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1 **Environmental-dependent proline accumulation in plants living on gypsum soils**

2  
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20  
21 **Abstract** Biosynthesis of proline – or other compatible solutes – is a conserved  
22 response of all organisms to different abiotic stress conditions leading to cellular  
23 dehydration. However, the biological relevance of this reaction for plant stress tolerance  
24 mechanisms remains largely unknown, since there are very few available data on  
25 proline levels in stress tolerant plants under natural conditions. The aim of this work  
26 was to establish the relationship between proline levels and different environmental  
27 stress factors in plants living on gypsum soils. During the 2-year study (2009-2010), soil  
28 parameters and climatic data were monitored, and proline contents were determined, in  
29 six successive samplings, in ten taxa present in selected experimental plots, three in a  
30 gypsum area and one in a semiarid zone, both located in the province of Valencia, in  
31 south-east Spain. Mean proline values varied significantly between species; however,  
32 seasonal variations within species were in many cases even wider, with the most

33 extreme differences registered in *Helianthemum syriacum* (almost 30  $\mu\text{mol g}^{-1}$  of DW in  
34 summer 2009, as compared to ca. 0.5 in spring, in one of the plots of the gypsum zone).  
35 Higher proline contents in plants were generally observed under lower soil humidity  
36 conditions, especially in the 2009 summer sampling preceded by a severe drought  
37 period. Our results clearly show a positive correlation between the degree of  
38 environmental stress and the proline level in most of the taxa included in this study,  
39 supporting a functional role of proline in stress tolerance mechanisms of plants adapted  
40 to gypsum. However, the main trigger of proline biosynthesis in this type of habitat, as  
41 in arid or semiarid zones, is water deficit, while the component of ‘salt stress’ due to the  
42 presence of gypsum in the soil only plays a secondary role.

43

44 **Key words** Abiotic stress, osmolytes, stress tolerance, seasonal variation, soil humidity,  
45 water deficit

46

#### 47 **Authors Contribution Statement**

48

49 Selection of experimental plots and plant species, and field samplings of plant material  
50 have been carried out by O. Mayoral, P. Donat and M. Boscaiu. Soil samplings and soil  
51 pre-treatments have been carried out by I. Bautista, A. Lidón and C. Lull. Installation of  
52 soil moisture and temperature sensors and processing of the data have been carried out  
53 by J.V. Llinares and A. Lidón. Analyses of plant material have been performed by O.  
54 Vicente and M. Boscaiu. Soil analyses have been performed by I. Bautista and C. Lull.  
55 Statistical analysis of the data has been realized by M. Boscaiu, I. Bautista and A.  
56 Lidón. M. Boscaiu, I. Bautista, O. Vicente and A. Lidón have collaborated in the  
57 elaboration of the manuscript. O. Vicente has been responsible for the general  
58 supervision of the work.

59

#### 60 **Introduction**

61

62 A conserved response of plants to different abiotic stress conditions causing cellular  
63 dehydration, such as drought and high soil salinity, is based on the synthesis of  
64 osmolytes, very soluble organic compounds that can accumulate at high concentrations

65 in the cytoplasm without interfering with the metabolism and are therefore considered  
66 'compatible' solutes (Flowers et al. 1977; Yancey 2005; Szabados et al. 2011). Apart  
67 from their contribution to osmotic adjustment, osmolytes also act as 'osmoprotectants',  
68 directly stabilising proteins and membrane structures under stress conditions and  
69 protecting plants from oxidative damage – a general secondary effect of abiotic stress –  
70 by their ROS scavenging activity (Yancey 2005; Ashraf and Foolad 2007; Flowers and  
71 Colmer 2008). Osmolytes are diverse from the chemical point of view, but one of the  
72 most common in plants is proline (Pro), which is considered a reliable biochemical  
73 marker of abiotic stress as it accumulates in response to soil water deficit, increasing  
74 salinity or low temperatures (Hare et al. 1998; Szabados and Saviouré 2010).

75 Most reports on the stress-induced biosynthesis of compatible solutes –  
76 including Pro – refer to experiments carried out using stress-sensitive model species  
77 such as *Arabidopsis thaliana*, under artificial laboratory or greenhouse conditions.  
78 Therefore, the relative importance of different environmental factors for the induction of  
79 osmolyte biosynthesis in stress-tolerant plants growing in their natural habitats is still  
80 largely unknown (Grigore et al. 2011).

81 Gypsum soils, which are characteristic of arid or semi-arid regions with an  
82 annual rainfall below 400 mm (FAO 1990), represent an adverse habitat for the  
83 establishment and development of plant communities (Palacio et al. 2007; Martínez-  
84 Duro et al. 2010), partly because of their chemical properties: they are generally poor in  
85 organic matter and contain very low levels of N and P (FAO 1990); the high  
86 concentration of soluble Ca interferes with the uptake by plants of other macronutrients  
87 (P, K, or Mg) and reduce the availability of several micronutrients, such as Zn, Fe and  
88 Mn. Some physical characteristics, such as weak aggregation of soil particles, poor  
89 water retention capacity or formation of hard gypsum crusts which impede penetration  
90 of roots, also limit plant growth (FAO 1990; Verheye and Boyadgiev 1997). However,  
91 gypsum habitats are extremely interesting from an ecological point of view: they are  
92 highly threatened by human activities and very sensitive to the foreseeable effects of  
93 global climate change, and the vascular flora colonising these zones includes many  
94 endemic and/or rare taxa (e.g., Meyer 1986). Therefore, it is somewhat surprising that  
95 there are still very few reports on the biochemical and physiological responses of plants  
96 adapted to gypsum environments (e.g., Alvarado et al. 2000; Palacio et al. 2007).

97 Concerning osmolyte biosynthesis in plants from gypsum areas, the only previous  
98 study, to our knowledge, was carried out by Alvarado et al. (2000), who determined Pro  
99 levels in five gypsophytes but did not analyse possible correlations between Pro  
100 accumulation and environmental stress factors.

101 In the frame of our studies on the physiological function(s) of osmolytes in plant  
102 stress tolerance mechanisms in nature, in the present work we have determined Pro  
103 contents in several species present in three experimental plots, defined by their position  
104 along a slope in a gypsum area, as well as in a fourth plot located in a calcareous, non-  
105 gypsiferous zone. Six samplings were carried out over a period of two years, and spatial  
106 and seasonal changes in Pro levels were correlated with several soil parameters and  
107 meteorological data. The specific aims of this study were to confirm the relationship  
108 between Pro contents and the degree of abiotic stress affecting the plants in their natural  
109 habitat, and to establish the relative importance of different environmental factors for  
110 Pro accumulation in the investigated species.

