Lettuce plants treated with L-pyroglutamic acid increase yield under water deficit stress

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Abstract

Water scarcity is one of the main challenges faced by modern agriculture, leading to a substantial drop in crop productivity and a threat to food security. Thus, novel agricultural approaches are necessary and urgent to face this problem. Some natural compounds such as amino acids have been shown to increase yield and mitigate the effects of drought stress. In this study, we demonstrate that the application of pyroglutamic acid (PG) is capable of increasing lettuce yield, under field conditions with 30% less than optimal irrigation. PG treatment showed a clearly protective effect in stressed plants, enhancing their fresh weight by 37% and yield by 31%, in comparison to untreated plants. PG appears to promote drought tolerance effects in deficit irrigated lettuce plants, with several advantages. It acts by enhancing photosynthesis rate and antioxidant defences, while maintaining osmotic and water balance, without toxicity to soil microorganisms. This illustrates the potential use of PG to combat productivity losses due to water scarcity.

Keywords

Drought, lettuce, pyroglutamic acid, water deficit stress, food security

1. Introduction

Water scarcity for crop irrigation is becoming a growing concern worldwide. However, research into crop response to deficit irrigation and resultant drought-stress is attracting more attention than research on the study of water deficit stress. This has affected agriculture from industrial, academic, and government perspectives,

largely owing to low financial support (Knepper and Mou, 2015). Deficit irrigation stress as a consequence of the progressive decrease in water availability has been a hot topic regarding food security during the last two decades (UNESCO, 2012). In fact, 56% of the areas under irrigated agriculture in the world were highly affected by this problem before 2013 (Gassert et al., 2013). Mitigating desertification and global warming will be there principal goals for agronomy in the future (UNESCO 2012). At present, 36% of earth's population inhabits zones with difficulty access to water sources (Safriel et al., 2005). Irrigation is the most important causes of water losses in the world, in certain areas, even 90% of the supply (Mekonnen and Hoekstra, 2012). It is therefore important to find new approaches to enhance plant productivity in 'limited fresh-water' areas. Finding feasible solutions to this problem is crucial to food security, to avoid crop productivity losses that may threaten a world population in constant growth.

Lettuce (*Lactuca sativa* L.) is an important salad crop grown almost worldwide. Among the world's largest producers are China, USA, Spain, Italy, India and Japan. Its leaves have high water content and it is extremely sensitive to deficit irrigation and drought due to its shallow root system (Jackson, 1995, Kizil et al., 2012). In lettuce, new strategies will become critical to enhance productivity under deficit irrigation (Malcom et al., 2012).

Plants have developed sophisticated mechanisms to cope with different types of stresses. For example, they are able to accumulate compatible solutes and activate antioxidant defences, which helps them face stress (Farooq et al., 2009). L-proline which is one of the compatible osmolytes most studied and documented in plants is

able to achieve an osmotic balance under stress to maintain turgidity, as well as detoxify reactive oxygen species (ROS) and protect plasma membrane, proteins and enzymes structures from denaturalization (Szabados and Saviouré, 2010). As a consequence of osmotic regulation in the cytoplasm, plants are able to grow normally (Subbarao et al., 2000) (Subbarao et al., 2000).

Research into applying natural compounds to increase yield and mitigate the effects of abiotic stresses has intensified in recent years. These compounds include plant hormones, biostimulants, amino acids and certain vitamins (Battacharyya et al., 2015; Lucini et al., 2015; Pushpalatha et al., 2007). Submitting plants to drought stress results in growth inhibition or retardation. However, L-proline treatment can provide osmoprotection and enhance plant growth under a range of stresses, among them water deficit (Hayat et al., 2012). Another strategy is to use protein hydrolysates as biostimulants that modulate plant molecular and physiological processes that favour growth, increase yield and alleviate the impact of abiotic stress on crops (Colla et al., 2017).

In plants, amino-acids are synthesized from three precursors, aspartate, glutamine and glutamate. The latter stands out as the amino acid into which nitrogen (N) is absorbed and assimilated by plants (Taiz and Zeiger, 2013). Several authors confirm their participation as stress-reducing substances, precursors of hormones and N carriers. (Zhao, 2010; DeLille et al., 2001; Maeda and Dudareva, 2012). However, researchers have studied the influence of a set of amino acids, but data on their efficacy alone is scarce. (Khan et al., 2009; Colla et al., 2014).

