

# 1 A diversity of traits contributes to salinity tolerance of wild Galapagos tomatoes

## 2 seedlings

3

## 4 Running title: Salinity responses in Galapagos tomatoes

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22 **Abstract:**

23 Traits of modern crops have been heavily selected in agriculture, causing the commercial  
24 lines to be more susceptible to harsh conditions, which their wild relatives are naturally better  
25 able to withstand. Understanding the developed mechanisms of tolerance present in wild  
26 relatives can enhance crop performance under stress. In this study, salinity tolerance traits of  
27 two species of wild tomato endemic to the Galapagos Islands, *Solanum cheesmaniae* and *Solanum galapagense*, were investigated. Since these tomatoes grow well  
28 despite being constantly splashed with seawater, they could be a valuable genetic resource for  
29 improving salinity tolerance in commercial tomatoes. To explore their potential, over 20  
30 traits reflecting plant growth, physiology and ion content were recorded in 67 accessions of *S.  
31 cheesmaniae* and *S. galapagense* and two commercial tomato lines of *Solanum lycopersicum*.  
32 Salt treatments of 200 mM NaCl were applied for ten days, using supported hydroponics.  
33 Great natural variation was evident in the responses of the Galapagos tomatoes to salt stress  
34 and they also displayed greater tolerance to salt stress than the commercial lines tested, based  
35 on multivariate trait analyses. Although Galapagos tomatoes in general exhibited better  
36 tolerance to salt stress than the commercial lines tested, the accessions LA0317, LA1449 and  
37 LA1403 showed particularly high salinity tolerance based on growth maintenance under  
38 stress. Thus, Galapagos tomatoes should be further explored using forward genetic studies to  
39 identify and investigate the genes underlying their high tolerance and be used as a resource  
40 for increasing salinity tolerance of commercial tomatoes. The generated data, along with  
41 useful analysis tools, have been packaged and made publicly available via an interactive  
42 online application ([https://github.com/mmjulkowska/La\\_isla\\_de\\_tomato](https://github.com/mmjulkowska/La_isla_de_tomato)) to facilitate trait  
43 selection and the use of Galapagos tomatoes for the development of salt tolerant commercial  
44 tomatoes.

46

47    **Keywords:**

48    salinity tolerance, salt stress, wild relatives, tomato, *S. cheesmaniae*, *S. galapagense*, seedling  
49    screen, hydroponics, phenotyping, Galapagos tomatoes.

## 50    **Introduction**

51    High soil salinity is one of the main agricultural challenges in the modern world (Rengasamy,  
52    2016). Salt stress affects the growth and development of plants, thus significantly reducing  
53    their yield and productivity (Arzani and Ashraf, 2016). Global cultivated lands cover ~1.5  
54    billion hectares, and an estimated 32 million hectares are damaged by salinity. Irrigated  
55    lands, having the highest productivity, comprise just 230 million hectares, of which an  
56    estimated 20% have yields significantly reduced by high soil salinity (Munns, 2005). Water  
57    availability for agriculture is another major concern, not only in desert regions but at a global  
58    level, as freshwater supplies are depleting (Famiglietti, 2014). Salt-affected areas worldwide  
59    are predicted to continue expanding at a rate of ~10% per year due to low precipitation, high  
60    surface evaporation, erosion of rocks, irrigation with saline water and poor cultural practices  
61    (Foolad, 2004).

62    Wild relatives of modern crops have been used for crop improvement for more than 60 years  
63    (Hajjar and Hodgkin, 2007). In their natural habitats, they are able to withstand harsh  
64    conditions such as high soil salinity (Muñoz *et al.*, 2017). Adaptation to wide-ranging  
65    environments has enriched the genetic pool of these wild relatives (Gruber, 2017), thus  
66    representing a rich source of potentially beneficial alleles that can be explored to improve  
67    salinity tolerance (Zamani Babgohari *et al.*, 2013). Therefore, performing phenotypic screens  
68    to capture the natural variation of the wild germplasm can help uncover new genetic sources  
69    for enhancing stress tolerance (Arzani and Ashraf, 2016).