111 .  
112

## 113 **Material and Methods**

114

### 115 **Selection of experimental sites and plant species, and sampling design**

116

117 The main study site is located near the village of Tuéjar, in the Province of Valencia  
118 (SE Spain) (39°47'28''N, 1°04'25''W) at 603 m.a.s.l. Three 10 x 10 m plots (P1, P2 and  
119 P3), located on a hillside with a SW orientation and a slope variable between 11.5° and  
120 19°, were selected according to the presence of plant species that were indicators of  
121 gypsum. Plot P1, situated at the top of the slope, was the driest, but had the lowest  
122 gypsum content, whereas plot P3, at the bottom, was the most humid and flattest, but  
123 contained more gypsum since soluble material, carried downhill by rains, is deposited  
124 and precipitated in the lowest part of the slope. A fourth plot (P4) was chosen in a non-  
125 gypsum area near Bétera (Province of Valencia) (39°39'44''N, 0°28'33''W), at 220  
126 m.a.s.l. on calcareous soils and under semiarid climate conditions, where the main  
127 restrictive factor for plant growth was water availability. The experimental work lasted

128 two years: 2009 and 2010. Plant material was collected six times in spring, summer and  
129 autumn in both years.

130

### 131 **Plant species**

132

133 The study area is characterised by the presence of gypsum indicator plants, such as  
134 *Ononis tridentata* subsp. *angustifolia* and *Gypsophila struthium* subsp. *hispanica*  
135 (included in the association *Ononidetum tridentatae* Br.-B. and O. Bolòs, 1858).  
136 According to Mota et al. (2009), these two species are considered severe gypsophytes  
137 and are included in the checklist of Iberian gypsophytes; both are classified in the scale  
138 ranking as 5, meaning that they are species exclusive of gypsum soils. The remaining  
139 taxa were either gypsovags – plants that often grow and are abundant on gypsum soils,  
140 but are also present on other soil types – or accidentals which, according to the  
141 definition by Mota et al. (2009), are indifferent to soil type or even exhibit optimal  
142 development in other habitats, and their presence on gypsum is accidental.

143 Altogether, ten taxa were selected (Table 1) according to several criteria: only  
144 perennial species were considered in order to collect plant material from the same  
145 individuals in all the samplings as far as possible; some species present in different plots  
146 were chosen for comparative analyses, along with gypsum indicators (gypsophytes),  
147 even if they were present only in one plot; finally, a few species not found in the  
148 gypsum area, but specific for arid and semiarid lands, were also included. In the area of  
149 Bétera, vegetation is dominated by Mediterranean scrub species and grasslands.

150

### 151 **Soil characterisation and soil and climate monitoring**

152

153 At the beginning of the study (spring 2009), soil characteristics were analysed in three  
154 random soil samples taken from each experimental plot at a depth of 0-15 cm, after they  
155 were air-dried and passed through a 2 mm sieve. Gypsum content was estimated by the  
156 reduction in sample weight between 60° and 105°C due to loss of hydration water  
157 (adapted from FAO, 1990). N mineral content was determined by extraction with 2 M  
158 KCl, followed by a colorimetric determination of nitric and ammoniacal nitrogen (FIA  
159 system). Soil samples were sieved (2 mm) and were extracted with 2 M KCl to

160 determine mineral nitrogen.  $\text{NO}_3\text{-N}$  was determined by the Griess-Alloway technique  
161 after reduction of  $\text{NO}_3$  to  $\text{NO}_2$  with a Cd column (Keeney and Nelson 1982) and  $\text{NH}_4\text{-N}$   
162 was determined by ammonia steam distillation in concentrated NaOH using flow  
163 injecting systems (Tecator 1984). Extraction of available P was carried out with a  
164 diluted acid solution (43 mM acetic acid containing 1 mM  $\text{H}_2\text{SO}_4$ ) according to Burriel  
165 and Hernando (1947), and P in the extract was determined colorimetrically by ascorbic  
166 acid method (Kuo 1996). Available K was determined by flame photometry after  
167 ammonium acetate extraction (Knudsen et al. 1982). All the soil samples, after being  
168 passed through a 0.5 mm sieve, were analysed for oxidable organic C by the Walkey-  
169 Blak method (Nelson and Sommers 1982). Water holding capacity was determined as  
170 the fraction of water retained in soil in a pressure chamber at 20 kPa. A 1:1 soil:water  
171 extract was prepared to determine electrical conductivity (EC) and soil solution  
172 composition:  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  by complexometry,  $\text{Na}^+$  and  $\text{K}^+$  by flame photometry, and  
173  $\text{Cl}^-$  using a Sherwood Chloride Analyzer 926.

174 To monitor the variables that were considered important for the induction of  
175 biochemical responses in plants, in each Tuéjar plot, several multiple sensors (5TE,  
176 Decagon) for salinity, humidity and temperature measurements were installed on 29  
177 April, 2009 at depths of 10 cm and 20 cm, and were connected to a datalogger (EM50,  
178 Decagon). In P4 (Bétera), four sensors for soil water content and four sensors for  
179 temperature were installed at depths of 10 cm and 20 cm. Additional sensors to measure  
180 air temperature and rainfall were also connected to the dataloggers in plots P2 and P4.  
181 Climatic data for the month previous to the first sampling were obtained from the  
182 nearest meteorological stations, located less than 3 km for the gypsum area and at about  
183 10 km from the semi-arid zone.

184

### 185 **Proline quantification**

186

187 Proline contents were measured in two gypsophytes and in six gypsovags present in the  
188 Tuéjar area and in five species from Bétera, three of which were common to the gypsum  
189 area (see Table 1). Young shoots were sampled separately from five individuals for each  
190 taxon, cooled on ice and transported to the laboratory, where leaves were separated  
191 from branches. Part of the leaf material was frozen and stored at  $-75^\circ\text{C}$ , and the rest was

192 dried in the oven at 65°C for 3-4 days until constant weight to obtain the percentage of  
193 dry weight (DW) of each individual. Pro was extracted from 0.1 g of frozen material in  
194 liquid nitrogen and quantified according to the method of Bates et al. (1973), as  
195 modified by Vicente et al. (2004); Pro content was expressed in  $\mu\text{mol}\cdot\text{gr}^{-1}$  of DW.

196

## 197 **Statistical analysis**

198

199 Data were analysed by the StatGraphic Centurion 16 programme. Significance of  
200 differences among seasons in the species sampled in only one plot was tested by  
201 applying a one-way ANOVA. Prior to ANOVA, the normality and homogeneity of  
202 variance were also checked. When the ANOVA null hypothesis was rejected, post-hoc  
203 comparisons were performed using the LSD test. For the taxa present in at least two  
204 plots, a two-way ANOVA was applied to check the interaction between plot and  
205 sampling season. In order to correlate ecological factors to proline levels, a multivariate  
206 approach of a principal component analysis (Martens and Maes 1989) was followed.  
207 The ecological variables that significantly correlated with proline content were  
208 subjected to the principal component option after a previous autoscale. In addition, a  
209 separate analysis between the mean proline levels in each plot and previous cumulative  
210 rainfall was performed for the species present in all the plots by applying non-linear  
211 regression.

212

## 213 **Results**

214

### 215 **Soil and climate data**

216

217 The three topographical positions in the Tuéjar area showed significant differences in  
218 gypsum and carbonate content (Table 2). As gypsum content increased, calcium  
219 carbonate content tended to decrease, and *vice versa*. The two higher plots (P1 and P2)  
220 showed similar gypsum and carbonate contents, but the lowest plot on the hill (P3)  
221 presented significantly higher gypsum content and lower carbonate content. The plot in  
222 the semiarid area in Bétera (P4) had no gypsum and very high carbonate content. The



223 soil in Bétera was more alkaline than in the gypsum area, due to its higher calcium  
224 carbonate content (Table 2).