Pyroglutamic acid (5-oxoproline) is a non-protein amino-acid derivative synthesized when glutamic acid or glutamine are cyclized as lactam, and becomes a metabolite in the glutathione cycle. Mazelis and Pratt (1978) demonstrated *in vivo* that L-pyroglutamic acid is converted into glutamate in plants. According to the studied effect of glutamate (ionized form of glutamic acid) application on the increasing of antioxidant capacity of plants under environmental adverse conditions and the role reactive oxygen species (ROS) on proline metabolism (Rejeb et al., 2014), we hypothesize that the exogenous application of PG could act as a precursor of proline under stress conditions to face an osmotic imbalance. The results obtained highlight the capacity of this amino acid to increase tolerance to water deficit.

2. Material and Methods

2.1 Plant material, treatments and experimental design

Thirty day old plants of Iceberg lettuce (*Lactuca sativa* L.) provided by a local nursery were used in this study. The field trials were conducted at Escuela de Capacitación Agraria de Tacoronte, Tenerife, Canary Islands (28°29'47.0"N 16°25'12.0"W) during three consecutive years. The experiment field area was covered with a waterproof roof, equipped with a drip irrigation system and sectorized into blocks according to treatment. During the experimental periods, average daily maximum and minimum temperatures were 25°C and 12°C, respectively. The soil of the site was classified as clay-loam (35% clay, 27% slime, 38% sand). The experiments were performed using totally randomized 20 m² blocks with two replications, each block containing 100,000 plants ha⁻¹. Irrigation volumes were calculated according to the FAO (2006), taking into account the

evapotranspiration (ET) provided by a weather station, property of Cabildo de Tenerife. Soil humidity was measured near the roots of the plants (10HS sensor from Decagon Devices, WA-US). Two irrigation regimes were established: a control (WW, 95% field capacity) and a deficit irrigation treatment (WD, 30% less than the control). Deficit irrigation treatments began two weeks after transplanting. The L-pyroglutamic acid (P7,520-2; Aldrich Chemical Co., St. Louis, MO, USA) treatments were performed by adding 0.5 kg ha⁻¹ through the irrigation system. Treatment began one week after transplanting and was repeated every week until the end of the experiment (when lettuce heads were harvested).

2.2 Production measurements, RWC and electrolyte leakage

All lettuces were harvested at the end of the experiment. Outer leaves were removed to simulate trimming in a commercial harvesting operation. Average lettuce head fresh weight (g) and production (kg m⁻²) were determined for each experimental treatment. Lettuce plants were sampled weekly after deficit irrigation treatment to determine Relative Water Content (RWC) in leaves. This was accomplished by excising twenty 1-cm diameter discs for each treatment. We weighed all leaf discs immediately to provide a measure of fresh mass (W_f), then soaked them 24 h in deionized water and re-weighed the resultant turgid mass (W_t). After drying at 85°C, discs were again weighed to establish the dry-mass (W_d). The RWC for each leaf was calculated according to: $RWC = (W_f - W_d) / (W_t - W_d)$.

Electrolyte leakage provided a measure of cell membrane permeability, was measured following the protocol in Lutts et al. (1995).

2.3 Gas exchange measurements

During the growing period, lettuce-leaf gas exchange was measured in an infrared gas analyser, LCpro-SD (BioScientific Ltd., England). Using climate conditions (the average chamber temperature was 23°C and relative humidity was between 70-80%) The photosynthetic photon flux density (PPFD) used was 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ which was optimized with a light PPFD curve. The cuvette air flow rate was 500 ml min^{-1} . Net photosynthetic rate, stomatal conductance, and transpiration rate were recorded simultaneously. Water-use efficiency was calculated as the ratio between net photosynthesis and transpiration rate.

2.4 Determination of total carbohydrates and proline

Total carbohydrates were quantified by the Phenol Sulphuric Acid method (Dubois et al., 1956) using a multiplate protocol described by Masuko et al. (2005). 100 mg of fresh lettuce leaves was ground in liquid N and hydrolysed with 5 ml of 2.5N HCl for 3 hours at 95°C. Then, the mixture was cooled to room temperature and neutralized with solid sodium carbonate until the reaction effervescence stops. The resulting mixture was diluted to 50% with water, and 50 μl was used for the Phenol Sulphuric Acid method. The sample was mixed with 30 μl of phenol 5% and 150 μl of sulphuric acid 96%, then incubated at 90 °C for 5 min and cooled at room temperature for another 5 min. Absorbance was measured at 490 nm in a multi-detection plate reader (FLUOstar Omega, BMG LABTECH). Total carbohydrate was calculated on a fresh-weight basis using L-glucose as standard.