70    The Galapagos Islands, an isolated environment close to the center of origin of the current  
71    domesticated tomato (Blanca *et al.*, 2012), hold a rich genetic diversity of tomato wild  
72    relatives, notably the two endemic species, *S. cheesmaniae* and *S. galapagense*, adapted to  
73    thrive in harsh environments and highly saline coastal habitats (Rick, 1956; Rush and  
74    Epstein, 1976). Previous studies hypothesized that at least some accessions of Galapagos

75 tomatoes, collected from both coastal and inland regions, are able to survive higher NaCl  
76 concentrations than the domesticated tomato (Rush and Epstein, 1976, 1981; Tal and  
77 Shannon, 1983). However, little is known about the specific mechanisms by which the  
78 Galapagos tomatoes thrive on saline soil.

79 The known mechanisms involved in plant salinity tolerance can be classified into three types:  
80 osmotic tolerance, involving the sensing and signalling modules occurring before shoot  $\text{Na}^+$   
81 accumulation and causing reductions in growth rate; ion exclusion, the limitation of ion  
82 accumulation in the shoot by ion sequestration in the roots; and tissue tolerance, where high  
83  $\text{Na}^+$  concentrations in the shoot are compartmentalized in the vacuoles to reduce their toxic  
84 effects (Munns and Tester, 2008; Roy *et al.*, 2014).

85 An in-depth characterization of Galapagos tomato accessions would allow to understand the  
86 mechanisms for salinity tolerance used within these species, as well as to identify the most  
87 tolerant accessions for further studies, thus helping to unlock their potential use as genetic  
88 resources for improved salinity tolerance of commercial tomato. However, the wild nature of  
89 these plants makes them difficult to compare with domesticated commercial lines, as they  
90 differ in growth rates and habit. Hence, developing a robust phenotyping method that is  
91 suitable for investigating wild germplasm, accounting for all variations in growth, was  
92 necessary. The application of salt treatments at the same growth stage for all accessions is  
93 important when growth rates vary significantly. To effectively deliver the salt treatment, a  
94 hydroponics growth system is preferred since it allows the precise control of salt  
95 concentration in the medium (Genc *et al.*, 2007; Munns *et al.*, 2010; Negrão *et al.*, 2017).  
96 Moreover, the effects on the ionic activity of micronutrients, such as calcium, by the  
97 interaction between salt and nutrients in the medium, can be calculated and balanced by  
98 adding supplemental nutrients (Tester and Davenport, 2003). Also, given so many tomatoes

99 are grown commercially in hydroponic systems, such results can even be of direct relevance  
100 to application.

101 In this study, the phenotypic traits related to salinity tolerance of 67 different accessions of  
102 wild tomato from the Galapagos Islands were scored, using a flood-and-drain hydroponic  
103 growth system. Traits reflecting growth, physiology and ion content were explored using  
104 multivariate analysis, leading to a better and more comprehensive understanding of the role  
105 of these traits contributing to salinity tolerance in Galapagos tomatoes. A wide variation in  
106 growth, physiology and ion content was observed across the accessions, demonstrating great  
107 natural diversity underlying the three main mechanisms of salinity tolerance within the  
108 Galapagos tomatoes.

109

## 110 **Materials and Methods**

### 111 **Plant material and seed treatments**

112 A collection of 67 Galapagos tomato accessions (Pailles *et al.*, 2017) was characterized and  
113 screened for salinity tolerance, of which 39 are *S. cheesmaniae* and 28 are *S. galapagense*.  
114 Two commercial *S. lycopersicum* varieties were also used for comparison: Heinz 1706 and  
115 Moneymaker. Heinz 1706 has a published reference genome sequence (The Tomato Genome  
116 Consortium, 2012), and Moneymaker was previously shown to have mild tolerance to salt  
117 stress (Cuartero *et al.*, 1992). The seeds were obtained from the Tomato Genetics Resource  
118 Center (TGRC), UC Davis, California, USA, and propagated in the greenhouse of King  
119 Abdullah University of Science and Technology (KAUST). Seeds from a single plant were  
120 used in the experiment.

