

1 **Soybean drought resilience: contributions of a brassinosteroid functional analogue.**

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16 **Highlight:** Brassinosteroid analogue DI-31 improves soybean growth, water economy,  
17 respiration, anti-stress response and nitrogen homeostasis under drought. Thus, they  
18 may be considered as a sustainable and environmentally-safe alternative for raising  
19 legumes climate resilience.

20 **Abstract**

21 Drought is one of the most important causes of severe yield loss in soybean worldwide,  
22 threatening food production for the coming years. Phytohormones such as  
23 brassinosteroids can increase response to water deficit. However, natural  
24 brassinosteroids low stability precludes large-scale field application, challenging  
25 research and development of more stable and cost-effective analogues. Seeking  
26 functional analogues capable of improving plant drought-response, we investigated for  
27 the first time the effect of DI-31 in *Arabidopsis* and soybean. We found that, in *A.*  
28 *thaliana*, the DI-31 increased root growth, biomass accumulation, leaf number *per*  
29 plant, triggered antioxidant response and dose-dependent stomatal closure, requiring

30 NADPH and peroxidase-dependent ROS production. In soybean, the relative water  
31 content, water use efficiency, biomass production and duration, root length, free proline,  
32 chlorophyll and carotenoid accumulation and enzymatic antioxidants activity were  
33 stimulated by DI-31 application after four and eight days of mild water shortage, while  
34 significantly reduced the lipid-peroxides content. Additionally, our results demonstrated  
35 that DI-31 diminishes the nodular senescence and successfully maintains the N  
36 homeostasis through a fine tune of biological/assimilative N<sub>2</sub>-fixation pathways. These  
37 findings support the DI-31 potential use as a sustainable alternative for integrative  
38 soybean resilience management under drought.

### 39 **Keywords**

40 Antioxidant response, brassinosteroid analogue DI-31, drought, growth promotion,  
41 nitrogen fixation, soybean

### 42 **Introduction**

43 Brassinosteroids (BRs) are a group of growth-promoting plant hormones, isolated and  
44 characterised from canola pollen (*Brassica napus* L.) (Moreno-Castillo et al. 2018). Up  
45 to date, nearly 70 kinds of natural BRs analogues have been isolated from tissues of  
46 various plant species (Tang et al. 2016). BRs possess beneficial pleiotropic effects due  
47 to its broad and highly coordinated cell modulation capacity (Sahni et al. 2016). They  
48 influence various developmental processes and agronomical traits (Gonzalez-Olmedo et  
49 al. 2004; González-Olmedo et al. 2005; Vriet et al. 2012). Moreover, BRs can increase  
50 plant response to a wide range of stresses (Moreno-Castillo et al. 2018). That is why  
51 researchers have been exploring strategies directed to increase crops yields and stress  
52 tolerance/resistance, like BRs exogenous application and the genetic manipulation of its  
53 endogenous levels. However, the BRs low stability in the field precludes large-scale  
54 application (Sakai et al. 1999), being replaced by functional or structural analogues with  
55 higher biological activity and field average life (Sasse 2003). The use of BRs analogues  
56 with a predominantly growth-promoting effect in a wide range of plant species  
57 constitutes an alternative directed to improving crop yields (Rao et al. 2002). The most  
58 commonly used is 24-epibrassinolide despite its high production fees (Moreno-Castillo  
59 et al. 2018), so the study of other analogue molecules with higher activity and lower  
60 costs constitute a promising alternative.

61 Among abiotic stresses, water deficit has the most severe effect in worldwide  
62 agriculture and shows detrimental effects on plants stomatal morphology,  
63 photosynthesis, growth rate and oxidation-reduction balance (Chai et al. 2016). Soybean  
64 (*Glycine max* (L.) Merrill) is one of the socio-economic crops affected by drought.  
65 Considered the most worldwide cropped legume, soybean is an essential source of oil  
66 and protein (Hungria and Mendes 2015; Wang et al. 2018). However, the occurrence of  
67 water scarcity periods significantly reduced the crop photosynthesis, growth and  
68 nitrogen (N) fixation, causing grain quality and yield losses (Jin et al. 2018). To  
69 mitigate the detrimental effects of water deprivation, researchers develop strategies such  
70 as genotypes selection, identification of tolerant genetic sources and establishment of  
71 cultural management practices, like bio-stimulant applications.

72 The synthetic spirostanic BRs functional analogue (25R)-3 $\beta$ ,5 $\alpha$ -dihydroxy-spirostan-6-  
73 one (DI-31) property of the CEPN (Center of Studies of Natural Products, Chemistry  
74 Faculty, Havana University) is characterized by the presence of a spiroketalic ring  
75 instead of the typical BR side chain (Coll et al. 1995; Mazorra et al. 2002) and BR-like  
76 activity (Furio et al. 2018). Up to date, the DI-31 has been studied only as a component  
77 of the commercial bio-stimulant Biobras-16, with beneficial effects on photosynthetic  
78 rate and yield of greenhouse-grown pepper (*Capsicum annuum* L.) (Serna et al.  
79 2012) and endive (*Cichorium endivia* L.) plants (Serna et al. 2013). Similarly, Biobras-  
80 16 application prevented the negative effect of salt stress in rice and lettuce plants  
81 (Serna et al. 2015).

82 Structurally, the DI-31 has an epoxy-oximic polar group, one of its major  
83 conformations, that interacts with the BRI1 BRs plant receptor with higher affinity and  
84 lower binding energy than 24-epibrassinolide (Moreno-Castillo et al. 2018), so it has a  
85 higher potential activity. Therefore, it could be useful to incorporate the DI-31 in a crop  
86 management strategy focused on increasing crop yield and to diminish the effect of  
87 abiotic stresses like osmotic imbalance and particularly drought. Besides, the  
88 application of hormonal molecules such as DI-31 could constitute an eco-friendly  
89 complement that stimulates soybean growth and N fixation maintenance under water  
90 scarcity. Nevertheless, in order to characterize this compound, it is very convenient to  
91 test its effects in a model plant such as *Arabidopsis thaliana* (L.) Heynh, and then to  
92 evaluate its potential in soybean plants.

93 In this study, we investigated for the first time the DI-31 molecule effect on *A. thaliana*  
94 growth, stomatal movement, oxidative burst and antioxidant enzymatic activity.  
95 Subsequently, we characterised the DI-31 action in soybean plants under water deficit  
96 for potential drought resilience, evaluating the compound effect on photosynthesis,  
97 water economy, biomass production and particularly N homeostasis under drought.  
98 These results support the potential use of this compound to crop management under  
99 drought conditions.

## 100 **Material and methods**

### 101 *Plant material and growth conditions*

102 All the experiments were conducted at the Estación Experimental Agroindustrial  
103 Obispo Colombres (EEAOC), Las Talitas, Tucumán, Argentina (S26°50', W65°12').  
104 The assays in wild type (WT) *A. thaliana* were developed using Columbia (Col-0)  
105 ecotype seeds. Seeds were disinfected for 5 minutes in a mixture of commercial bleach,  
106 distilled water and ethanol (1:1:8). Subsequently, they were washed three times with  
107 96% ethanol under sterile conditions and seeded in Petri dishes containing MS medium  
108 (Murashige and Skoog 1962), with 1% (w/v) sucrose, 0.5 g/L of MES pH 5.8 (Duchefa,  
109 Holland) and 0.8% (w/v) agar (Sigma, USA). Seeds in the plates were stratified in the  
110 dark for three days at 4°C and then transferred to a growth chamber at 22-23°C  
111 temperature and 16 h light- 8 h darkness photoperiod. Seed germination was monitored  
112 for five days, and then seedlings were transferred to new Petri dishes with fresh MS  
113 medium or plastic glasses (diameter: 5.5 cm, height: 9 cm) filled with GrowMix®  
114 Multipro commercial substrate (Terrafertil S.A., Argentina), according to each test  
115 requirements. Only the DI-31 curve dose-response assay was carried out in Petri dishes  
116 using 5-day-old seedlings transferred to plates with fresh medium and supplemented  
117 with the compound at different doses. The rest of the experiments were performed using  
118 3-week-old plants (except stomatal assays, performed with 4-week-old plants) grown in  
119 plastic glasses filled with commercial substrate and irrigated with Hoagland Complete  
120 Solution (Hoagland and Arnon 1950).

121 The experiments in soybean were performed in glasshouse conditions using the  
122 commercial cv. Munasqa RR, selected from previous comparative trials with soybean  
123 cv. TJ2049 and MG/BR 46 Conquista (Perez-Borroto unpublished, ongoing). An

124 amount of 1000 Munasqa RR homogeneous seeds (EEAOC Germplasm Bank),  
125 manually harvested and with a high germination potential were selected and planted in 4  
126 L plastic pots (diameter: 18 cm, height: 21 cm) filled with GrowMix® Multipro  
127 commercial substrate. Before sowing, seed were inoculated with *Bradyrhizobium*  
128 *japonicum* E109 ( $9 \times 10^9$  viable cells  $\text{kg}^{-1}$  of seeds) in order to guarantee maximum  
129 soybean plant performance. Four seeds *per* pot were placed to ensure germination.  
130 When plants reached V1 vegetative stage (open leaf at the unifoliate node) according to  
131 (Fehr et al. 1971), the number of plants *per* pot was reduced by half. Trials were  
132 performed using V3 (second open trefoil) or V5 (fourth open trefoil) vegetative stage  
133 plants. During the experiments, the environmental (ET) and substrate temperatures  
134 (ST), relative humidity (RH) and the photosynthetically active radiation (PAR) were  
135 measured every 15 min, averaged and recorded at one h intervals with data loggers  
136 (Cavadevices.com, Buenos Aires, Argentina). The environmental variables evaluated in  
137 the period of experiments presented the following average values: ET = 28°C ( $\pm 7^\circ\text{C}$ );  
138 ST = 22.4°C ( $\pm 4^\circ\text{C}$ ); RH = 90.2%; PAR = 648.37  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Plants grew with a 12 h  
139 average photoperiod. Pots were distributed in a completely randomized design and  
140 weekly moved and rearranged to minimize possible environmental effects.