225 Gypsum-rich soils are normally poor in organic matter (FAO 1990);  
226 accordingly, the lowest level of organic matter was found in P3. When comparing the  
227 chemical characteristics of the soil in the gypsic habitat in Tuéjar and the semiarid zone  
228 in Bétera, the highest differences (significant at the 99% confidence level) were found  
229 in the  $\text{CaCO}_3$  content (more than 8-fold higher in Bétera than the average value in  
230 Tuéjar), but levels of soluble Ca and Mg, and electrical conductivity in the 1:1 water  
231 extract, were considerably higher in gypsum *vs.* non-gypsic soils. Significant  
232 differences, at the 95% confidence level, were recorded in the amount of available P (4-  
233 fold higher in Tuéjar) and K; the latter was higher only in P1, located at the top of the  
234 slope in Tuéjar, but was similar in the remaining three plots from both areas (Table 2).  
235 When comparing only the three plots in Tuéjar, P3 had more than double the amount of  
236 gypsum than P1 and P2, had much less  $\text{CaCO}_3$  – which was expected – but also less  
237 organic C and less available K. P1, situated at the top of the slope, had almost a 4-fold  
238 higher amount of  $\text{Mg}^{2+}$  and more than double  $\text{Cl}^-$  in a 1:1 water extract. P1 was the  
239 nearest to the interface between the geological strata of gypsum and the upper strata,  
240 accounting for its higher soil fertility, as indicated by its mineral N content, available K  
241 and water soluble Mg. This plot also presented slightly higher levels of soluble salt in a  
242 1:1 water extract: 2.61 dS/m *vs.* 2.43 in P2 and P3 (Table 2). These values are similar to  
243 those reported by other authors (Pueyo et al. 2007).

244 A quite different hydrological behaviour was noted in the two study years: 2009  
245 and 2010 (Fig. 1). The sensors installed in Bétera showed that water content varied  
246 between values close to 0 (summer 2009) to about  $0.35 \text{ m}^3/\text{m}^3$  of soil (spring 2010); in  
247 Tuéjar, the corresponding values ranged from 0.1 to  $0.27 \text{ m}^3/\text{m}^3$ . A stronger response to  
248 water loss by evapotranspiration was found in P4 (Bétera), where soil was very shallow.  
249 When comparing the three plots in Tuéjar, soil humidity was generally much lower in  
250 P1 and the variation in humidity was also higher. This is explained by the topographic  
251 position of P1, situated in the upper part of the slope with less soil depth, but there was  
252 also a higher degree of stoniness in this plot. P3, located at the bottom of the slope, was  
253 the zone that maintained higher levels of humidity. The soil humidity values reveal that  
254 the annual soil drought pattern in summer, typical of the Mediterranean climate, was

255 more rigorous in 2009 than in 2010. Spring 2009 was dry, with scarcely any rainfall  
256 from May to August; therefore, the 2009 summer sample collection was carried out  
257 after a considerable soil water deficit period. In the same year, rainfall in September was  
258 about 80 mm, the result of a significant precipitation which filled the soil water reserve.  
259 Both the winter and spring of 2010 were wet. The dry period began later in July, but  
260 lasted until November; therefore, the 2010 summer sampling was carried out after a  
261 rainy period, while the autumn sampling followed a 4-month drought period. Table 3  
262 summarises the mean climatic variables for the month previous to each sampling. Since  
263 the effect of rain on plant water availability is not immediate, as it depends not only on  
264 the amount of rain, but also on evaporation and soil water holding capacity, the rainfalls  
265 from the previous two months were also included. Soil humidity data, especially in the  
266 two summers, provided by the sensors, better correlated with the 2-month period than  
267 with the 1-month period. In general, even though the sampling dates in the two areas  
268 were not exactly the same, the rainfall in Tuéjar was more abundant than in Bétera.

269

### 270 **Proline quantification**

271

272 The mean Pro values per species (including all the samplings and all the plots) varied  
273 from a minimum of 0.6  $\mu\text{moles}\cdot\text{gr}^{-1}$  of DW in *Stipa tenacissima* to a maximum of 3.92  
274  $\mu\text{moles}\cdot\text{gr}^{-1}$  of DW in *Ononis tridentata*. As expected when including species of  
275 different genera and families, Pro showed broad individual variation, ranging from a  
276 minimum of 0.18  $\mu\text{moles}\cdot\text{gr}^{-1}$  of DW recorded in *S. tenacissima* from P4 (Bétera) to a  
277 maximum of 29.54  $\mu\text{moles}\cdot\text{gr}^{-1}$  of DW in *H. syriacum* from P2 in Tuéjar (individual  
278 data not shown). When considering Pro level variation within one species, the least  
279 variation was again found in *S. tenacissima* (individual values ranging from 0.25 to 0.91  
280  $\mu\text{moles}\cdot\text{gr}^{-1}$  of DW) and the maximum was recorded in *H. syriacum* (from 0.48 to 29.54  
281  $\mu\text{moles}\cdot\text{gr}^{-1}$  of DW). The seasonal mean values for the species present in only one plot  
282 are summarised in Table 4, whereas those present in more than one plot are shown in  
283 Fig. 2.

284 *Gypsophila struthium* showed relatively low mean Pro values which peaked in  
285 the spring of 2009, followed by a second higher value in the spring of 2010 (Table 4). It  
286 is worth mentioning that this plant species was present in the study area exclusively in

287 P3, this being the plot that best maintained moisture throughout the year; hence, water  
288 stress was not so accentuated in summer. Proline variation was again significant in the  
289 three species present only in the semiarid zone (Table 4): *Stipa offneri* and *Dorycnium*  
290 *pentaphyllum* had considerably larger amounts of proline in summer 2009, but *Stipa*  
291 *tenacissima* showed lower values in all the seasons.

292 Fig. 2 illustrates seasonal proline variation in the taxa present in more than one  
293 plot. Very high proline values were recorded in *Helianthemum syriacum* in the summer  
294 of 2009, especially in plots P1 and P2. A similar pattern was also detected in  
295 *Rosmarinus officinalis*, *Cistus clusii*, *Anthyllis cytisoides* and *Thymus vulgaris*, which  
296 again presented the highest proline contents in summer 2009. During that extremely dry  
297 period, collection of the plant material of the last cited species was possible only in P3,  
298 which best maintained soil humidity, since the plants from drier areas (P1 and P2 in  
299 Tuéjar, and P4 in Bétera) had completely lost all their leaves; this is an adaptation  
300 strategy of several Mediterranean genera to the hot, dry summers characteristic of this  
301 type of climate. In contrast, *Ononis tridentata* showed a relatively high mean Pro value  
302 and a different accumulation pattern, with a notably higher content in autumn than in  
303 the previous summer in both years.