Proline content was analysed as in Bates et al. (1973). Proline concentration was determined using a standard curve and calculated on a fresh-weight basis.

2.5 Antioxidant capacity

The antioxidant status of plant tissues was assayed according to the multiplate Oxygen Radical Absorbance Capacity (ORAC) method described by Gillespie et al. (2007). The assay is performed in a microplate assessed with a 96-well multi-detection plate reader (Fluorstar Omega CBMC LABTECH, Germany). ORAC was calculated on a fresh weight basis using Trolox as antioxidant standard.

2.6. Microbiological determinations

Microbiological determinations were performed using a 1 g of soil, extracted and directly surface sowed on selective culture media. Colony formation units (CFU) were counted by *Analytical Biotech* (analyticalbiotech@infulab.com). CFU data were the average of 3 replicates.

2.7 Nutritional analyses

The lettuce leaf samples from each treatment were collected at the end of the experiment. Para realizar macronutrients analyses (Ca, K, Mg) se tomaron the lettuce leaf samples de los diferentes tratamientos y se secaron en estufa de aire forzado a una temperatura constante de 80 °C. After drying, they were ground with an IKA M20 grinder. They were then kept in a stove at 105 ° C for 5 hours and then transferred to a desiccator to proceed with the weighing of the samples. One g of ground powder was taken from

each lettuce sample, it was mineralized by dry way with 6N hydrochloric acid, after have been converted in ashes in a muffle stove at 480°C. From this extract was determined by ICP OES Avio 500. All measurements were done in triplicate. The laboratory techniques described in Official Methods of Analysis were followed (MAGRAMA, 1994).

2.8 Statistical procedure

Production statistical analyses used one-way ANOVA and a Tamhane *post hoc* test was applied to the significance of differences between experimental groups. A two-way ANOVA was then run to test the differences among factors. PG treatment was the first factor, deficit irrigation the second, and interaction the third. Significances were provided by IBM-SPSS20 software. Other measures were analysed using a T-student test. Significations are indicated in the legends.

3. Results

3.1 Lettuce heads fresh weight, productivity and leaves RWC

Lettuce plants grown under control irrigation conditions for five consecutive weeks (WW and PG) exhibited no significant differences between treatments in fresh-weight and production of trimmed heads (Figure 1A; 1B). However, significant differences were found between deficit irrigation treatments (WD and WD+PG), showing a 37% increase in head fresh-weight and a 31% increase in production when lettuce plants were treated with PG (Figure 1A;1B).

Moreover, the distribution of lettuce head fresh-weight between treatments (shown in Figure 2) showed notable differences between

control and deficit irrigated plants (WW and WD). In fact, deficit-irrigated plants showed a 47% increase in head fresh-weight values below 300 g and no production or below 5% in the other two groups (above 400 g and $\pm$ 350 g). However, PG-treated plants under control irrigation (WW+PG) showed almost a constant distribution of head fresh-weight between groups (above 400 g, $\pm$ 350 g and below 300 g). This was almost replicated in PG-treated plants under deficit irrigation (WD+PG), despite a 10% loss in head fresh-weight above 400 g (Figure 2).

Furthermore, leaf RWC showed a 10% drop in deficit-irrigated plants (WD) two weeks after irrigation deficit was imposed, when compared to the other three treatments. These differences were maintained until the end of the experiment (Figure 3A). Percentages of soil relative humidity according to each treatment, monitored during overall field trials, are shown in Figure 3B. Lastly, the effect of deficit irrigation and PG were individually tested, and also their mutual interaction, using a two-way ANOVA comparing head fresh-weight and leaf RWC, as shown in Table 1. Statistically, deficit irrigation and PG treatment significantly affect lettuce head fresh-weight and their interaction also showed differences. Also, deficit irrigation began to affect leaf RWC after two weeks, while PG treatments had no apparent effect. From the second week on, the two factors showed significant differences in their interaction.