#### 141 *Water availability treatments*

142 The estimation of substrate water content (SWC) in each pot was performed as  
143 described by (Pereyra-Irujo et al. 2012). Briefly, the weight of each empty pot and the  
144 commercial substrate at the beginning of the experiment were determined. Also, we  
145 quantified the fresh weight of the plants every five days. Then, the data was used to  
146 calculate the water content of each pot and the amount of water that had to be added  
147 every day to reach the desired SWC. Subsequently, the relationship between SWC and  
148 substrate water potential ( $\Psi_s$ ) was determined according to (Richards 1965). All pots  
149 were watered to a 22% SWC corresponding to a  $\Psi_s$  of -0.05 MPa until the imposition of  
150 water deficit treatments. Stress imposition was performed according to (Pardo et al.  
151 2015) drought phenotyping protocol, which consists in maintaining the SWC at 14%  
152 corresponding to a  $\Psi_s$  of -0.65 MPa during eight days. The  $\Psi_s$  corresponding to the  
153 water deficit treatment was reached in a 1-2 days interval. The plant's water status  
154 (relative water content) was monitored throughout the water shortage period to ensure  
155 stress occurrence. Additionally, the experimental design also included six pots filled

156 with substrate distributed in the two SWC treatments ( $\Psi$ s of -0.05 and -0.65 MPa). The  
157 pots were distributed randomly and daily watered and weighed to quantify the amount  
158 of water evaporated from the substrate.

#### 159 *DI-31 dose-response curve and growth promotion*

160 5-day-old WT *A. thaliana* seedlings were transferred to new Petri dishes and divided in  
161 four groups (10 plants *per* treatment): (i) MS medium (control treatment), (ii) MS  
162 medium + DI-31 (0.22  $\mu$ M; 0.1 mg/L), (iii) MS medium + DI-31 (1.12  $\mu$ M; 0.5 mg/L)  
163 and (iv) MS medium + DI-31 (2.23  $\mu$ M; 1 mg/L). Five days after treatments, we  
164 photographed the seedlings and measured morphological growth indicators such as the  
165 number of leaves, root length and biomass production. The DI-31 concentration of 2.23  
166  $\mu$ M was chosen to perform further assays. The experiment was performed twice with  
167 similar results.

#### 168 *Stomatal measurements*

169 Stomatal assays (Gudesblat et al. 2009) were carried out using *A. thaliana* 4-week-old  
170 plants (non-flowered). We placed the epidermal peels in well plates with 500  $\mu$ L of a  
171 10:10 buffer solution (10 mM KCl and 10 mM MES-KOH, pH = 6.15) under the  
172 normal culture conditions for 2 h. Then, different treatments were applied and the  
173 epidermis were incubated for an additional 1.5 h. To assess the DI-31 effect on stomata  
174 four treatments were defined: (i) untreated buffer solution, (ii) buffer solution + ABA  
175 (20  $\mu$ M), (iii) serial dilutions of 24-epibrassinolide and (iv) DI-31 (0.1; 0.5; 1; 5; 10 and  
176 20  $\mu$ M). To test whether DI-31-induced stomatal closure is dependent on ROS  
177 production, specific inhibitory compounds treatments were defined: (i)  
178 Diphenyleneiodonium (DPI) 10  $\mu$ M (Sigma, USA) + ABA (20  $\mu$ M), (ii) DPI + DI-31  
179 (10  $\mu$ M), (iii) Salicylhydroxamic acid (SHAM) 2mM (Sigma, USA) + ABA (20  $\mu$ M)  
180 and (iv) SHAM acid + DI-31 (10  $\mu$ M). DPI and SHAM were incubated 30 min before  
181 DI-31 treatments. We performed 40 measurements *per* treatment and presented the data  
182 as the average of 80 measurements collected from four independent experiments.

#### 183 *Oxidative burst assay*

184 To determine whether DI-31 can activate defence mechanisms as the respiratory burst in  
185 well-watered *A. thaliana* plants, we evaluated the appearance of superoxide radicals.

186 Three-week-old WT plants were grouped according to the following treatments: (i)  
187 control with distilled water (DW) and (ii) DI-31 (2.23  $\mu$ M). Treatments were foliarly  
188 applied (by sprinkling) to the plant rosettes until they reached the drip point. To  
189 determine the oxidative burst, we collected three plants for each treatment and harvest  
190 timing (total of 24 plants). From each plant collected, the sixth and seventh rosette  
191 leaves were detached at 6, 12, 24 and 48 h after DI-31 treatment and subjected to NBT  
192 (Nitroblue tetrazolium) staining protocol (Doke 1983). We selected the sixth and  
193 seventh leaves (counting from the youngest leaf) representing fully expanded leaves.

#### 194 *Antioxidant response measurements*

195 WT *A. thaliana* plants were used to assess the DI-31 ability to stimulate enzymatic  
196 antioxidants activity such as the superoxide dismutase (SOD, EC 1.15.1.1) (Li 2012),  
197 catalase (CAT, EC 1.11.1.6) (Chance and Maehly 1955), ascorbate (APX, EC  
198 1.11.1.11) (Nakano and Asada 1987) and phenol peroxidase (POX, EC 1.11.1.7) (Kar  
199 and Mishra 1976) as well as the protein accumulation (Bradford 1976). We performed  
200 an enzymatic uniform extraction (Liu et al. 2010; Singh et al. 2014). The DI-31 (2.23  
201  $\mu$ M) was sprinkled to drip point. Two experiments were performed in a growth chamber  
202 using 3-week-old plants, distributed in two treatments: (i) well-irrigated plants sprinkled  
203 with distilled water (control) and (ii) well-irrigated plants sprinkled with DI-31. Ten  
204 plants *per* treatment were collected and sampling times (60 plants *per* experiment) were  
205 at 0, 24 and 48 hours after the compound application.

206 Subsequently, we determined the effect of DI-31 foliar application in soybean  
207 antioxidant activity under well-irrigated and water scarcity conditions in two glasshouse  
208 experiments. When the Munasqa RR plants reached the V3 phenological stage were  
209 distributed into four groups corresponding to the following treatments: (i) well-irrigated  
210 plants ( $\Psi_s = -0.05$  MPa) sprinkled with distilled water (DW), (ii) well-irrigated plants  
211 sprinkled with DI-31, (iii) stressed plants ( $\Psi_s = -0.65$  MPa) sprinkled with DW and (iv)  
212 stressed plants sprinkled with DI-31. Then, we induced the water deficit in the  
213 treatments (iii) and (iv). Once the treatments reached the SWC and  $\Psi_s$  corresponding to  
214 moderate water stress, the DW and the DI-31 (2.23  $\mu$ M) were applied using the same  
215 method previously described for *A. thaliana* trials. Ten soybean plants *per* treatment  
216 were used and V2, V3 and V4 leaves from each plant (total of 120 plants *per*  
217 experiment) were collected at 0, 4 and 8 days after the DW and DI-31 application.

218 Besides the antioxidant enzymes and protein measurements, the content of MDA  
219 (Hodges et al. 1999), free proline (Bates et al. 1973), chlorophyll (Porra 2002) and  
220 carotenoids (Riemann 1978) were determined.

#### 221 *Soybean growth, water economy and N fixation measurements*

222 The experiments were carried out with V5 Munasqa RR plants (20 plants *per* treatment  
223 and sampling time, a total of 240 plants *per* experiment), distributed into the same four  
224 groups corresponding to the previously described treatments. We performed the stress  
225 imposition and DW and DI-31 application, as we described in the previous section. At  
226 0, 4 and 8 days after the application of the compound, the whole plants were collected.  
227 For each sampling time, 15 collected plants were used to determine  
228 morphophysiological characters associated with growth, nodulation and N fixation. The  
229 remaining five plants, collected at each time, were used to determine the water status  
230 according to the (Weatherley 1950) relative water content (RWC) method. The water  
231 use efficiency (WUE), defined as the ratio between the above-ground biomass and the  
232 water consumed (Van Halsema and Vincent 2012) was also measured. As growth  
233 indicators, we quantified the biomass production (Porcel et al. 2003) and then calculated  
234 the biomass duration (BMD) over time (Hunt 1978). The number of leaves, stem length  
235 and thickness and primary root length, were also quantified. As nodulation parameters,  
236 we collect the nodules located in the root crown (an imaginary cylinder of 2.5 cm of  
237 diameter and length) according to (Burton 1976). Subsequently, the nodules were cut to  
238 visualize their activity status according to the Leghemoglobin colouration, then were  
239 labelled and photographed together with a scale for further morphological analysis. The  
240 number of active nodules *per* plant was quantified. In order to determine if the DI-31  
241 have any effect on nodules development under stressful conditions, morphological  
242 parameters such as equatorial and polar diameter, the thickness of periderm and cortex  
243 area (outer, middle and inner) and the estimated area of the infected central medulla  
244 were measured in all the active nodules (Kanu and Dakora 2017), using the image  
245 processing and analysis program *ImageJ* (version 1.52). The N fixation parameters were  
246 measured in extracts obtained from the aerial portion of the plants. Indicators such as  
247 the *in vivo* activity of Nitrate Reductase (NR) enzyme (Jaworski 1971), nitrate (Cataldo  
248 et al. 1974),  $\alpha$ -amino acids (Herridge et al. 1990) and ureide content (Young and  
249 Conway 1942) were determined. Additionally, we calculated the ureide relative



250 abundance (Takahashi et al. 1992) and the percentage of biological N fixed (Herridge  
251 et al. 1990). The experiments were performed twice with similar results.