121 To sterilize the surface of the seeds and to break their dormancy, all seeds were treated with  
122 10% bleach solution for 10 to 30 minutes, until the seed coat was softened and become

123 transparent, then they were washed several times in tap water. This treatment was necessary  
124 for the germination of most of the Galapagos tomato seeds (Rush and Epstein, 1976).  
125 Although the commercial varieties did not require bleaching to germinate, the same treatment  
126 was applied to all the seeds.

127 **Experimental hydroponics setup**

128 Eight-centimeter square pots were filled with plastic pellets as a substrate to support the  
129 roots. The pellets were chosen for their inert quality and dark color to protect the roots from  
130 light. The plastic pellets were made of 20% talc-filled polypropylene, black, and with a  
131 density of  $1.05 \text{ g/cm}^3$  to sink in water (Edwards Industrial Repair, Robards, KY). Treated  
132 seeds were germinated directly in the pots, on 0.8% agar plugs (8 mm diameter, 12 mm deep)  
133 containing  $\frac{1}{4}$  Murashige and Skoog (MS) salts inserted in the plastic pellets (**Figure 1A**).  
134 Two agar plugs, each with one seed, were placed in each pot to increase the chances of  
135 germination of at least one seed per pot. Sown pots were placed in nursery trays filled with  
136 fresh water and covered with a transparent plastic cover, then kept at  $26^\circ\text{C}$ . Germination took  
137 between three and eight days. After germination, the two seedlings per pot were thinned to  
138 one, by choosing plants with even size and healthy appearance. Treatment with 10% bleach  
139 was repeated for those seeds that did not germinate one week after the first treatment  
140 (Darwin, 2009). Six biological replicates were used for each control and salt treatment.  
141 Because different species with different growth habits were being compared, another six  
142 replicates were included, to be harvested before the salt treatment was started – thus, the  
143 effects of salinity on growth that occurred only during the time of the salt treatment could be  
144 calculated, correcting for differences in growth that occurred prior to the salinity treatment.

145 When the cotyledons had emerged fully and the radicle was long enough, the pots were  
146 transferred from the nursery to a supported hydroponics system (EconoTray by American  
147 Hydroponics, Inc.), which uses an ebb-and-flow scheme for root aeration (**Figure 1B**). This

148 system consists of a grow tray, a tray frame, a 100 L nutrient reservoir tank, and a  
149 submersible aquarium-pump (ViaAqua 360 by Commodity Axis, Inc.). The tray frame height  
150 was modified, from 0.5 to 1.06 m, to hold the plants above the greenhouse walls and thus  
151 avoid shading. Each growth tray was able to hold up to 108 pots (8 cm<sup>2</sup>) with seedlings at the  
152 cotyledon stage and 96 pots (8 cm<sup>2</sup>) growing tomato plants up to the 7<sup>th</sup> or 8<sup>th</sup> leaf stages. The  
153 growth tray rests on the tray frame above the reservoir tank, which contains the nutrient  
154 solution. The solution was pumped to the grow tray to deliver nutrients to the plants, then  
155 drained back into the reservoir tank allowing root aeration. Aquarium pumps inside the  
156 reservoir tanks were controlled by a programmed timer to be on for 15 min, pumping nutrient  
157 solution up to fill the grow tray, and off for 15 minutes, allowing the solution from the grow  
158 tray to drain back into the tank. The nutrient solutions were prepared using 100 L of tap water  
159 from the greenhouse and 33 ml of each nutrient stock, FloraGro, FloraMicro and FloraBloom  
160 (General Hydroponics), as suggested by the manufacturer. The tap water was tested for  
161 calcium, chloride, potassium, sodium, ammonium and nitrate ion content using Multi-Ion Kit  
162 (CleanGrow Europe), before preparing the solutions. These measurements were recorded for  
163 future normalization. Nutrient depletion was monitored weekly using the Multi-Ion Kit ion-  
164 sensitive electrodes (CleanGrow Europe). The nutrient solution was changed at the start of  
165 the salt stress treatment and no further significant nutrient depletion occurred throughout the  
166 rest of the experiment (10 days).

