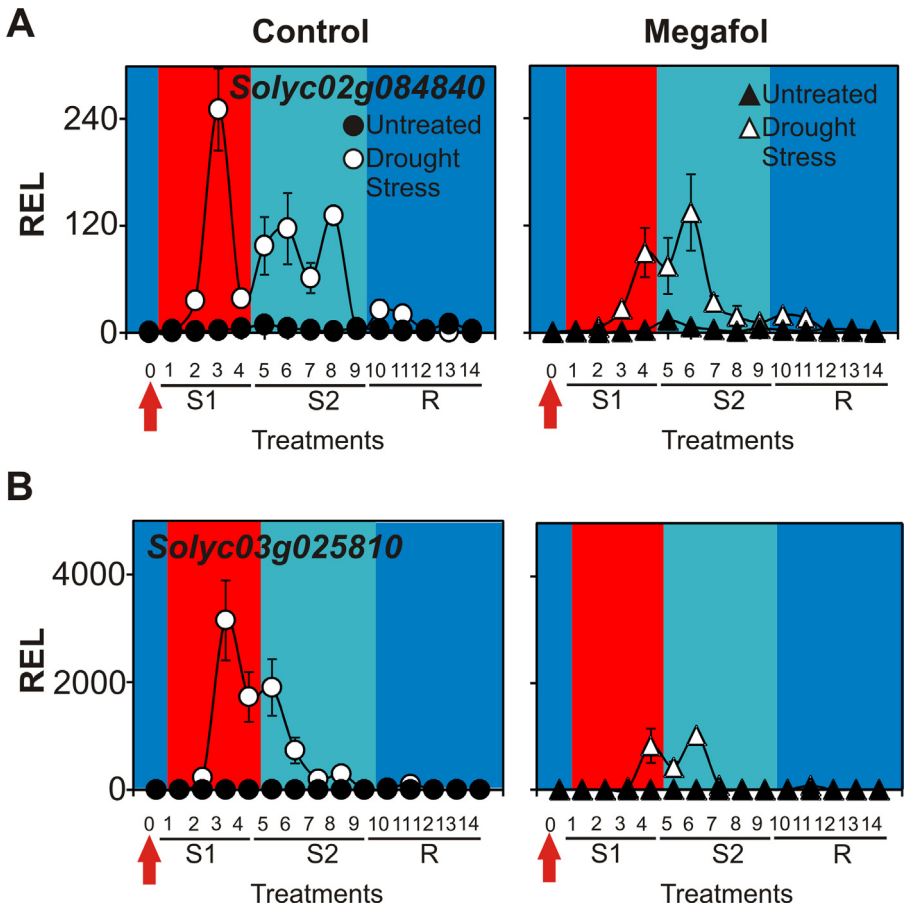

Fig. 6. Pattern of expression of *Solyc02084840* (A) and *Solyc03g025810* (B) in tomato plants under drought stress. On the left for each gene the comparison between stressed (*empty circles*) and non-stressed plants (*filled circles*). On the right the comparison between samples treated with Megafol® (time 0 time of Megafol® treatment) before the water stress induction (*empty triangles*) and stressed without biostimulant treatment (*filled triangles*). The pattern is analysed during a time course, splitting the total time of treatments into three phases: S1 – total stop of watering, S2 – partial replacement of watering (50 mL day^{-1}) and *R* – total watering recovery (200 mL day^{-1}). The stressed tomato plants pre-treated with Megafol® showed a lower gene induction, suggesting a hardening effect induced by the biostimulant.

were much better at increasing and maintaining their leaf water content. This was probably due to an increased metabolic capacity of the Megafol® treatment, resulting in a greater leaf area (digital biomass in Fig. 4A) and a greater water distribution in the leaves (Fig. 5). Finally, during the recovery at normal irrigation (*R*), the drought stressed plants reached almost the same fraction of high water content class as the unstressed plants. At the end of the recovery phase, the water content of the stressed plants not treated with Megafol® was slightly but still significantly lower than the other experimental conditions.

3.5. Gene expression analysis

Plants respond actively to stress conditions through the perception of stress signals, resulting in the enhanced expression of related genes leading to the activation cascade of stress-responsive genes (Shinozaki and Yamaguchi-Shinozaki, 2007). The study of the behaviour of these genes might help in understanding the molecular mechanisms governing stress responses and tolerance in tomato plants after treatment with a biostimulant product, such as Megafol®. In order to study the role of Megafol®, we analysed in tomato plants treated with the biostimulant and then subjected to water stress the transcript levels of two drought stress marker genes: (1) the dehydration-inducible gene *Solyc02g084840*, encoding for a low-temperature-induced protein, and (2) *Solyc03g025810*, a dehydrin. These genes are orthologous of Arabidopsis *RAB18* and *RD29B* genes, respectively (Lang and Palva, 1992; Cutler et al., 2010). We then compared these results with those obtained for stressed plants without pre-treating them with the biostimulant. Intriguingly, the expression of both genes dramatically increased (Fig. 6A and B) on the third day of water stress (S1), when the maximum stress perception is achieved. Subsequently, relative transcript levels progressively decreased during emergency watering (S2), until they disappeared in the recovery phase (*R*). The expression pattern of the two genes was very similar under the control conditions. Overall, the lower expression levels of both genes in the Megafol®-treated plants (Fig. 6) suggest that these plants were experiencing a much lower drought stress. On the whole, these results concerning the Megafol® treatment are in full agreement with the phenomic data described above.