## 252 *Statistical analysis*

253 All data were analyzed in GraphPad Prism 5.01, using ANOVA and Tukey's test  
254 (Tukey's HSD). Each treatment value is presented as the arithmetic mean  $\pm$  S.E.  
255 (standard error) marked with letters in the graphs.

## 256 **Results**

### 257 *DI-31 enhances A. thaliana growth and triggers antioxidant response*

258 To assess DI-31 effect on growth, we measured root length, the number of leaves and  
259 biomass increase in *A. thaliana* WT plants (Fig. 1). The results showed that DI-31  
260 promotes root length in a dose-dependent manner, reaching a ~46% length increase in  
261 the plants treated with the highest concentration (Fig. 1b). Furthermore, compared to the  
262 control and the DI-31 lowest dose (0.22  $\mu$ M), the treatments with DI-31 highest  
263 concentrations (1.12 and 2.23  $\mu$ M) showed a significant effect on the number of leaves  
264 *per* plant (Fig. 1c) and biomass accumulation (Fig. 1d), five days after the compound  
265 application.

266 Furthermore, the DI-31 effect on enzymatic antioxidants activity over time was assessed  
267 and compared to control. The DI-31 application progressively stimulated the activity of  
268 the SOD (Fig. 2a), APX (Fig. 2b) and POX (Fig. 2c) enzymes. The POX reached the  
269 highest activity value 24 h after the compound application, while the SOD and APX did  
270 it at 48 h. CAT enzyme remains unmodified (Fig. 2d). The protein content (Fig. 2e) did  
271 not statistically differ among the treatments and harvest timings. Additionally, we  
272 assessed the DI-31 effect on superoxide radicals production (Fig. 2f). After 6 hours, was  
273 detected the appearance of respiratory burst symptoms in DI-31- treated rosettes, which  
274 reached the highest formation of blue formazan points 48 h after the compound  
275 application.

### 276 *DI-31-mediated stomatal closure requires ROS production in A. thaliana*

277 As it was previously described for 24-epibrassinolide (Shi et al. 2015), we decided to  
278 test whether DI-31 can induce stomatal closure. Stomatal closure patterns induced by  
279 24-epibrassinolide and DI-31 were very similar in all treatments applied. DI-31

280 significantly induced stomatal closure in a dose-dependent manner similarly to 24-  
281 epibrassinolide (Fig. 3a), in agreement with previous results.

282 We additionally conducted a stomatal assay to test whether inhibition of ROS could  
283 impair the DI-31-mediated stomatal closure. For this purpose, we used DPI to inhibit  
284 NADPH oxidases and SHAM for suppressing cell-wall peroxidase activity. Our results  
285 showed that DI-31 stomatal closure was partially inhibited by both compounds, being  
286 more remarkable the effect of DPI. These results suggest that inhibition of NADPH  
287 oxidases prevents DI-31 from promoting stomatal closure and, to a lesser extent,  
288 peroxidases are also required (Fig 3b) for DI-31 action on stomata.

### 289 *DI-31 enhances soybean growth, water economy and stress response under drought*

290 To validate DI-31 effect observed in *A. thaliana*, we decided to test this compound in a  
291 crop of agronomic importance. Thus, we conducted experiments in soybean cv.  
292 Munasqa RR. A wide range of morphophysiological and biochemical parameters  
293 associated with growth, water economy and stress response under water shortage were  
294 measured. Our results showed the protective effect of DI-31 in soybean plants subjected  
295 to water scarcity. The stressed plants treated with the compound showed an attenuated  
296 defoliation phenotype (Fig. 4f), evidencing still green and hydrated young leaves, with  
297 less typical drying symptoms such as curling. In agreement with these findings, our  
298 results indicated that application of DI-31 attenuated the RCW (Fig. 4a) and WUE (Fig.  
299 4b) reduction under stress. Well-watered plants (both with DW and DI-31 application)  
300 evidenced a RWC of ~91% during the experiment. Otherwise, under water shortage,  
301 DI-31-treated plants exhibited RWC values of 83 and 76.2%, while untreated plants  
302 showed 70.5 and 58.3%, on the fourth and eighth day of the trial, respectively.

303 On the other hand, at the fourth and eighth day of the experiment, the WUE, under well-  
304 watered conditions, increased 4.3 and 11.5% in DW and DI-31-treated plants,  
305 respectively. Under water shortage, plants with DW application showed a 26.4 and 42%  
306 of WUE reduction, in comparison with DW-treated and well-watered plants at the  
307 fourth and eighth day of the experiment. Meanwhile, during the same period, the  
308 stressed plants treated with DI-31 showed a 13.6 and 27% of WUE reduction, compared  
309 to the control plants. The BMD under drought was also favoured by DI-31, compared to  
310 DW-treated plants subjected to water scarcity (Fig. 4c). Other indicators associated with  
311 growth, such as stem thickness and length, remained unmodified. Meanwhile, the DI-31

312 treatment stimulated the biomass production in the root system (Fig. 4d) and leaves  
313 development (Fig. 4e) in well-irrigated plants during the first four days after  
314 application.

315 The compound application over time also stimulated the specific activity of SOD (Fig.  
316 5a) and APX enzymes (Fig. 5b), while the POX (Fig. 5c) and CAT enzymes (Fig. 5d)  
317 remained unmodified. No variations in protein content were observed (Fig. 5e).  
318 Findings that agree with the results showed in *A. thaliana* plants. Under water  
319 deprivation, treatment with DI-31 partially prevented chlorophyll pigments degradation  
320 (Fig. 5f-h), increased the total carotenoids (Fig. 5i) and free proline content (Fig. 5j)  
321 and limited the MDA accumulation (Fig. 5k) in leaf tissue.

### 322 *Nodulation and nitrogen homeostasis under drought and DI-31 treatments*

323 Under water shortage, the treatment with DI-31 showed beneficial effects in Munasqa  
324 RR plants active/functional nodules (Fig. 6b) located in the imaginary root cylinder  
325 (Fig. 6a). During the first four days after the compound was applied, no significant  
326 decrease in the number of active nodules was observed, while at the eighth day we  
327 quantified a reduction of ~31% (Fig. 6d). In contrast, DW-treated plants subjected to  
328 water shortage showed reductions in the number of active nodules by ~53 and 57% on  
329 the fourth and eighth day of stress, respectively. From a more detailed analysis of the  
330 active nodules collected on the eighth day after DI-31 treatment (trial last day),  
331 significant differences were quantified in several morphological parameters (Fig. 6c). In  
332 well-watered and stressed plants, the compound application stimulated the infected  
333 central medulla estimated area, the equatorial and polar diameter of active nodules (Fig.  
334 6e-g). While, in plants under water scarcity, significant reductions of these parameters  
335 were quantified, resulting in visually smaller nodules. Interestingly, under water  
336 shortage, the compound caused a thickening of the periderm (Fig. 6h) and a thinning of  
337 the nodular cortex area (Fig. 6i).

338 The evaluated physiological and biochemical parameters associated with the N fixation  
339 showed significant alterations in the plants treated with the DI-31. Under well-watered  
340 conditions, the compound did not affect the *in vivo* NR activity (Fig. 7a) or the nitrate  
341 content (Fig. 7b); while the content of  $\alpha$ -amino acids (Fig. 7c) increased at the fourth  
342 and eighth day after treatment. However, at the fourth day of water shortage, the *in vivo*  
343 NR activity and nitrate content increased due to DI-31 action, as well as the  $\alpha$ -amino

344 acids levels, that also increased at eighth days after treatment. Plants subjected to DW  
345 and water shortage treatment showed a constant increase of these three indicators. On  
346 the other hand, the ureide content in Munasqa RR leaves (Fig. 7d) significantly  
347 increased over time, in well-watered and stressed plants treated with DI-31. Finally, the  
348 compound application increased in ~15% the ureides relative abundance (Fig. 7e) and  
349 ~16% the percentage of biological N fixed (Fig. 7f) in well-watered plants, while  
350 Munasqa RR plants, submitted to water shortage and DI-31 treatments, showed the  
351 maintenance of both indicators.