304 A two-way ANOVA was carried out for the species present in more than one  
305 plot (Table 5) by taking 'plot' and 'sampling date' as factors. Differences according to  
306 sampling date were significant at the 99% confidence level in all the taxa. When  
307 considering the 'plot' factor and the interaction between plot and sampling date, the  
308 differences found were significant for all the taxa, except *Ononis tridentata*. A plot and  
309 season interaction was found for the species sampled from all the plots, but also in  
310 *Cistus clusii* and *Anthyllis cytisoides*, present only in the gypsum area. With *C. clusii*,  
311 which was sampled from plots P1 and P3, the climatic factor response pattern was  
312 strikingly different, with considerably high proline values in summer in the plants from  
313 plot P1. These differences between proline contents in plants from different plots in the  
314 gypsum area were also detected in other species and can be explained by the  
315 aforementioned differences in soil humidity.

316 The increase in Pro contents in summer 2009, as compared to other seasons, was  
317 due to specific Pro biosynthesis, and probably also to inhibition of Pro degradation, but  
318 not to non-specific protein degradation under stress conditions since a parallel increase

319 in the general pool of free amino acids was not detected in these species (data not  
320 shown).

321 To confirm the apparent relationship between environmental factors and proline  
322 levels, a principal component analysis was carried out, which included the soil and  
323 climatic variables that significantly correlated with proline content: previous month  
324 mean temperature (Mean T), previous month soil humidity at a depth of 10 cm (Hum  
325 10), previous month soil humidity at a depth of 20 cm (Hum 20), cumulative rainfall  
326 from two previous months (Rain 2 month) and electrical conductivity in the 1:1 soil  
327 water extract (salinity). Two components with an eigenvalue equal to or greater than 1  
328 explain a cumulative percentage of variance of 68%. These two components were  
329 applied to obtain the interrelation between variables and objects. The loading vectors  
330 plot shows the relationship between variables (Fig. 3). The first component, which  
331 explains 48% of variance, positively correlated with the climatic variables associated  
332 with water loss (mean temperature), and negatively correlated with the variables  
333 associated with water availability (previous two months' rainfall and soil humidity at  
334 depths of 10 cm and 20 cm). The second component, which explains an additional 20%  
335 of variance, related to the soil electrical conductivity value. Thus, proline accumulation  
336 in plants from gypsum environments is influenced mostly by soil water deficit, but also,  
337 to a lesser extent, by salinity.

338 Finally, to establish the response of proline accumulation to the soil water  
339 reserve, for the species present in all the plots, the mean proline content was correlated  
340 with previous cumulated rainfall for periods ranging from 15 days to 4 months. For  
341 most species, the best fit was an exponential correlation with rainfall from the two  
342 months previous to sampling of plant material. These correlations are shown only in the  
343 case of *Rosmarinus officinalis* (Fig. 4), but the trend was similar in all the taxa analysed.  
344 The P1 plants showed a higher proline content under low rainfall conditions if  
345 compared to those in P3, which is more humid since it is a drainage area and there was  
346 an additional entry of runoff water from upper hill parts. The best fit was noted in plot  
347 P4 (semiarid zone). With the exception of the very dry summer in 2009, Pro levels were  
348 generally lower in this plot, but their variation also correlated very well with  
349 accumulative rainfall. The lower Pro values recorded in this area can be explained by  
350 the presence of different species, but is also due to the fact that soil solution did not

351 include high levels of soluble salts; therefore, there was no ‘ionic stress’ to be added to  
352 water stress.

353

## 354 **Discussion**

355

356 The cellular accumulation of Pro – or other compatible solutes, such as glycine betaine  
357 or different soluble carbohydrates – is well established as a general response of plants to  
358 abiotic stress (Ashraf and Foolad 2007; Szabados and Savouré 2010). This notion is  
359 mostly based on experiments in which plants are subjected to stress treatments under  
360 controlled – but artificial – conditions in laboratory set-ups. However, field studies  
361 correlating changes in osmolytes contents with the type and degree of environmental  
362 stress affecting the plants in their specific habitats are very scarce, and these few reports  
363 have usually dealt with plants adapted to saline environments (e.g., Murakeözy et al.  
364 2003; Gil et al. 2011). Therefore, the relative contribution of different environmental  
365 stress conditions to osmolyte biosynthesis, and the biological relevance of this response  
366 for plant tolerance mechanisms in nature remain largely unknown.

367         Studies on plants growing in gypsum areas have mostly focused on restrictive  
368 ecological factors characteristic of these habitats, such as the formation of hard gypsum  
369 crusts, which hinder seedling establishment and growth (Meyer 1986; Escudero et al.  
370 1999; Romão and Escudero 2005), or the importance of terrain topography for  
371 gypsophile vegetation patterns (Meyer et al. 1992; Pueyo et al. 2007); there are also  
372 several studies dealing with seed germination of gypsophytes (e.g., Escudero et al.  
373 1997; Caballero et al. 2003; Ferriol et al. 2006; Moruno et al. 2011). Yet there are very  
374 few reports on the physiological and biochemical responses of plants adapted to gypsum  
375 environments, including for example those by Palacio et al. (2007), who found  
376 differences in the chemical composition of ash between gypsophytes and gypsovags,  
377 and by Alvarado et al. (2000) on nitrogen metabolism in five species growing on  
378 gypsum. The latter publication also reports Pro contents in those plants, but without  
379 addressing possible correlations with environmental stress factors. Therefore, the work  
380 reported here constitutes the first systematic study on osmolytes accumulation in  
381 response to abiotic stress in plants of gypsum habitats.

382           We have focused our studies on Pro, which turned out to be a good indicator of  
383 environmental stress in most taxa under study, and is very likely involved in stress  
384 tolerance mechanisms. We also quantified other osmolytes – glycine betaine and total  
385 soluble sugars – in four of the selected taxa (*Gypsophila struthium*, *Helianthemum*  
386 *syriacum*, *Ononis tridentata* and *Rosmarinus officinalis*), but did not find any  
387 meaningful correlation between their patterns of variation and abiotic stress factors  
388 (data not shown).

389           We also found that Pro levels strongly vary among the samples collected in  
390 different seasons; that is, under different climatic conditions. In fact, in many cases,  
391 variation within one species is similar to, or even greater than variation among species.  
392 This broad variability in Pro levels depending on environmental factors should be taken  
393 into account when quantifying Pro in plants collected in the field. However, most  
394 previous studies have been based on single samplings of plant material, for example  
395 from saline habitats (e.g., Briens and Larher 1982; Tipirdamaz et al. 2006), and it is  
396 doubtful that the information they provide can be generalised; in addition, it seems  
397 extremely difficult to reach meaningful conclusions when comparing quantitative data  
398 on osmolyte levels obtained independently in plants growing in the field.