## 167 Salt stress treatment and considerations

168 The use of 8 units of the hydroponics system allowed the salt treatment of a total of 69  
169 accessions of three different tomato species (*S. cheesmaniae*, *S. galapagense*, and *S.  
170 lycopersicum*), with 6 biological replicates per accession per treatment , plus 6 seedlings per  
171 accession that were harvested as a baseline before treatment. The plants were subjected to salt  
172 stress treatment at the same developmental stage, when the 4<sup>th</sup> leaf started to emerge. Given

173 that *S. lycopersicum* plants were bigger than Galapagos tomatoes throughout all  
174 developmental stages (as leaves were larger and stems were thicker), they were treated when  
175 the 3<sup>rd</sup> leaf started to emerge, to compensate for the size difference (Figure S1), since bigger  
176 plants are often better able to tolerate salt stress than smaller plants.

177 The salt stress treatment was administered gradually to the plants. As each plant reached the  
178 desired developmental stage (i.e. the 4<sup>th</sup> or 3<sup>rd</sup> leaf emergence), it was moved from the  
179 nutrient-only hydroponics systems to the systems supplemented with NaCl. Plants were first  
180 moved to a 75 mM NaCl hydroponic system and 12 hours later, moved to a 200 mM NaCl  
181 hydroponic system. Seedlings remained in the 200 mM NaCl hydroponic system for 10 days.

182 Supplemental CaCl<sub>2</sub> was added to the solutions to compensate for the decrease in Ca<sup>2+</sup>  
183 activity arising from the addition of NaCl (Tester and Davenport, 2003). The amount of  
184 CaCl<sub>2</sub> added to the NaCl solutions was calculated using GEOCHEM-EZ software (Shaff *et*  
185 *al.*, 2010) to maintain Ca<sup>2+</sup> activity at 0.4 mM, which was the normal Ca<sup>2+</sup> activity in the  
186 nutrient solution prior to NaCl addition (Table S1).

#### 187 **Sample collection and recording traits related to salinity tolerance**

188 Plants were photographed and tissues harvested to measure traits related to plant growth, leaf  
189 area, and ion allocation (**Figure 1A**). Photographs of each plant were taken at the start and  
190 end of the salt treatment, using a Photosimile 200 light-box and a Nikon D5100 digital  
191 single-lens reflex camera. The in-camera white balance calibration function was used with a  
192 reference photo of the empty light-box white background, taken at the intended light  
193 intensity. This ensured a consistent and accurate colour capture in all photographs taken. The  
194 images were used to test a non-destructive approach to estimate the salinity tolerance of  
195 Galapagos tomato seedlings. The photographs were processed using a Matlab script for green  
196 pixel count (green\_finder\_V2.m), which can be found in the Supplementary Data. For