3.2 Photosynthesis, plant total carbohydrates and nutritional analysis

The results obtained in head fresh weight, production and RWC were consistent with values of photosynthesis rates in deficit-irrigated plants. Weekly measurements were performed to assess

the effect of PG treatments in control and deficit-irrigated plants. As shown in Figure 4, plants exposed to deficit irrigation (WD) reduced their net photosynthesis rates from the first week of the experiment and kept these values for three consecutive weeks. This trend was also found in the other two variables analysed: stomatal conductance and water use efficiency (Figure 4). However, PG-treated plants under both treatments (WW+PG, WD+PG) showed higher net photosynthesis rates, stomatal conductance and water-use efficiency during the monitoring period. In fact, during the second and third weeks, PG-treated plants showed higher stomatal conductance and water-use efficiency than the control plants, independently of the water regime imposed (Figure 4).

This inhibition of net photosynthesis detected in WD-treated plants when compared with WW ones was accompanied by a significant reduction in plant total carbohydrates (an average of 40%), once the water regimes were imposed. Similarly, PG-treated plants under deficit irrigation also showed a decline in total carbohydrates. However, as an average over the monitoring period, these differences were never below 10% when compared with the WW-PG treatment (Figure 5).

The nutritional analysis of lettuce leaves under control irrigation conditions showed significant differences between WW and WD treatments in Ca, K and Mg levels (Table 2), but no significant differences in foliar N (Data not shown). PG-treated plants under control irrigation showed no statistical differences in foliar Mg and N content. However, they exhibited a 1.3-fold increase in Ca and a significant but slight reduction in K levels when compared to WW lettuce plants. In addition, PG-treated plants under deficit irrigation

showed no significant differences in N levels, but a significant accumulation of Ca, K, and Mg levels compared to WW-PG and WW treatments (Table 2).

3.3 Proline, antioxidant capacity and electrolyte leakage

Proline accumulation in lettuce leaves during the first three weeks of the irrigation treatment showed no significant differences between WW and WD lettuce plants (Figure 6A). However, WD-PG treated plants exhibited significant differences in leaf proline accumulation when the values were compared with the other three groups since the second week of the experiment. In fact, this metabolite content of WD-PG treated plants increased 40% in the last week of analysis, when compared to the rest of the treatments (Figure 6A).

The ORAC analysis, a measure of the antioxidant status of the lettuce leaves and expressed as Trolox equivalents, showed significant but slight differences between WW and WD treatments during the third week of the experiment. However, PG-treated plants did show clearly significant differences compared to untreated ones, but not between the PG-treatments (Figure 6B).

Electrolyte leakage did not show any differences in our experimental conditions after submitting plants to deficit irrigation stress (Data not shown).

3.4 Soil microorganisms study

Data from a soil microbiological study performed at the end of the experiment are presented in Table 3. Comparing control and PG-treated soils, significant changes in the soil microorganism groups with high counts of CFUg⁻¹ were found. Examples are total

heterotrophic bacteria, anaerobic endospore-forming bacteria, heterotrophic fungi and phosphate-solubilizing microorganisms (Table 3). However, some microbial groups dropped in numbers, such as bacteria classified as *Pseudomonas*, *Enterobacter*, Non-symbiotic N-fixing microorganisms and *Actinomyces* (Table 3).

4. Discussion

Increasing water deficit and drought conditions will lead to serious consequences for food security in the near future, since the world population may easily grow to ten billion. The scenario is under the influence of worldwide climatic disturbances, likely to intensify the impact of such stress. Therefore, to search for solutions we must expand our understanding of water deficit and drought stress in plants (Hossain et al., 2016). Various strategies have been tested to improve drought tolerance in crop plants, such as the use of more tolerant transgenic lines. The wide range of drought-related plant genes offers amazing opportunities for crop improvement, but generally the success rate is low (Khan et al., 2011). Some drought-tolerant crop plants have already been obtained through selective breeding, which is usually time-consuming (Farooq et al., 2009; Xoconostle-Cazares et al., 2010). Among other strategies to face this problem, the use of compounds capable of inducing abiotic stress tolerance is still little explored. Amino-acid treatments (usually as registered mixtures) have been shown to reduce or mitigate plant stress, besides other applications (Teixeira et al., 2017; DeLille et al., 2001; Maeda and Dudareva, 2012).

As mentioned, lettuce is particularly and extremely sensitive to drought due to its short root system and the high-water content held in the leaves (Jackson, 1995). Previous studies demonstrated that a

water deficit of 40% drastically reduces the lettuce weight and increases oxidative damage (Sayyari et al., 2013). However, under our experimental conditions, a 30% water deficit also considerably reduced lettuce head fresh weight and the final production. Moreover, PG showed a clearly protective effect on yield parameters, inducing a 37% increment in weight and 31% in production in water-deficit treated plants. Environmental factors such as drought may cause nutrient deficiencies, even in fertilized fields, as the physiochemical properties of the soil can lead to reduced mobility and absorbance of individual nutrients (Amtmann and Blatt 2009). In our study, Ca accumulation in lettuce leaves was significantly higher in PG-treated plants, while lettuce plants under WD treatment showed lower Ca values. These results indicate a direct effect of PG treatment on Ca uptake, which could be important in future post-harvest studies and lettuce shelf life.