399           There are several environmental factors that may affect variation in Pro levels in  
400 plants from gypsum zones, especially those relating to salt and water stress. If gypsum  
401 itself were the most relevant stressful factor, one would expect to detect higher Pro  
402 levels in the plants present in those areas with higher gypsum contents in soil. Our  
403 results, however, indicate precisely the opposite: the plants from P3, the experimental  
404 plot with a larger amount of gypsum which roughly doubles P1 or P2, present generally  
405 lower Pro contents when considering the mean values of all the taxa per plot or if  
406 considering those taxa present in all three plots separately. On the other hand, although  
407 soil electric conductivity is, on average, 7-fold higher in the gypsum area than in the  
408 semiarid zone (P4), we found only slightly higher Pro values in the plants from Tuéjar  
409 when compared to those from Bétera. In fact, the salinity levels in the gypsum area are  
410 moderate and steady throughout the year since the soil solution composition is regulated  
411 by the low solubility of gypsum; this means that the soil solution remains gypsum-  
412 saturated irrespectively of humidity. These findings are in agreement with previous  
413 reports (Rubio and Escudero 2000; Romão and Escudero 2005) suggesting that the

414 chemical toxicity of gypsum soils is not a major restrictive factor for plants in such  
415 habitats, contrary to what had been proposed by other authors (Ruiz et al. 2003).

416 According to this reasoning, environmental factors other than salt stress should  
417 be more relevant for induction of osmolyte biosynthesis in gypsum zones and, indeed,  
418 we found a clear negative correlation between the Pro levels in plants and water content  
419 in soil. These data suggest that the major trigger of Pro biosynthesis is water deficit in  
420 soil, not only in plants from the semiarid zone, but also in those from gypsum habitats.  
421 In the latter case, however, the ‘salt stress’ component plays an additional secondary  
422 role. The combination of water stress and ionic toxicity can partly explain why the  
423 plants from gypsum areas show relatively higher Pro levels as a general pattern, except  
424 during severe drought periods. Water availability in summer is the major restrictive  
425 factor for many Mediterranean-type habitats; actually, the very definition of the  
426 Mediterranean climate is based on the presence of at least two consecutive months  
427 characterised by summer drought (Rivas-Martínez and Rivas-Sáenz 2009), and the  
428 importance of soil-water relations has already been revealed in former studies (Parsons  
429 1977). In our study, water balance represents the major ecological factor in relation to  
430 Pro synthesis in plants from gypsum habitats; this comes over quite clearly when  
431 considering the relatively lower Pro levels during drought periods in plants from P3, the  
432 plot that has a higher gypsum content but is more humid. According to Meyer and  
433 García-Moya (1989), water penetrates more deeply in gypsum due to its low water-  
434 retention capacity, but it moves upwards in response to the gradient created by surface  
435 drying to result in a more continuously moist near-surface environment.

436 The correlation of Pro amounts in the plants with environmental factors becomes  
437 more evident when considering temporal variations: in general, higher Pro levels are  
438 found during drought periods, as inferred from the precipitation and mean temperature  
439 data obtained from the area, and also from the local rainfall and soil humidity data  
440 recorded by the sensors installed in the experimental plots. Over the 2-year study  
441 period, the strongest water deficit was observed early in the summer of 2009;  
442 accordingly, most taxa presented significantly higher Pro content values in the plants  
443 sampled in July 2009. In 2010, the drought period was not as intense as in the previous  
444 year, and lasted from late summer to late autumn, as reflected in the higher Pro values in  
445 those plants sampled in November if compared to those collected in July.

446 Interestingly, a different Pro accumulation pattern was detected in the two  
447 gypsophytes present in the gypsum area: significantly higher Pro values were recorded  
448 in spring in *G. struthium* and in the autumn sampling in *O. tridentata* for both years.  
449 Several authors have discussed different ecological strategies (Rubio and Escudero  
450 2000; Pueyo et al. 2007; Martínez-Duro et al. 2010), and even different chemical  
451 compositions (Palacio et al. 2007), between gypsophytes and gypsovags. We believe,  
452 however, that the different Pro accumulation pattern probably does not relate to these  
453 two categories, but is more likely associated with genetic differences or morphological  
454 traits, such as succulence in *O. tridentata* and *G. struthium*

455 In short, the results presented and discussed herein clearly show a correlation  
456 between environmental factors and the Pro level in most of the taxa included in our  
457 study, supporting a functional role of Pro in stress tolerance mechanisms. Although  
458 some species may not follow the general pattern – probably because they are not typical  
459 Pro accumulators, but instead use a different compatible osmolyte such as glycine  
460 betaine or some sugar(s) – we conclude that Pro may be considered a reliable  
461 biochemical marker of abiotic stress in plants adapted to gypsum. However, the main  
462 trigger of Pro biosynthesis in this type of habitat, as in arid or semiarid zones, is water  
463 deficit, and not ‘salt stress’, due to the presence of gypsum, which only plays a  
464 secondary role.

465

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469 Development Fund.

470

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577

578 FIGURE LEGENDS

579

580 **Fig. 1** Rainfall and soil water content recorded by the rain gauges and the two sensors  
581 installed at a depth of 10 cm in all three experimental plots (P1, P2, P3) in Tuéjar (a)  
582 and in the one plot (P4) in Bétera (b)

583

584 **Fig. 2** Seasonal variation of proline content on the taxa present in more than one plot:  
585 *Helianthemum syriacum* (Hs), *Ononis tridentata* (Ot), *Rosmarinus officinalis* (Ro),  
586 *Thymus vulgaris* (Tv), *Cistus clusii* (Cc) and *Anthyllis cytisoides* (Ac). Bars indicate  
587 mean values and standard deviation calculated in 3-5 individuals per plot and season,  
588 and per species

589

590 **Fig. 3** Biplot from the principal component analysis showing the relationship between  
591 proline content and ecological variables: previous month mean temperature (Mean T),  
592 previous month soil humidity at a depth of 10 cm (Hum 10), previous month soil  
593 humidity at a depth of 20 cm (Hum 20), cumulative rain from two previous months  
594 (Rain 2 month) and electrical conductivity in the 1:1 soil water extract (salinity)

595

596 **Fig. 4** Exponential correlation between the mean proline and rainfall accumulated in a  
597 60-day period prior to plant material sampling in *Rosmarinus officinalis* for the four  
598 experimental plots (n=5)

599

600

601 **Table 1** Location (plot number) of the plant material. P1, P2 and P3 are located on  
 602 gypsum substrate and P4 is a comparative plot on calcareous soils under semiarid  
 603 climate conditions  
 604

Taxa under study	Abb. <sup>a</sup>	Sampling zone
<i>Anthyllis cytisoides</i> L.	Ac	P1, P2
<i>Cistus clusii</i> Dunal	Cc	P1, P3
<i>Dorycnium pentaphyllum</i> Scop.	Dp	P4
<i>Gypsophila struthium</i> L. in Loefl. subsp. <i>hispanica</i> (Willk.) G. López	Gs	P3
<i>Helianthemum syriacum</i> (Jacq.) Dum.-Cours	Hs	P1, P2, P3, P4
<i>Ononis tridentata</i> L. subsp. <i>angustifolia</i> (Lange.) Devesa López	Ot	P1, P2, P3
<i>Rosmarinus officinalis</i> L.	Ro	P1, P2, P3, P4
<i>Stipa offneri</i> Breistr.	So	P4
<i>Stipa tenacissima</i> L.	St	P4
<i>Thymus vulgaris</i> L.	Tv	P1, P2, P3, P4