## 352 **Discussion**

353 There are two frameworks which identify drought tolerance characteristics in crop  
354 plants, including soybean (Bhatia et al. 2014). One takes into account yield variation in  
355 terms of traits affecting water use and especially the water use efficiency. The other is  
356 related to specific physiological and biochemical characteristics that lead to  
357 improvement under drought (escape, avoidance and tolerance). We took into  
358 consideration these two frameworks to discuss the applicability of DI-31 to mitigate the  
359 detrimental effects of water scarcity. As plant growth promoters, BRs participate in  
360 many developmental processes, such as cell elongation-division, assimilate  
361 translocation (Müssig 2005), the increase of shoot fresh and dry weight, plant height,  
362 petioles length and leaves size and number (Anjum et al. 2011). In this study, we  
363 corroborated that both in *A. thaliana* and soybean plants, the number of leaves, primary  
364 root length and biomass production increased due to DI-31 application; pointing out the  
365 compound capability to enhance plant photosynthetic area and growth rate.  
366 Interestingly, we observed that pot-grown soybeans treated with DI-31 and under water-  
367 limited conditions showed hydrated, expanded and young green leaves, unlike the plants  
368 subjected only to water stress that exhibited wilted, wrinkled and chlorotic phenotype.  
369 This result suggests that the compound modulates mechanism related to the dehydration  
370 postponement under water-limited conditions. Additionally, we analyzed the hydric  
371 status of Munasqa RR plants and found that well-irrigated plants, with DW and DI-31  
372 application, evidenced a hydrated water status (91.3% average RWC). Meanwhile, the  
373 stress application reduced the RWC of DW-treated plants in a ~28 and ~45% at the  
374 fourth and eighth day of trial, respectively. This result corroborates the effectiveness of  
375 the stress induction by water shortage. Meanwhile, Munasqa RR plants treated with DI-

376 31 and subjected to water shortage showed a ~78% average RWC, and registered RWC  
377 decreases in ~15 and ~25% at the fourth and eighth day of stress, respectively. These  
378 results evidenced that the Munasqa RR plants subjected to stress maintained a hydrated  
379 water status due to DI-31 action.

380 Plant biomass is defined as the source of storable energy, mobilizable according to  
381 metabolic requirements (de Freitas Lima et al. 2017) and is highly dependent on Carbon  
382 (C) and N gathering (Zheng 2009). In a previous study, (Yamori and Shikanai 2016)  
383 correlated chlorophylls and carotenoids accumulation with an increase in dry weight  
384 production. In accordance, our results showed an increase of BMD and carotenoid  
385 levels in stressed plants treated with DI-31, strengthening the contribution of this BR  
386 analogue to energy production maintenance under water deficit conditions. There are  
387 significant correlations in soybean between the ability to cope with drought and root  
388 traits such as dry weight increase (Bhatia et al. 2014). In accordance, we observed that  
389 the DI-31 also exerts a positive effect on Munasqa RR roots, stimulating the biomass  
390 production under water shortage, which leads to an increase in water-nutrient absorption  
391 capacity to withstand the stress.

392 Additionally, the production of biomass represents a water cost that can be estimated  
393 through the WUE, allowing to establish a relationship between water consumption and  
394 plant production (Van Halsema and Vincent 2012). In accordance, we observed that,  
395 under water deficit, the WUE reduction was attenuated in soybean plants when DI-31  
396 was applied. While, under conditions of proper irrigation, the application of DI-31  
397 stimulated the WUE over time; suggesting that the compound favours the hydric status  
398 maintenance and the biomass conversion with lower water cost. Crop genetics, nutrient  
399 availability and the regulation of evapotranspiration, determine the plant WUE (Van  
400 Halsema and Vincent 2012). Besides, partial closure of stomata at a certain level of soil  
401 water deficit might lead to an increase in WUE (Miransari 2015). Plant stomata regulate  
402 key processes like CO<sub>2</sub> exchange and transpiration. Thus, we examined DI-31 capacity  
403 to modulate stomatal movements, like its analogue 24-epibrassinolide, that was reported  
404 to promote stomatal closure in a dose-dependent manner (Shi et al. 2015). As we  
405 expected, DI-31 was able to induce stomatal closure in the range in which 24-  
406 epibrassinolide exerted its effect, also in a dose-dependent way. At ~5-10 µM  
407 concentration of DI-31, the plant stomata reached 50% of closure, in agreement with  
408 previously published results. We also demonstrated that DI-31-mediated stomatal

409 closure is partially abolished by NADPH and peroxidase inhibitors, which suggest that  
410 this compound requires ROS production to exert its action. As part of its protective  
411 effect, DI-31-mediated stomatal closure contributes to reducing transpiration. These  
412 results are also in agreement with RWC and WUE maintenance and the hydrated  
413 phenotype observed in DI-31-treated soybean plants under water shortage. An efficient  
414 relationship between dry matter production and water consumption depends on the  
415 photosynthetic efficiency and the stomata movement that regulates transpiration (Bhatia  
416 et al. 2014). Thus, the DI-31 effect on stomatal closure, water status maintenance and  
417 roots development constituted promising indicators of the compound effect on yield  
418 potential (biomass) improvement in water-limited environments.

419 There are a plethora of physiological and biochemical traits involved in the water stress  
420 response. In this paper, we decided to evaluate the effect of DI-31 on stress response  
421 markers associated with osmotic adjustment, antioxidant activity, respiratory burst and  
422 chlorophyll and photoprotective pigments accumulation. BRs can stimulate ROS  
423 production, which acts as second messengers in several processes like photosynthesis,  
424 respiration and tolerance/resistance to environmental stresses (Tripathy and Oelmüller  
425 2012). Cells tightly control ROS level to prevent oxidative injuries, partially through  
426 enzymatic antioxidants like SOD and a wide range of peroxidases among which stand  
427 out APX and POX enzymes (Sharma et al. 2012). We observed that DI-31 foliar  
428 application triggers *A. thaliana* respiratory burst. Besides, under well-watered  
429 conditions the compound caused an increased in SOD, APX and POX activities, in *A.*  
430 *thaliana* and soybean cv. Munasqa RR plants. While, under water deficit, DI-31 effect  
431 on antioxidant enzymes was even more pronounced. These findings indicate the  
432 compound intrinsic ability to activate mechanisms like oxidative burst, ascorbate-  
433 glutathione (Asa-GSH) cycle and phenols synthesis. We analysed lipid peroxidation  
434 through the MDA level and found that water stress, as expected, provoked an increase  
435 in MDA content. However, the MDA formation decreased in stressed plants due to DI-  
436 31 treatment, suggesting that the compound diminish the lipid-peroxides accumulation.  
437 Related to osmotic adjustment, we quantified an increase of free proline content in  
438 Munasqa RR plants subjected to DI-31 treatment, especially under water shortage  
439 conditions. In response to water deficit, plants can activate or increase a major defence  
440 mechanism composed, among others, by enzymatic antioxidants (Sajedi et al. 2011).  
441 Thus, a high antioxidant capacity is linked to increased crop stress tolerance (Sharma et

442 al. 2012). Overall, our data indicate that the DI-31 promote the degradation of  $O_2^-$ ,  
443  $H_2O_2$  and lipoperoxidation products under stress. Additionally, in soybean plants under  
444 water scarcity, the compound also contributes to chlorophyll content maintenance and  
445 carotenoids and free proline overproduction. These findings agree with the obtained in  
446 wheat, rice and potato cultivars, where the accumulation of non-enzymatic antioxidants  
447 and the lipid-peroxides reduction resulted in a higher survival rate, yield and tolerance  
448 to drought/osmotic stresses (Amini et al. 2015).

449 BRs pleiotropic effects in plant growth and development, as well as resistance/tolerance  
450 to biotic and abiotic stresses, offer exciting potentialities for enhancing crops  
451 productivity and quality (Ali and Ashraf 2008; Baghel et al. 2019; Gill et al. 2017).  
452 Nevertheless, few studies address the effect of these hormones on the balance of  
453 macronutrients such as N, even though (Shu et al. 2016) reported that foliar application  
454 of 24-epibrassinolide significantly enhanced the activity of assimilative  $N_2$ -fixation  
455 critical enzymes in tomato plants. While, (Wang et al. 2019) suggest that the  
456 transcriptional factor BZR1, a BRs positive regulator, possibly plays a critical role in  
457 tomato N-starvation response.

458 It's known that plant N regulation is needed to maintain optimum photosynthetic rate  
459 and therefore biomass production since most of leaf N is a constituent element of the C  
460 assimilation protein ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO)  
461 strongly involved in photosynthesis (Rotundo and Cipriotti 2017; Sinclair and Horie  
462 1989). Soybean demands high N concentrations, and it is often grown on soils with low  
463 N availability thus the biological  $N_2$  fixation (BNF) makes significant contributions to  
464 the plant growth, yield and high-protein seed and forage production (Peoples et al.  
465 2009). Few reports exist of low BNF contributions in soybean, and most of these results  
466 come from breeding and cropping-managements with high inputs of N-fertilizers  
467 (Hungria and Mendes 2015).