A similar trend was observed for K and Mg; both macronutrients are important in opening and closing the stomata and chlorophyll biosynthesis (Mengel and Kirby, 2001). Under drought stress, the limitation of photosynthesis debilitates the efficiency of all other biological processes, leading to lower growth and yield (Hammad and Ali, 2014). Our results show how 30% lower irrigation levels considerably reduced net photosynthesis and stomatal conductance, which have a clearly negative effect on plant growth (Figure 1C). However, PG prevents these negative effects with higher values of the mentioned parameters which were consistent with higher total carbohydrate content (Fig. 5). This behaviour is similar to the results of Hammad and Ali (2014). Net photosynthesis rates are positively correlated with stomatal conductance under optimal and osmotic

stress conditions, thus effective stomatal control promotes rapid growth and tolerance under drought conditions (Haworth et al., 2018). Comparing WUE parameters, stressed and non-stressed PG-treated plants had higher values than untreated plants (Figure 4).

Osmolyte accumulation is frequently cited as a key putative mechanism for increasing yields of crops subjected to drought conditions (Serraj and Sinclair, 2002). Among them, L-proline is the most studied under stress conditions. Cytoplasmic and intercellular accumulation of proline is able to protect cells from damage; it acts as both a radical scavenger and as osmo-protection agent (Szabados & Saviouré, 2010). Under our experimental conditions, PG promoted proline accumulation under water deficit stress from the third week on, probably to counteract the osmotic pressure induced by water deficit in the soil, while untreated plants did not show significant changes. Mazelis & Pratt (1976) demonstrated how PG is converted into glutamic acid in plants. In fact, glutamic acid is a precursor in proline biosynthesis under stress conditions (Szabados & Saviouré, 2010). Direct treatment with glutamic acid did not increase proline accumulation in soybean (Teixeira et al., 2017). In this study, proline accumulation kinetics in PG-treated plants may be explained by the conversion of PG to glutamate and finally to proline, but only under stress conditions (see hypothetical schema, Figure 7). However, further studies are necessary to confirm this hypothesis.

Reactive oxygen species (ROS) are produced by exposure of plants to environmental stresses, e.g. water shortage. These ROS can react with proteins, lipids, and other important macromolecules and denature their structure and function (Meloni et al., 2003). In the

experiments, similar electrolyte leakage levels were found in all lettuce treatments (data not shown), suggesting a low oxidative stress in lettuce leaves. This could be caused by the high humidity and mild temperatures they underwent. Anyhow, PG seems to specifically improve ROS scavenging efficiency, by raising the total antioxidant capacity. For that reason, it is interesting to test if PG treatment is capable of inducing tolerance against water deficit in the harshest environments, because the ability of plants to balance ROS production and scavenging is associated with a higher tolerance to a range of environmental stresses (Martinez et al., 2016).

Finally, in order to test the effect of PG treatment on different groups of soils we performed a microbial analysis (Table 1). Overall, it showed that PG treatment did not adversely affect soil microorganisms. Nevertheless, the positive relation of PG soil-treatment with an increase in phosphate-solubilizing microorganisms (PSM) is worth noting. Such bacteria are very important for the soil because phosphorous (P) is the second most fundamental fertilizing element after N, governed by plant requirements (Sharma et al., 2013). However, P is abundant in soils in both inorganic and organic forms, but its insoluble or otherwise unassimilable compounds (99.9%) are unavailable for root uptake (Rengel & Marschner, 2005, Illmer and Schinner, 1995). (In this regard, PSM have been shown to be an environmentally friendly P source for crops (Sharma et al., 2013). Further studies are required, to elucidate if PG promotes PSM.

5. Conclusions

PG shows a clearly protective effect on yield parameters, inducing a 37% increment in weight and 31% in yield in water-deficit treated

plants. PG appears to promote water deficit tolerance by acting at different levels: photosynthesis rate, osmotic and water balance, and antioxidant defences, without evidence of toxic effects on the soil. This study provides evidence for the protective effect of PG against water supply limitations, using root treatment. It highlights the potential use of PG as inducer for enhancing crop tolerance to adverse environmental conditions, and its practical application in agriculture.