605

606 <sup>a</sup> *abbreviation*

607 **Table 2** Soil characteristics of the three plots from the gypsum area (P1, P2 and P3) and  
 608 of the plot from the semiarid zone (P4) corresponding to the spring 2009 sampling. Data  
 609 represent mean values  $\pm$  SD of three samples per plot  
 610

Soil property	Zone				Significance level
	P1	P2	P3	P4	
Gypsum content (%)	34 $\pm$ 21 <sup>a</sup>	33 $\pm$ 11 <sup>a</sup>	71 $\pm$ 9 <sup>b</sup>	-	*
CaCO <sub>3</sub> content (g kg <sup>-1</sup> )	165 $\pm$ 54 <sup>b</sup>	189 $\pm$ 17 <sup>b</sup>	76 $\pm$ 28 <sup>a</sup>	435 $\pm$ 31 <sup>c</sup>	***
pH	7.82 $\pm$ 0.05 <sup>b</sup>	7.79 $\pm$ 0.04 <sup>ab</sup>	7.74 $\pm$ 0.05 <sup>a</sup>	8.42 $\pm$ 0.02 <sup>c</sup>	***
Organic carbon (g kg <sup>-1</sup> )	28.5 $\pm$ 12.6 <sup>b</sup>	26.7 $\pm$ 11.4 <sup>b</sup>	16.5 $\pm$ 3.3 <sup>a</sup>	24.2 $\pm$ 4.3 <sup>b</sup>	*
Mineral nitrogen (mg kg <sup>-1</sup> )	5.3 $\pm$ 3.7 <sup>b</sup>	2.0 $\pm$ 0.9 <sup>a</sup>	2.1 $\pm$ 1.4 <sup>a</sup>	1.2 $\pm$ 0.7 <sup>a</sup>	*
Available P (mg kg <sup>-1</sup> )	3.9 $\pm$ 1.0 <sup>a</sup>	3.6 $\pm$ 1.4 <sup>a</sup>	4.6 $\pm$ 2.8 <sup>a</sup>	1.0 $\pm$ 0.5 <sup>a</sup>	**
Available K (mg kg <sup>-1</sup> )	325 $\pm$ 98 <sup>b</sup>	209 $\pm$ 69 <sup>a</sup>	191 $\pm$ 62 <sup>a</sup>	206 $\pm$ 27 <sup>a</sup>	**
EC 1:1 extract (dS m <sup>-1</sup> )	2.61 $\pm$ 0.06 <sup>c</sup>	2.44 $\pm$ 0.08 <sup>b</sup>	2.43 $\pm$ 0.04 <sup>b</sup>	0.36 $\pm$ 0.03 <sup>a</sup>	***
Ca 1:1 extract (mM)	16.6 $\pm$ 0.4 <sup>b</sup>	16.5 $\pm$ 0.3 <sup>b</sup>	16.6 $\pm$ 0.4 <sup>b</sup>	1.7 $\pm$ 0.2 <sup>a</sup>	***
Mg 1:1 extract (mM)	2.55 $\pm$ 0.34 <sup>b</sup>	0.64 $\pm$ 0.26 <sup>a</sup>	0.67 $\pm$ 0.18 <sup>a</sup>	0.28 $\pm$ 0.04 <sup>a</sup>	***
Cl 1:1 extract (mM)	0.46 $\pm$ 0.23	0.21 $\pm$ 0.02	0.21 $\pm$ 0.00	0.2 $\pm$ 0.04	NS
Na 1:1 extract (mM)	1.86 $\pm$ 0.75 <sup>b</sup>	0.67 $\pm$ 0.33 <sup>a</sup>	1.07 $\pm$ 0.04 <sup>a</sup>	0.31 $\pm$ 0.05 <sup>a</sup>	**
K 1:1 extract (mM)	0.87 $\pm$ 0.43 <sup>b</sup>	0.28 $\pm$ 0.15 <sup>a</sup>	0.54 $\pm$ 0.06 <sup>ab</sup>	0.15 $\pm$ 0.04 <sup>a</sup>	*

611  
 612 \*\*\*, \*\*, \* or NS, indicate significant differences at the 0.001, 0.01 and 0.05 probability  
 613 levels or not significant, respectively. Values with different lower-case letters show  
 614 significant differences at the 0.05 probability level.  
 615  
 616

617 **Table 3** The sampling dates and means of mean, maximum and minimum daily  
 618 temperature and accumulative rainfall. For temperature, the previous month to sampling  
 619 data is considered; for rainfall, both one and two months are taken into account  
 620

Sampling dates	Variable				
	Mean T	Max T	Min T	Accumulative rainfall (mm)	
	(°C)	(°C)	(°C)	30 days	60 days
<b>Tuéjar</b>					
29/04/09	11.6	17.7	5.5	34	100
13/07/09	24.8	33.3	16.4	7	17
11/12/09	11.7	18.3	5.1	11	35
26/04/10	12.0	18.4	5.6	113	193
19/07/10	23.4	31.4	15.4	3	129
26/11/10	11.1	17.2	5.0	37	91
<b>Bétera</b>					
06/05/09	14.9	22.4	7.9	38	81
31/07/09	25.9	31.6	19.8	3	3
18/12/09	11.5	18.7	7.0	36	46
29/04/10	14.1	20.6	7.7	35	75
20/07/10	24.3	30.6	17.4	3	76
18/12/10	11.9	18.9	5.6	10	49

621



622 **Table 4** Seasonal variation of proline content ( $\mu\text{mol}\cdot\text{gr}^{-1}\text{DW}$ ) for the different species present in only one plot. Mean values  $\pm$  SD (n=5)

Species	Sampling date						Significance level
	Spring 2009	Summer 2009	Autumn 2009	Spring 2010	Summer 2010	Autumn 2010	
<b>Gs</b>	$3.03 \pm 0.59^c$	$0.56 \pm 0.12^a$	$0.76 \pm 0.12^{ab}$	$1.07 \pm 0.35^b$	$0.43 \pm 0.09^a$	$0.51 \pm 0.12^a$	***
<b>So</b>	$0.23 \pm 0.06^a$	$5.79 \pm 1.52^d$	$0.66 \pm 0.04^{ab}$	$0.48 \pm 0.23^a$	$2.56 \pm 0.67^c$	$1.74 \pm 0.68^{bc}$	***
<b>St</b>	$0.26 \pm 0.14^a$	$0.76 \pm 0.19^{bc}$	$0.92 \pm 0.54^c$	$0.25 \pm 0.05^a$	$0.31 \pm 0.11^{ab}$	$0.40 \pm 0.28^{bc}$	**
<b>Dp</b>	$0.67 \pm 0.06^a$	$13.23 \pm 3.29^b$	$2.15 \pm 0.08^a$	$0.70 \pm 0.06^a$	$0.31 \pm 0.12^a$	$2.06 \pm 0.18^a$	***

623

624 For each species, \*\*\* or \*\* indicate significant differences at the 0.001 and 0.01 probability levels, respectively. Values with different  
625 letters present significant differences at the 0.05 probability level.