468 Soybean BNF depends on the formation of the nodule (Denton et al. 2017).  
469 Interestingly, soybean nodules are determined; there is no permanent meristem, so its  
470 growth depends on expansion instead of cell division. In consequence, the nodular N-  
471 fixing capacity depends on the nodules size and the number of bacteroids (fixing  
472 centres) located in the infected medulla (de Felipe Antón 2007). The nodule  
473 development and therefore the BNF are strongly related and also are severely affected  
474 during periods of moisture deficiency (Purcell et al. 2004; Sinclair et al. 2007); mainly

475 through ROS and/or reactive N species (RNS) accumulation leading to the  
476 Leghemoglobin self-oxidation and the Nitrogenase complex inactivation (Puppo et al.  
477 2005). Water deficit also provokes nodule dehydration and wrinkling, with a marked  
478 reduction of the infected central medulla and finally the nodular senescence activation  
479 (Hernández-Jiménez et al. 2002). Our findings demonstrate that the DI-31 retarded the  
480 active nodules senescence. The presence of numerous light pink nodules, after eight  
481 days of water scarcity, allowed us to assume that the DI-31 reduced the Leghemoglobin  
482 self-oxidation; either by activating (i) ROS/RNS-scavenging or exclusion mechanism,  
483 (ii) water economy or (iii) Leghemoglobin recycling pathways. On the other hand, the  
484 shrinking in the cortex area, observed in all the DI-31-treated nodules, might be  
485 explained by the expansion of the infected central medulla, which suggests the  
486 compound ability to increase the bacterial colonization area and therefore the nodules  
487 N-fixing capacity. Moreover, the significant periderm thickness, quantified in DI-31  
488 treated nodules under stress, indicates an outer layers reinforcement to cope with water  
489 deficit.

490 To complement the results obtained in the nodulation morphology analysis, we  
491 evaluated several N homeostasis biochemical markers and found that DI-31 regulates  
492 the N homeostasis mechanism, especially under stress. According to (Rodríguez-  
493 Navarro et al. 2011), the Nitrate Reductase (NR) is the first enzyme in nitrates  
494 assimilative reduction (NAR) pathway, catalyzing the nitrate ( $\text{NO}_3^-$ ) conversion into  
495 nitrite ( $\text{NO}_2^-$ ) which is subsequently transformed in ammonia ( $\text{NH}_3$ ) and then in  
496 assimilable ammonium ( $\text{NH}_4^+$ ). The NR is synthesized and degraded continuously, so  
497 the control of its activity is through a substrate regulation (Rajasekhar and Oelmüller  
498 1987). Thus high levels of  $\text{NO}_3^-$  increase the enzyme activity. Our results show that  
499 soybean plants under water scarcity present high levels of nitrate and NR activity.  
500 Therefore, plants NAR was possibly stimulated to satisfy the N demand caused by the  
501 nodular senescence and BNF deficit due to stress occurrence. On the contrary, stressed  
502 and DI-31 treated plants showed a reduced loss of active nodules, lower NR activity and  
503 nitrate content, so we speculate that the DI-31 also modulates the mineral N absorption  
504 pathways.

505 In soybean, the  $\text{NH}_4^+$  resulting from the symbiosis is converted into ureides, which are  
506 synthesized in the nodules and transported to the leaves through the xylem (King and  
507 Purcell 2005; White et al. 2007). In contrast, the  $\text{NH}_4^+$  produced by NAR is converted



508 into  $\alpha$ -amino acids, mainly asparagine (White et al. 2007). The  $\alpha$ -amino acids  
509 accumulation observed in Munasqa RR leaves, due to water shortage, agrees with an  
510 increase in NAR pathway indicators. On the other hand, ureides have four C and four N  
511 atoms in their chemical structure, therefore are more efficient for N transport than  $\alpha$ -  
512 amino acids, which only have two N atoms. Besides, the nodules require high amounts  
513 of C, before an active BNF, which agrees with the ureides efficiency in transporting N  
514 atoms equivalent to their C number (Freixas et al. 2010). Nitrogen metabolism under  
515 water deficit plays a crucial role in the BNF regulation, which occurs through a negative  
516 feedback mechanism (King and Purcell 2005). It is not clear if the accumulation of  
517 ureides in plants leaves constitutes a stress tolerance or susceptibility response. (Vadez  
518 and Sinclair 2002) report a decrease in Nitrogenase activity and the content of ureides in  
519 the leaves in drought-sensitive soybeans subjected to water scarcity and manganese  
520 treatments.

521 Meanwhile, a research performed by (King and Purcell 2005) also in drought-  
522 susceptible soybeans correlates a BNF decrease with the ureides accumulation in leaves.  
523 In a subsequent study, the same authors state that there is no evidence of tolerance when  
524 soybean varieties, sensitive or tolerant to water deficit, increase the ureides  
525 concentration in roots and decrease in leaves during drought (King and Purcell 2005).  
526 Ureides accumulation in the nodule directly inhibits the BNF during stress (Charlson et  
527 al. 2009), therefore, if their proper transport from the nodules to leaves is guaranteed,  
528 the BNF inhibition could be prevented, at least temporarily. Thus, the increase in ureide  
529 content and relative abundance in Munasqa RR leaves due to DI-31 application, could  
530 contribute to the BNF maintenance under water scarcity. (Santachiara et al. 2018)  
531 reported that BNF in soybean represents ~60% of the total N uptake. Whereas, in  
532 Argentina, the range of N derived from BNF varied from 46 to 71% in farmers' fields  
533 (Collino et al. 2015) . In agreement, our results demonstrate that the percentage of N  
534 fixed biologically in Munasqa RR plants, covers ~50.4% of the total N demand.  
535 Interestingly, the significant BNF increase in well-watered and stressed plants, both  
536 treated with DI-31, reinforced the hypothesis of probable BRs-plant-nodule crosstalk  
537 that positively modulates the N homeostasis.

538 Authors like (Sinclair and Horie 1989), (Rotundo and Cipriotti 2017) and (Santachiara  
539 et al. 2018) actively discuss that N plays a central role in the proteins homeostasis,  
540 photosynthesis, respiration, leaf area generation and water use efficiency. Thus, we

541 considered that the DI-31 action on NAR and BNF could link with other physiological  
542 traits such as photosynthesis, antioxidants activation, water preservation, stomatal  
543 movement, growth and biomass production/duration, previously discussed in the paper.  
544 The source-to-sink N partitioning directly influences the grain production, and soybean  
545 shows a strong positive correlation between seed yield and N uptake (Rotundo and  
546 Cipriotti 2017; Salvagiotti et al. 2008; Tamagno et al. 2017). In accordance, it is  
547 conceivable to speculate that the DI-31 application, beyond the short and middle-term  
548 effect on the growth, respiration, anti-stress response and N homeostasis, might have  
549 also a long-term effect on soybean seed quality and yield.

## 550 **Conclusions**

551 The exogenous application of DI-31 stimulates leaves and roots development,  
552 photosynthetic and water/nutrient absorption capacity, water economy partially through  
553 stomatal closure induction, WUE and biomass production and duration. Also, the  
554 compound promotes respiratory burst, osmotic adjustment, the synthesis of  
555 photoprotective pigments and enzymatic antioxidants, improving the ROS-scavenging  
556 and preventing the accumulation of damaging products like MDA. Moreover, the  
557 compound showed a remarkable protective effect in soybean nodulation and N  
558 homeostasis. Accordingly, plants treated with DI-31 showed a higher number of active  
559 nodules, with a larger size, reinforced periderm and larger medulla, as well as higher  
560 BNF values under stress. Thus, we propose that DI-31 represents a practical value as a  
561 promising bio-stimulant that might help to alleviate stress-derived impacts on soybean  
562 production. Moreover, the potential use of DI-31 to promote growth and regulate stress-  
563 response could represent a sustainable and environmentally safe alternative for  
564 integrative crop resilience management amidst climate change threats.

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## Figure legends

**Fig. 1** DI-31 application promotes *A. thaliana* Col-0 growth. **(a)** Representative image of plants growing proportionally to DI-31 concentration (0.22  $\mu$ M (0.1 mg/L), 1.12  $\mu$ M (0.5 mg/L) and 2.23  $\mu$ M (1 mg/L)). Morphological parameters such as **(b)** root length (cm), **(c)** number of leaves and **(d)** biomass increase (mg) measured in 10-day-old plants grown in MS medium with and without DI-31 at different doses. Data represent the mean ( $\pm$ SD) of two independent experiments (n=80). Different letters on top of the bars indicate significant difference as determined by ANOVA with *post hoc* contrasts by Tukey's test: (P<0.05).

**Fig. 2** DI-31 application stimulates the respiratory burst and antioxidant response of *A. thaliana* Columbia 0 plants under well-watered conditions. **(a)** Superoxide dismutase (SOD), **(b)** Ascorbate peroxidase (APX), **(c)** Phenol peroxidase (POX) and **(d)** Catalase (CAT) enzymatic activities, and **(e)** protein content determined in WT plants leaves (n=120) collected at the trial beginning (0), 24 and 48 h after distilled water (DW) and DI-31 (2.23  $\mu$ M) foliar applications. **(f)** Representative image of the oxidative burst induced by DI-31 application, measured through superoxide radicals accumulation by NBT staining on the sixth and seventh plant leaves, collected at 6, 12, 24 and 48 h after treatment (n=24). Different letters on top of the bars indicate significant difference as determined by ANOVA with *post hoc* contrasts by Tukey's test: (P<0.05).

**Fig. 3** DI-31 induce *A. thaliana* dose-dependent stomatal closure, requiring NADPH and peroxidase-dependent ROS production. **(a)** DI-31 and 24-epibrassinolide stomatal closure comparative experiments in 4-week-old plants, using an opening control (untreated KCl-MES-KOH buffer), a closing control with ABA (20  $\mu$ M) and treatments with serial dilutions (0.1; 0.5; 1; 5; 10 and 20  $\mu$ M) of DI-31 and 24-epibrassinolide. Stomatal apertures were measured 1.5 hours after the compound application. Data represent the mean ( $\pm$  SE) of two independent experiments (40 stomata *per* treatment, a total of 560 stomata). **(b)** Stomatal closure assay with DPI and SHAM ABA inhibitors carried out in 4-week-old plants, using an opening control (untreated KCl-MES-KOH buffer), a closing control with ABA (20  $\mu$ M) and treatments with specific inhibitors of NADPH-oxidases (DPI: 10  $\mu$ M) and cell-wall peroxidases (SHAM: 2 mM) with and without DI-31 (10  $\mu$ M). Stomatal apertures were measured 1.5 hours after the application of the treatments. Data represent the mean ( $\pm$  SE) of two independent experiments (n=480 stomata). Different letters on top of the bars indicate significant difference as determined by ANOVA with *post hoc* contrasts by Tukey's test: (P<0.05).