4. Discussion

Biostimulants represent a class of products used in agriculture that is attracting the interest of the market and the research community (Santaniello et al., 2013). As defined by Du Jardin (2012), "plant biostimulants are substances and materials, with the exception of nutrients and pesticide, which when applied to plants, seeds or growing substrate in specific formulations, have the capacity to modify physiological processes of plants in a way that provides potential benefits to growth, development and/or stress response". Although the scientific literature reports evidence for the efficacy of biostimulants (Paradiković et al., 2011), little is known about their mode of action. Recently, the use of microarrays allowed to dissect

the effects of biostimulants at the molecular level (Santaniello et al., 2013), highlighting the ability of raw materials that are used to formulate biostimulants to induce the expression of various sets of genes. Remarkably, among the gene families that were shown to be up-regulated, several belonged to functional categories related to abiotic stress tolerance and responses to ABA (Santaniello et al., 2013). These evidences suggest that biostimulants may act by priming the treated crops against abiotic stresses. To test this hypothesis we performed the integrated molecular/phenotypic analysis we described here. To our knowledge this is the first experimental evidence for the mechanism behind the function of biostimulants such as Megafol®. Analysis of the results from the NIR, fluorescence, and white light imaging, demonstrated that the Megafol® pre-treatment of the plants confers resistance to the effects of drought stress, in agreement with a priming effect of this treatment. In addition to drought stress resistance, the Megafol®-treated plants were able to recover more quickly when they had access to water. The biostimulant appears to directly and positively influence plant response to drought, an effect that persists until the end of the drought stress. Photosynthetic efficiency and a greater level of plant water content under drought stress conditions indicate an improved metabolic activity of plants under difficult conditions. This results in plants with a greater biomass. By delaying the negative effects of drought stress, the Megafol®-treated plants are able to use the scarce water resource available more efficiently. Under normal irrigation conditions, Megafol® treatment appears to have no influence on the plant growth, photosynthetic efficiency (stress index), or plant water content.

The positive effects of the Megafol® treatments on the stressed plants were confirmed at the molecular level. By analysing the expression levels of drought stress marker genes in tomato plants, we observed that plants pre-treated with Megafol® showed a lower expression of drought-related genes even when strongly water-stressed. Considering the functional role of the marker genes analysed, which typically accumulate and up-regulate during drought stress (Ismail et al., 1999), the association between the accumulation of members of the LEA protein family and tolerance to stress is reasonable and has already been shown in several species (Cellier et al., 1998; Lopez et al., 2003).

The lower expression of these genes in the Megafol®-treated tomato plants highlights the fact that these plants are indeed experiencing a lower level of water stress, as a consequence of the Megafol® treatment itself. This result was confirmed in our analysis of the stress-related parameters. Megafol® contains betaines which could explain the results described in this set of experiments. The role of these molecules in reducing drought stress cellular damage in plants is well documented (Ashraf and Foolad, 2007; Hoque et al., 2007; Park et al., 2006; Chen and Murata, 2008). Of the various protective mechanisms against cellular damage induced by drought stress, the accumulation of small organic metabolites is important. This differs among species, but is mostly represented by polyols, sugars, aminoacids, betaines and related compounds. Betaines are synthesized in abiotic stress responses and their level is correlated with the increased tolerance by plants (Rhodes and Hanson, 1993). Tomato plants do not naturally accumulate betaines (Wyn Jones and Storey, 1981), but there is much evidence that exogenous applications improve the growth and survival of numerous species under stress (Zhao et al., 1992; Alia et al., 1998; Allard et al., 1998; Mäkelä et al., 1998; Jokinen et al., 1999; Chen et al., 2000) and in tomato, when applied to the leaves, they are taken up readily (Park et al., 2006). The betaine components of Megafol® probably reduce the sensitivity of the plants to water deficiency, resulting in a lower perception of the stress signal and in weakened negative physiological consequences. Although additional research is necessary to further clarify the molecular and physiological mechanisms which exploit biostimulants, such as Megafol®, to overcome high levels of abiotic stress, the present research demonstrated that biostimulants exert a clear, measurable alleviation of abiotic stress injuries.

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