626 Gs: *Gypsophila struthium* (P3); So: *Stipa offneri* (P4); St: *Stipa tenacissima* (P4); Dp: *Dorycnium pentaphyllum* (P4).

627

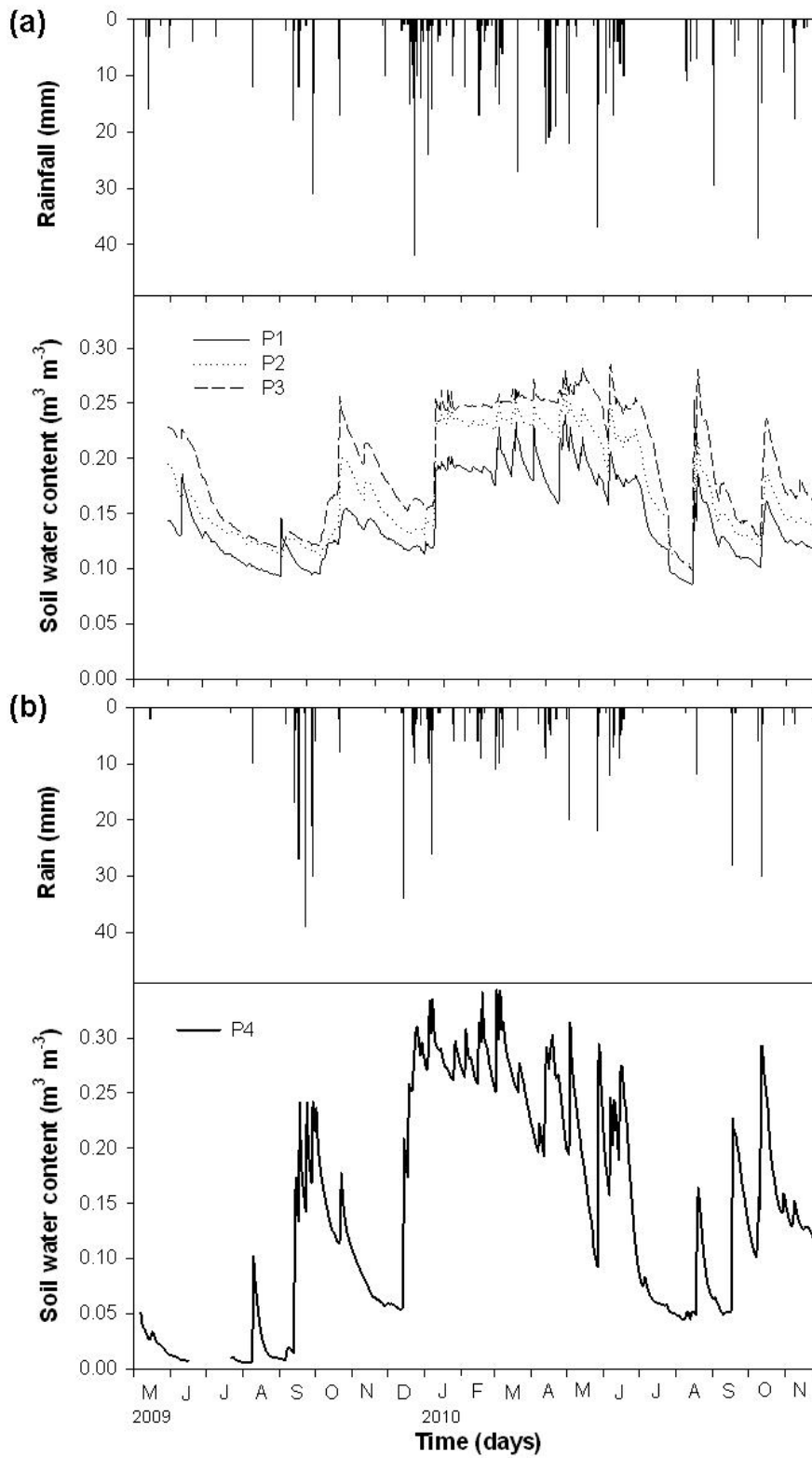
628 **Table 5** P-values from the two-way ANOVA indicating the statistical significance of  
629 the plot and season factors in the taxa present in more than one plot

630

<b>Species</b>	<b>A. Plot</b>	<b>B. Season</b>	<b>AxB Interaction</b>
Ro	0.0278	0.0000	0.0189
Hs	0.0000	0.0000	0.0000
Ot	0.0509	0.0000	0.0646
Tv	0.0179	0.0000	–
Cc	0.0000	0.0000	0.0000
Ac	0.0001	0.0000	0.0191