**Fig. 4** DI-31 contributes to soybean growth and water preservation under water shortage. Morphophysiological parameters such as (a) plant relative water content (RCW), (b) water use efficiency, (c) biomass duration, (d) roots biomass and (e) leaves number, were measured in V5 soybean cv. Munasqa RR plants. (f) Representative image of the highly contrasting phenotype observed in the Munasqa RR plants treated with distilled water (DW) and DI-31 and submitted to eight days of water shortage. Four treatments were defined: (i) well-irrigated plants (substrate water potential ( $\Psi_s$ ) =  $-0.05$  MPa) sprinkled with DW, (ii) well-irrigated plants sprinkled with DI-31, (iii) stressed plants ( $\Psi_s$  =  $-0.65$  MPa) sprinkled with DW and (iv) stressed plants sprinkled with DI-31. Once the stress treatments reached the substrate water content and  $\Psi_s$  corresponding to moderate water stress, the DI-31 ( $2.23 \mu\text{M}$ ) and DW treatments were performed by sprinkling to the drip point. Whole plants were collected at 0, 4 and 8 days after the water shortage. Data represent the mean ( $\pm$  SE) of two independent experiments  $n=60$  (a-b) and  $n=180$  (c-e). Different letters on top of the bars indicate significant difference as determined by ANOVA with *post hoc* contrasts by Tukey's test: ( $P<0.05$ ).

**Fig. 5** DI-31 application stimulates soybean anti-stress response under water shortage. Physiological and biochemical indicators such as (a) Superoxide dismutase (SOD), (b) Ascorbate peroxidase (APX), (c) Phenol peroxidase (POX) and (d) Catalase (CAT) specific activities, as well as the content of (e) protein, (f) chlorophyll a, (g) b and (h) total, (i) total carotenoids, (j) free proline and (k) malondialdehyde (MDA), were determined in V5 soybean cv. Munasqa RR plants. Four treatments were defined: (i) well-irrigated plants (substrate water potential ( $\Psi_s$ ) =  $-0.05$  MPa) sprinkled with distilled water (DW), (ii) well-irrigated plants sprinkled with DI-31, (iii) stressed plants ( $\Psi_s$  =  $-0.65$  MPa) sprinkled with DW and (iv) stressed plants sprinkled with DI-31. Once the stress treatments reached the soil water content and water potential corresponding to moderate water stress, the DI-31 ( $2.23 \mu\text{M}$ ) and DW treatments were performed by sprinkling to the drip point. The V2, V3 and V4 leaves from each plant were collected at 0, 4 and 8 days after the application of the treatments. Data represent the mean ( $\pm$  SE) of two independent experiments  $n=240$ . Different letters on top of the bars indicate significant difference as determined by ANOVA with *post hoc* contrasts by Tukey's test: ( $P<0.05$ ).

**Fig. 6** DI-31 treatment modulates soybean nodulation parameters under water shortage. Nodules located in the root crown imaginary cylinder (**a**) were collected and classified in active nodules according to the Leghemoglobin pink colouration (**b**), then the total of active nodules was quantified (**d**) and cut for morphological analysis (**c**). Nodulation parameters such as equatorial (**e**) and polar (**f**) diameter, the estimated area of the infected central medulla (**g**) and the thickness of periderm (**h**) and (**i**) cortex area (outer, middle and inner) were measured in V5 soybean cv. Munasqa RR active nodules using through image processing and analysis (*ImageJ* 1.52v). Four treatments were defined: (i) well-irrigated plants (substrate water potential ( $\Psi_s$ ) =  $-0.05$  MPa) sprinkled with distilled water (DW), (ii) well-irrigated plants sprinkled with DI-31, (iii) stressed plants ( $\Psi_s$  =  $-0.65$  MPa) sprinkled with DW and (iv) stressed plants sprinkled with DI-31. Once the stress treatments reached the substrate water content and  $\Psi_s$  corresponding to moderate water stress, the DI-31 ( $2.23 \mu\text{M}$ ) and DW treatments were performed by sprinkling to the drip point. Whole plants were collected at 0, 4 and 8 days after the application of the treatments. Data represent the mean ( $\pm$  SE) of two independent experiments ( $n= 180$ ). Different letters on top of the bars indicate significant difference as determined by ANOVA with post hoc contrasts by Tukey's test: ( $P<0.05$ ).

**Fig. 7** DI-31 regulates soybean nitrogen homeostasis under well-watered and water shortage conditions. Biochemical N fixation parameters such as (**a**) Nitrate Reductase (NR) *in vivo* activity, the content of (**b**) Nitrate and (**c**)  $\alpha$ -amino acids, (**d**) ureides content and (**e**) relative abundance, and (**f**) the percentage of biological N fixed were measured in V5 soybean cv. Munasqa RR leaves. Four treatments were defined: (i) well-irrigated plants (substrate water potential ( $\Psi_s$ ) =  $-0.05$  MPa) sprinkled with distilled water (DW), (ii) well-irrigated plants sprinkled with DI-31, (iii) stressed plants ( $\Psi_s$  =  $-0.65$  MPa) sprinkled with DW and (iv) stressed plants sprinkled with DI-31. Once the stress treatments reached the substrate water content and  $\Psi_s$  corresponding to moderate water stress, the DI-31 ( $2.23 \mu\text{M}$ ) and DW treatments were performed by sprinkling to the drip point. Whole plants were collected at 0, 4 and 8 days after the application of the treatments. Data represent the mean ( $\pm$  SE) of two independent experiments ( $n= 180$ ). Different letters on top of the bars indicate significant difference as determined by ANOVA with post hoc contrasts by Tukey's test: ( $P<0.05$ ).