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Table 1. Results of a two-way analysis of variance of lettuce head fresh weight and relative water content (RWC), due to the factors ‘deficit irrigation’ and ‘pyroglutamic acid’ and their interaction. Numbers represent F values: ***p < 0.001; n.s., non-significant.

Dependent variable	WD	PG	WD+PG
Lettuce head fresh weight	40.52***	14.07***	21.95 ***
RWC after 1-week	0.347 n.s	0.027 n.s	0.081 n.s
RWC after 2-weeks	16.62 ***	0.003 n.s	10.898 ***
RWC after 3-weeks	30.9 ***	0.053 n.s	27.39***
RWC after 4-weeks	21.95***	0.226 n.s	25.69***

Table 2. Analysis of 3 nutrients in leaves, according to treatment.

	g.Kg-1		
	Ca	K	Mg
WW	7.3 ± 0.014 a	71.0 ± 2.4 a	4.1 ± 0.016a
WD	4.7 ± 0.003 b	42.2 ± 1.2 b	2.7 ± 0.053 b
WW+PG	9.1 ± 0.16 c	63.4 ± 1.3 c	4.1 ± 0.016 a
WD+PG	11.2 ± 0.015 d	80.1 ± 2.1 d	5.3 ± 0.011 c

Table 3. Analysis of total microorganisms found in the soil among the different treatments. CFU.g-1 means colony forming units per gram.

Microorganisms	Control soil	Pyroglutamic treated soil	CFU.g-1 Change
Total heterotrophic bacteria	1.29E+08	3.10E+08	1.81E+08
Anaerobic endospore-forming bacteria	3.07E+08	5.28E+08	2.21E+08
Heterotrophic fungi	2.38E+05	1.41E+07	1.38E+07
Phosphate-solubilizing microorganisms	1.22E+07	2.00E+08	1.88E+08
Pseudomonas	6.52E+05	7.90E+04	-5.73E+05
Enterobacter	4.50E+06	1.50E+06	-3.01E+06
Non-symbiotic nitrogen-fixing microorganisms	8.65E+06	3.95E+06	-4.70E+06
Actinomycetes	1.79E+06	1.76E+06	-3.50E+04

Figure legends

Figure 1.- Analysis of lettuce head fresh weight (**A**), productivity (**B**) and appearance (**C**) of lettuce plants after different experimental treatments. Results presented are the average of three independent field trials. Values within each treatment marked by the same letter are not significantly different at $p < 0.05$, according to the ANOVA statistical procedure.

Figure 2.- Distribution of lettuce plants by percentage of each weight category among the different treatments.

Figure 3.- Analysis of relative water content percentages of lettuce leaves among the different treatments (**A**). Percentages of soil relative humidity according to treatment during the field trials (**B**). The results represent the average of three independent field trials. Values within each treatment marked by the same letter are not significantly different at $p < 0.05$, following T-Student test procedure.

Figure 4.- Analysis of several photosynthetic parameters of PG-treated and untreated lettuce plants under well-watered and deficit irrigation stress conditions: net photosynthesis (**A**), stomatal conductance (**B**), and water use efficiency (**C**). The results represent the average of three independent field trials. Values within each treatment marked by the same letter are not significantly different at $p < 0.05$, according to T-Student test procedure.

Figure 5.- Changes in total carbohydrate in PG-treated and untreated lettuce plants during a 4 week period, under normal and deficit irrigation stress conditions. The results represent the average of three independent field trials. Values for the same time-point

marked by the same letter are not significantly different at $p < 0.05$, according to the ANOVA statistical procedure.

Figure 6.- Kinetics of proline accumulation (**A**) and antioxidant capacity (**B**) in leaves of PG-treated and untreated lettuce plants under normal and deficit irrigation stress conditions. The results represent the average of three independent field trials. Values within the same time-point marked by the same letter are not significantly different at $p < 0.05$, according to T-Student test procedure

Figure 7.- Hypothetical schema of the effect of PG on defence mechanisms against water-deficit stress in lettuce plants. PG stays as reservoir under well-watered conditions but acts promoting its conversion to glutamate which is finally converted in proline under water-deficit conditions. Abbreviations: WW, well-watered; WD, water deficit.

Figure 1

A

B

C

Figure 2

Figure 3

A

B

Figure 4

Figure 5

Figure 6

A

B

Figure 7

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.