197 destructive sample harvesting before and after salt treatment, the plant was carefully  
198 extracted from the pot, roots were rinsed in 10 mM MgCl<sub>2</sub> solution with the excess solution  
199 dried off using tissue paper. Plant root, shoot, and 3<sup>rd</sup> or 4<sup>th</sup> leaf tissues were each weighted  
200 separately. The 3<sup>rd</sup> and 4<sup>th</sup> leaves, taken from *S. lycopersicum* and Galapagos accessions  
201 respectively, were further characterized as they had developed under salt stress conditions.  
202 Each leaf was scanned using an EPSON scanner to calculate leaf area, perimeter, height and  
203 width using the WinFolia software (Regent Instruments Inc.). In terms of physical  
204 measurements, stem thickness was measured at the base using a caliper, while the stem and  
205 root length were measured using a ruler. The different tissues were then stored in paper  
206 envelopes and dried at 60°C for three days to then measure their dry mass. Dry leaf samples  
207 (without petiole) and root samples were digested in 50 mL Falcon tubes with 5 mL of 1%  
208 (v/v) nitric acid in a HotBlock<sup>TM</sup> (Environmental Express) at 80°C for four hours. Sodium  
209 content was measured in leaf and root samples using a flame photometer (model 420;  
210 Sherwood Scientific Ltd., Cambridge, UK).

211 **Data analysis**

212 A multivariate analysis was performed to assess the effect of the salt-induced changes in the  
213 plants under salt stress conditions. The mean of six replicates was calculated for all the  
214 measured traits, with the exception of leaf number, for which the mode was calculated  
215 instead.

216 All traits were corrected by subtracting the initial measurement (before salt stress treatment)  
217 to measure only the differences that occurred during treatment (Figure S2), except for traits  
218 measured in leaves 3 or 4. To facilitate comparison of different species and accessions with  
219 large differences in early growth (Figure S3), only traits of salt-treated plants relative to traits  
220 of control treated plants were used for analysis (Negrão *et al.*, 2017):

= —

221 To determine the salt stress effects on the different accessions, correlations between all of the  
222 traits measured were analysed using the *corrplot* package (Wei and Simko, 2016) in R (R  
223 Core Team, 2017). A total of 11 representative traits were selected for further analyses.

224 The variability in traits related to salinity tolerance was described using a principal  
225 component analysis (PCA) on a matrix of 11 traits × 64 Galapagos and commercial tomato  
226 accessions. Note that of the 67 accessions screened, five had no survivors to the salt stress  
227 treatment: LA0526, LA0930, LA1141, LA1411, and LA1815. Since the variables have  
228 different units, they were scaled to have a variance of 1 and a mean of 0, by subtracting the  
229 mean and dividing by the standard deviation, using the *scale* function in R. PCA analysis was  
230 carried out using the *FactoMineR* package (Lê *et al.*, 2008) in R (R Core Team, 2017).

231 The two Galapagos tomato species were observed to have distinctly different morphologies,  
232 hence the phenotypic data for each species were analyzed separately. The K-means clustering  
233 method (MacQueen, 1967) was used to reveal groups within the data. The clustering method  
234 was run with different numbers of clusters (2 to 6) and it was found that two clusters  
235 provided the most interpretable output, in terms of accession clustering by traits. The  
236 accessions were grouped based on 11 traits related to salinity tolerance: Na and K  
237 concentration in root and leaf, leaf area, leaf elongation (width/length), leaf succulence (leaf  
238 water/leaf area), leaf number, stem and root length, and total fresh mass. K-means were  
239 calculated using the *stats* package in R (R Core Team, 2017).

240 To identify possible tolerance mechanisms, all trait measurements from the accessions of  
241 each species were compared using a heat map, drawn by the function *heatplot* of the *made4* R  
242 package (Culhane *et al.*, 2005), which also draws dendograms of the traits and accessions  
243 using correlation similarity metric and average linkage hierarchical clustering (Eisen *et al.*,  
244 1998).

245 All the phenotypic data was integrated into an Isla\_Tomate App, available at  
246 [https://mmjulkowska.shinyapps.io/La\\_isla\\_de\\_tomato/](https://mmjulkowska.shinyapps.io/La_isla_de_tomato/). The App allows interactive  
247 exploration of the correlations between individual traits as well as cluster analysis of the  
248 accessions based on the chosen traits. The App was developed with the shinyapp package.  
249 The code used for the App is available at  
250 [https://github.com/mmjulkowska/La\\_isla\\_de\\_tomato](https://github.com/mmjulkowska/La_isla_de_tomato), and the instructions on how to use the  
251 App can be found at [https://mmjulkowska.github.io/La\\_isla\\_de\\_tomato/](https://mmjulkowska.github.io/La_isla_de_tomato/).