Fig 1

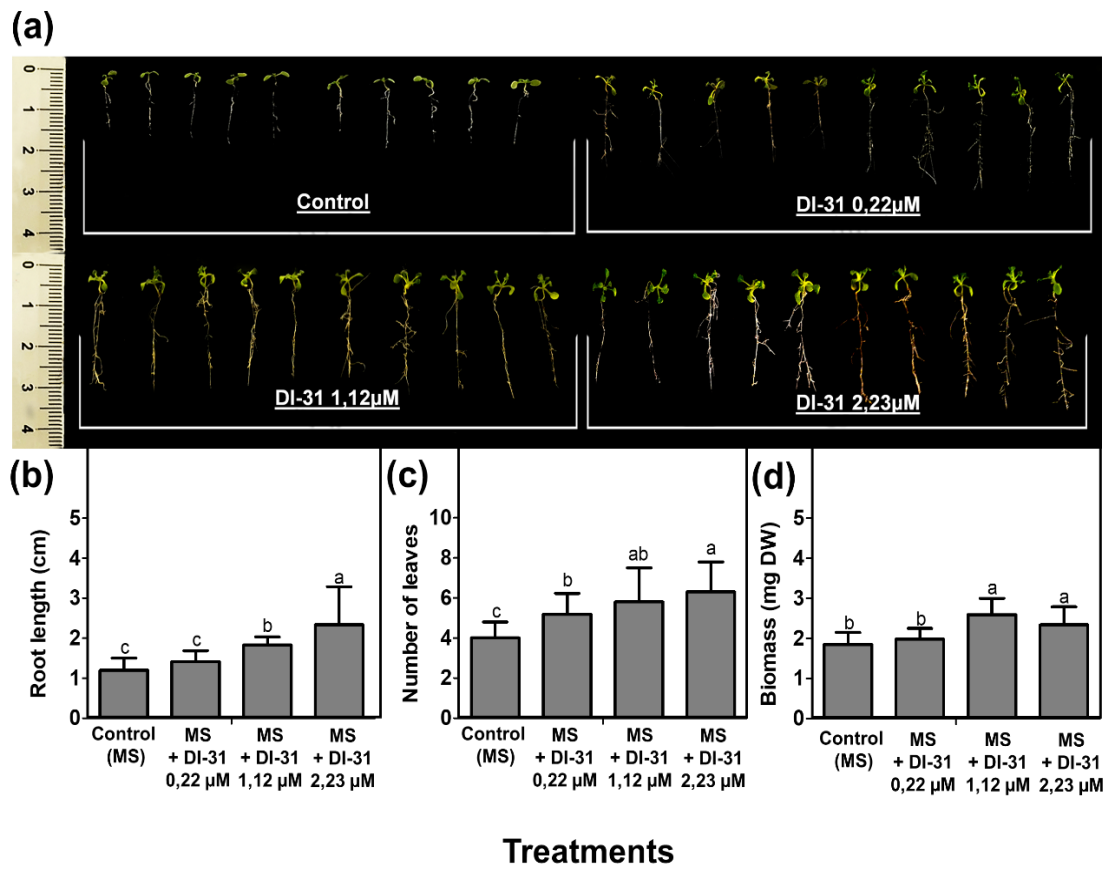


Fig 2

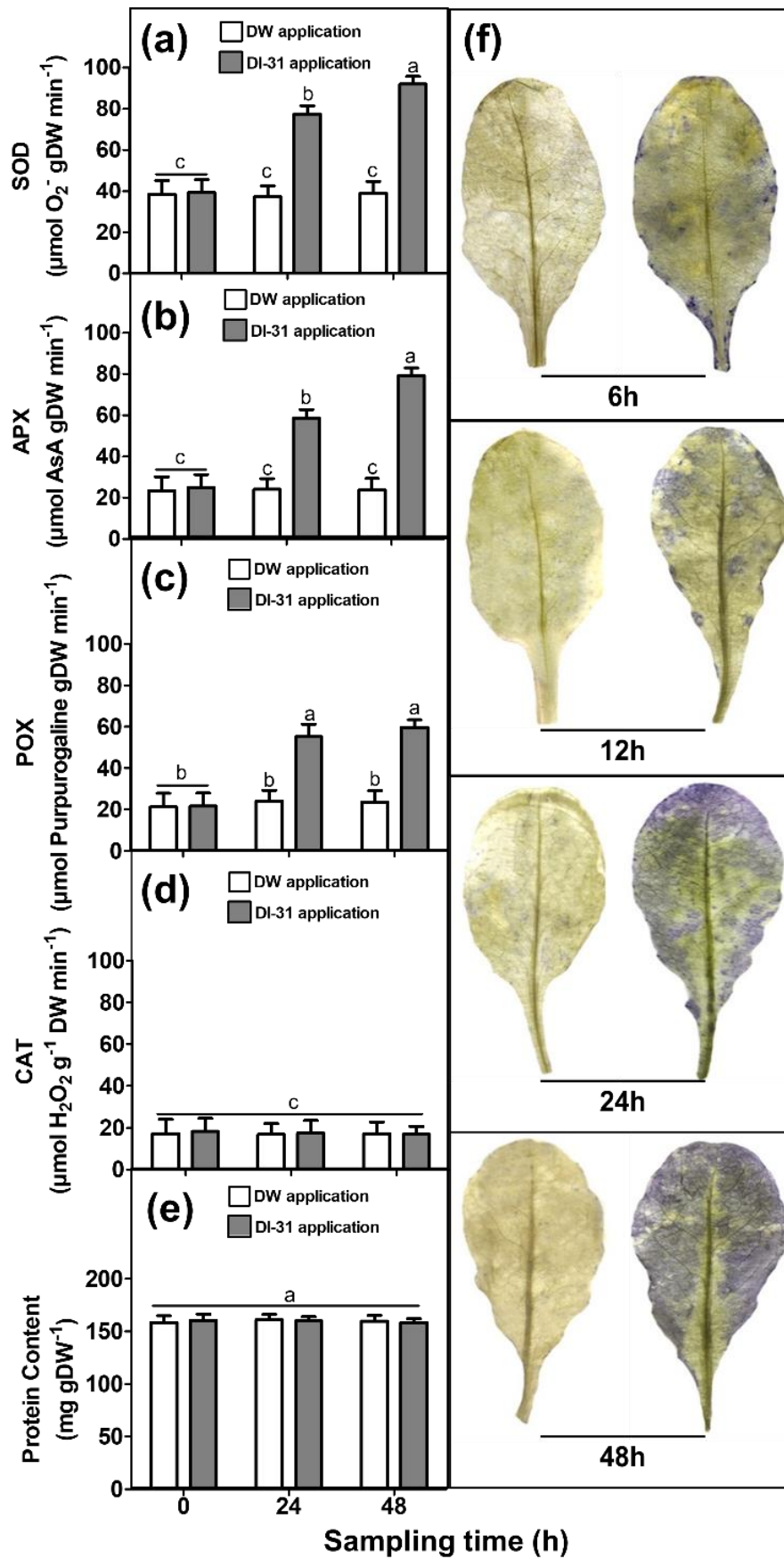
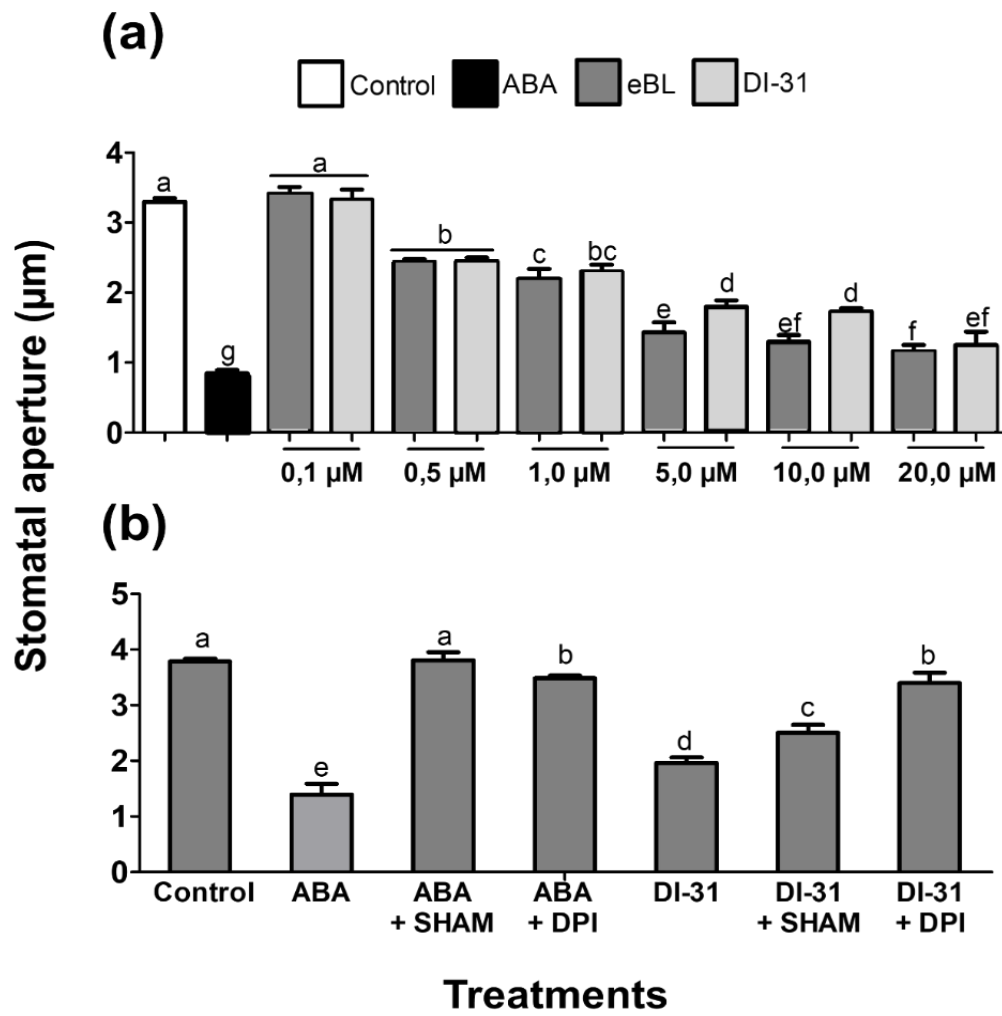