631

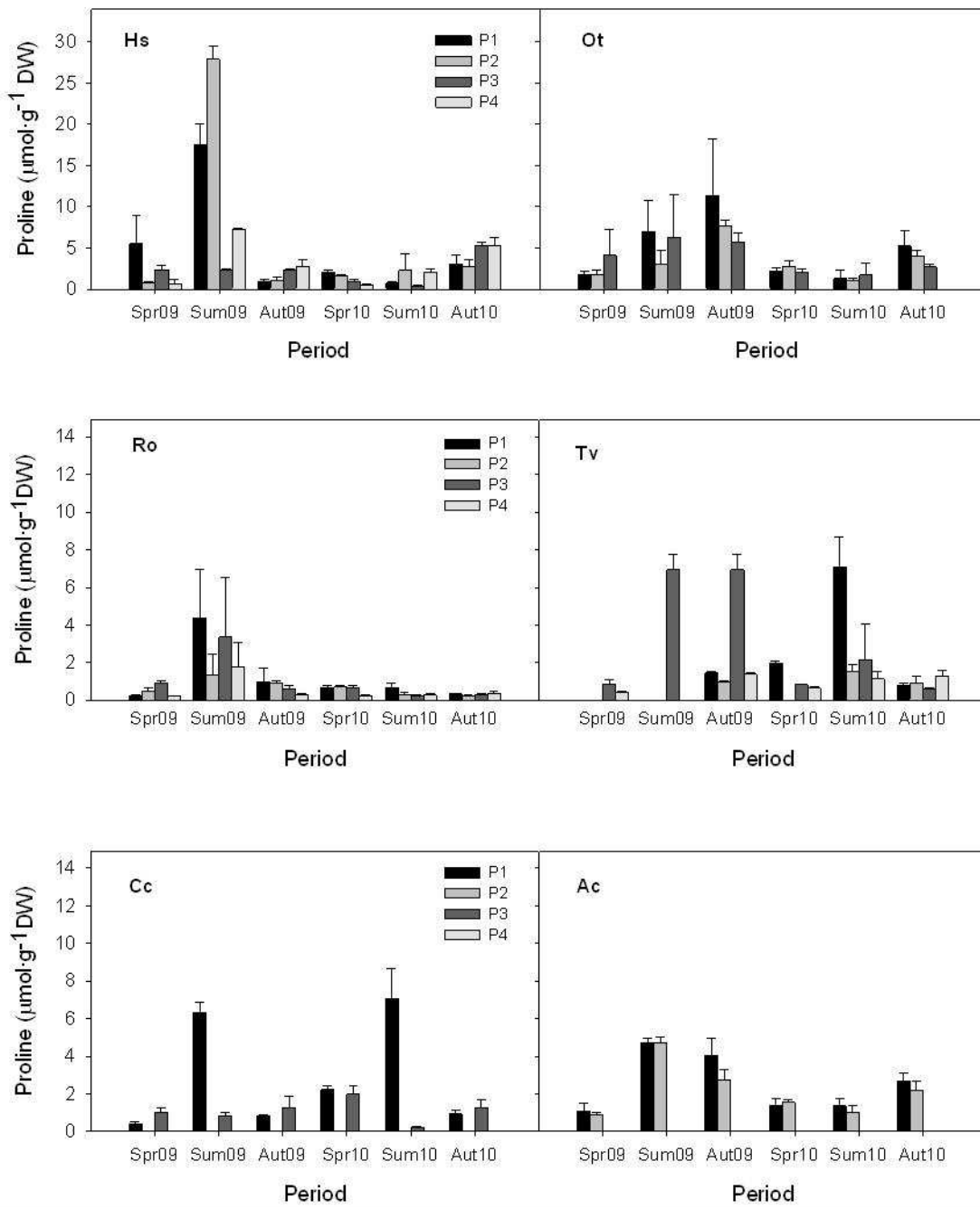
632 Figure 1



633

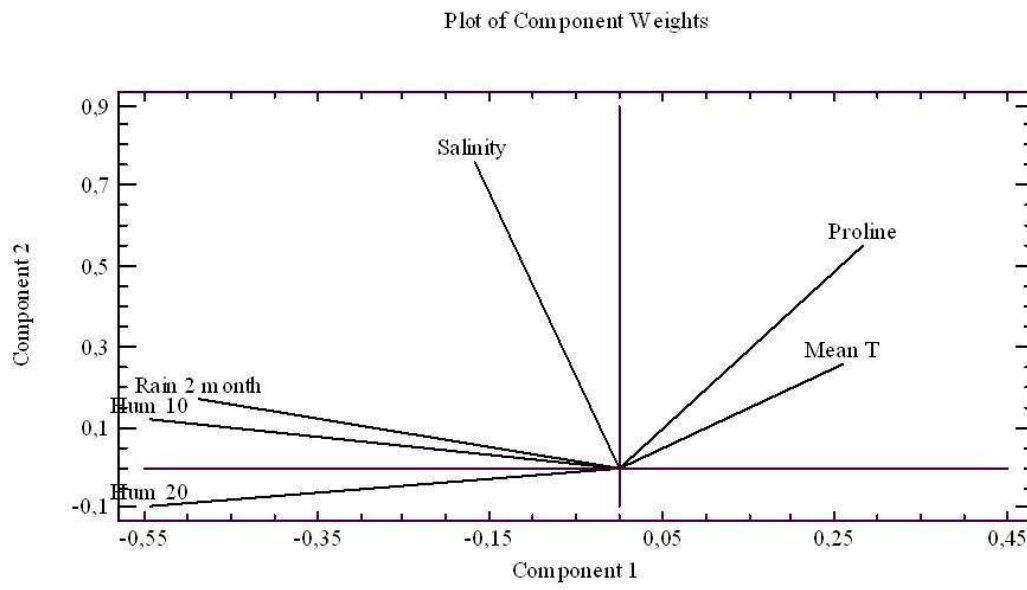
634

635 Figure 2



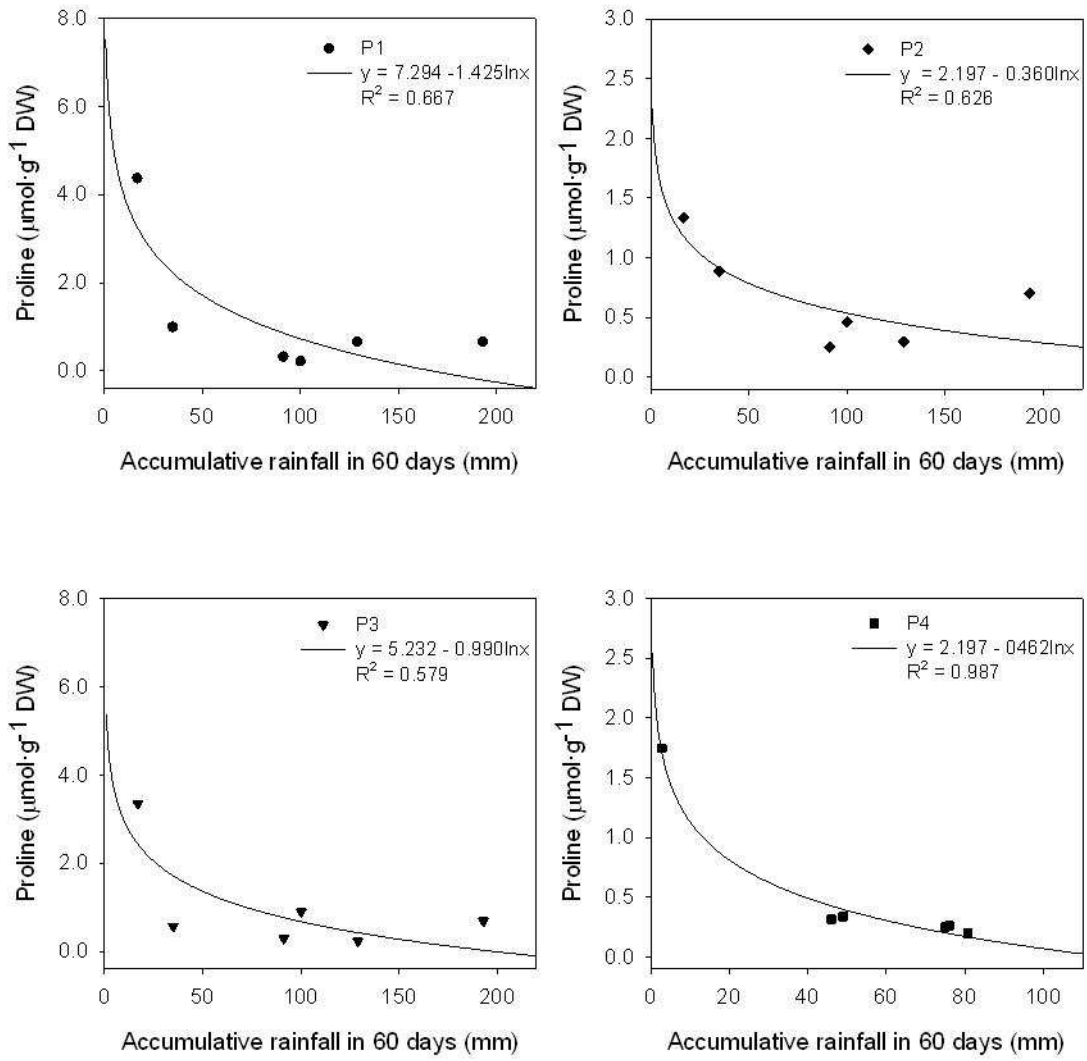
636

637 Figure 3



638

639 Figure 4



640

641