Fig 3



**Fig 4**

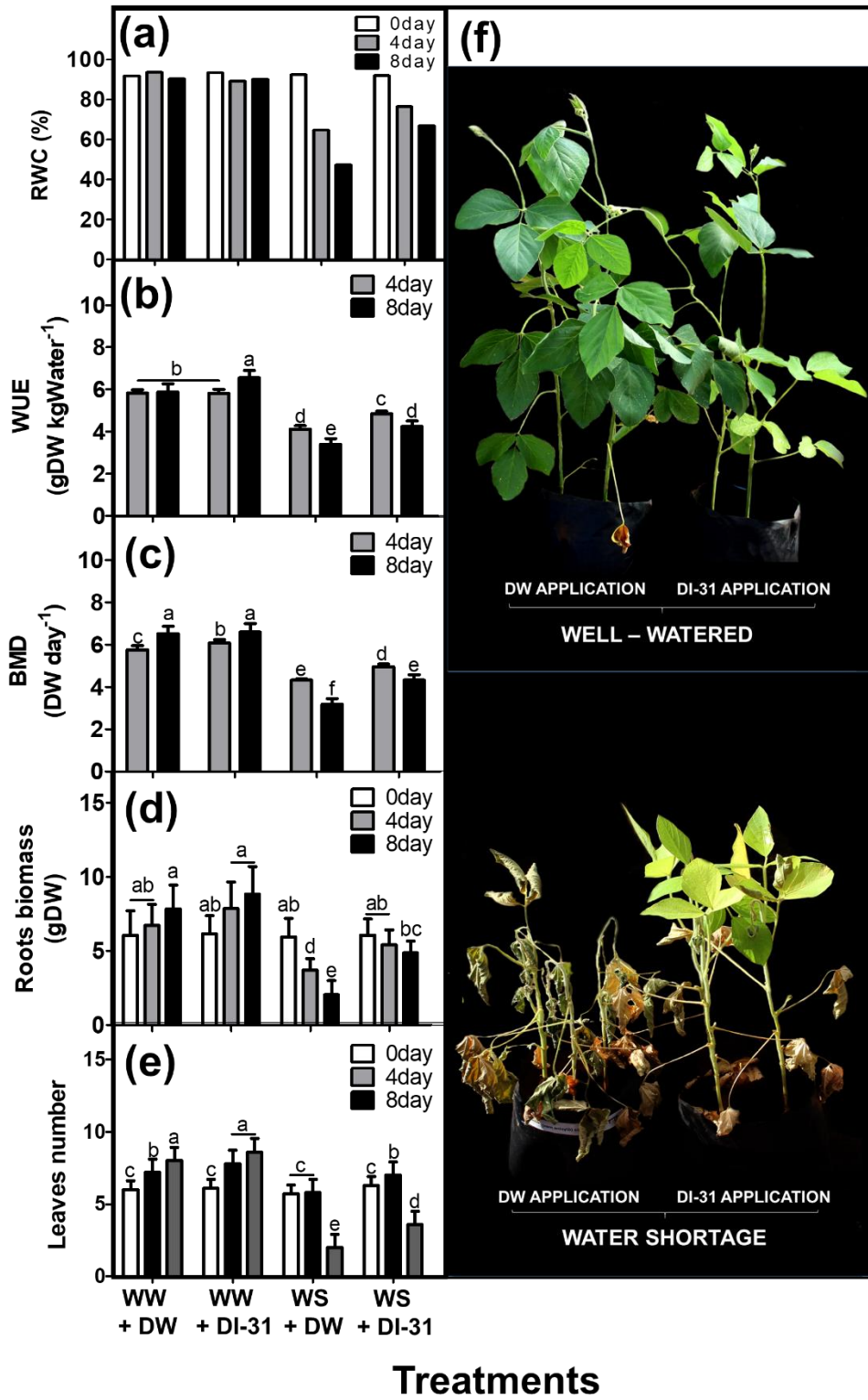


Fig 5

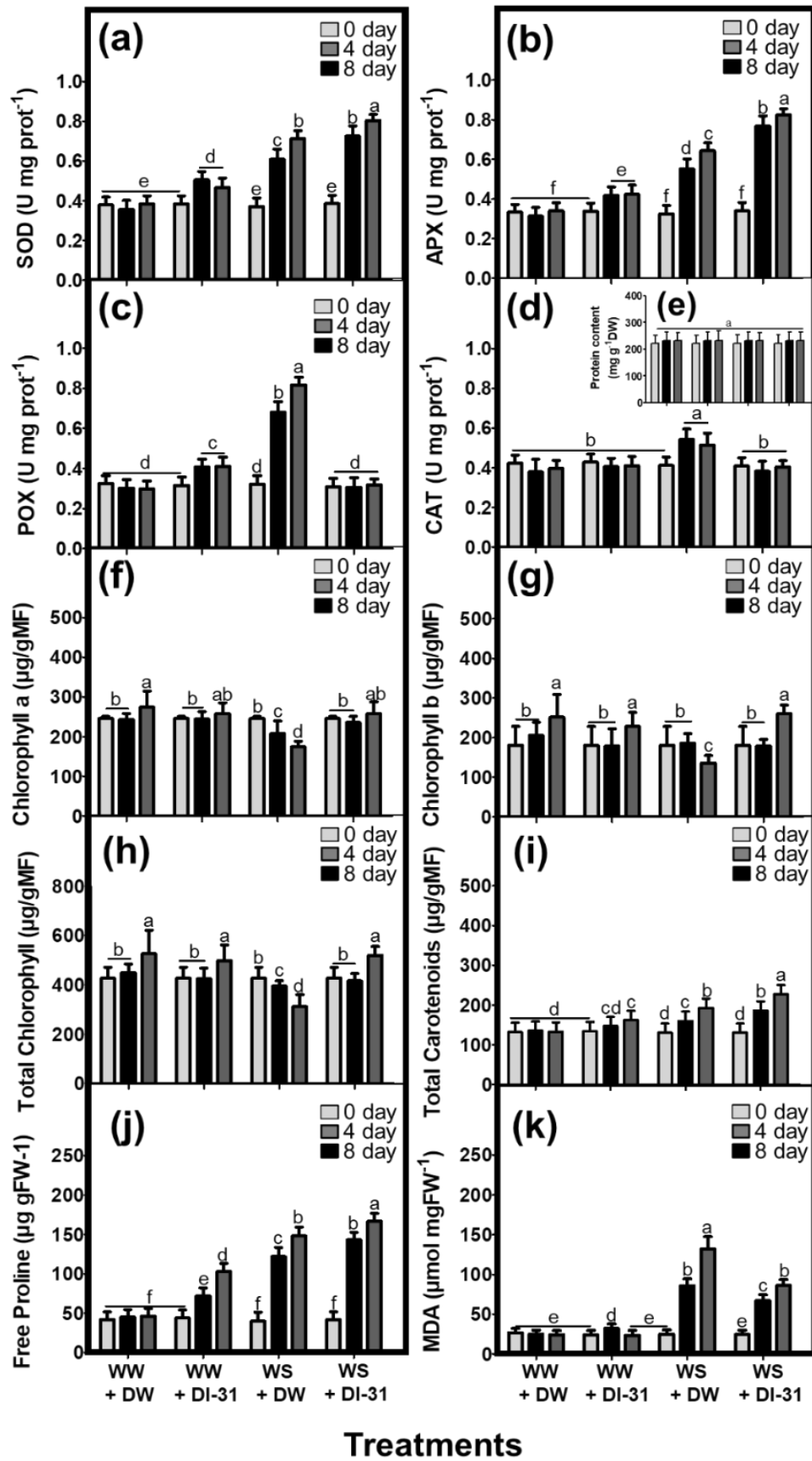


Fig 6

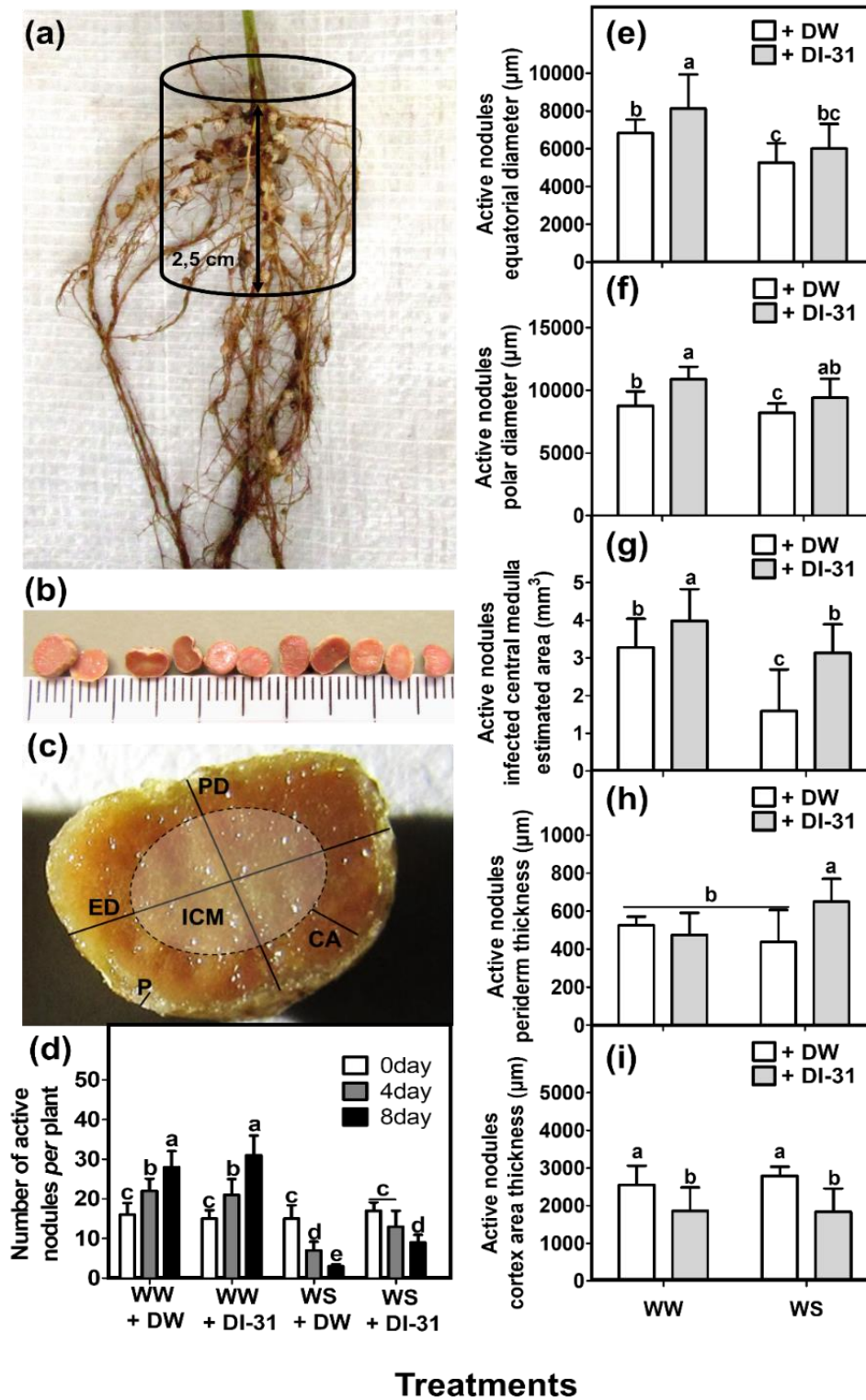


Fig 7