252

## 253 **Results**

254 **Galapagos tomatoes are more salt tolerant than the commercial tomato varieties tested**  
255 As suggested by Negrão et al. (2016), responses to salinity stress were measured only for the  
256 time when the plants were stressed (by taking measurements before and after the stress  
257 treatment). *S. cheesmaniae* and *S. galapagense* accessions were better able to maintain  
258 growth (based on dry mass) during the salt stress period than the *S. lycopersicum* varieties  
259 tested (**Figure 2**). This effect is less apparent when biomass is determined only at the  
260 endpoint of the experiment (because of the size advantage of *S. lycopersicum* accessions)  
261 (Figure S2). There was a large variation in salinity tolerance between accessions, ranging  
262 from a difference in dry mass in saline conditions relative to control conditions of 12 to 55%  
263 (**Figure 2**).

## 264 **Correlation analysis of different seedling traits revealed trait groups**

265 The correlation matrix (**Figure 3**) shows that leaf traits such as perimeter, vertical length, dry  
266 and fresh mass, horizontal width and area are all positively and significantly correlated  
267 (correlation coefficients 0.79-0.93, *p*-value=0.001: Figure S4). Plant growth-related traits,  
268 such as shoot fresh and dry mass, root fresh and dry mass, total fresh and dry mass and total

269 water content, are also positively and significantly correlated (correlation coefficients 0.69-  
270 1.00,  $p$ -value=0.001) (Figure S4).

271 Interestingly, leaf K concentration in salt-treated plants relative to control plants is negatively  
272 correlated with all the leaf traits, some plant growth-related traits, and Na and K in the root.  
273 On the other hand, leaf Na concentration in salt-treated plants, relative to control plants, did  
274 not have a significant correlation with any other of the measured traits. In most cases, leaf Na  
275 concentration in control plants was negligible, so the leaf Na concentration in salt-treated  
276 plants, relative to control plants, was very similar to the leaf Na concentration in salt-treated  
277 plants. Na and K in the root have a slight positive correlation with leaf and plant growth-  
278 related traits.

279 Green pixel count was significantly correlated with most of the salinity-tolerance related  
280 traits (**Figure 3**). For most of the accessions tested, the green pixel count had a positive  
281 correlation with the shoot fresh mass of salt-treated plants relative to control plants (Figure  
282 S5A). However, green pixel count proved a more useful measure of plant growth in *S.*  
283 *cheesmaniae* ( $r^2=0.85$ ), than in *S. galapagense* ( $r^2=0.64$ ) (Figure S5B-D). Based on the  
284 correlation analyses, a set of plant traits representing each of the groups of traits that have  
285 been identified as potentially useful predictors of salinity tolerance in plants, were selected  
286 for further analyses: Na and K concentration in root and leaf, leaf area, leaf elongation, leaf  
287 succulence, leaf number, stem and root length, and total fresh mass. To compare the different  
288 species, the traits in salt stress relative to control conditions of the same accession were used.

#### 289 **Principal component analysis revealed selected traits tendencies and contributions**

290 A PCA was performed to reduce data dimensionality and reveal the potential relationships  
291 among representative salinity-tolerance traits. In this study, the four main PCA axes had  
292 eigenvalues larger than 1 (Table S2), which indicates that each principal component (PC)

293 accounts for more variance than accounted-for by one of the original variables in the  
294 standardized data. This was used as a cut-off to determine the number of PCs to retain.

295 The PC1 explained 33.8% of the total variability between traits/individuals and was  
296 associated with most traits, except leaf Na concentration and leaf succulence (**Table 1 and**  
297 **Figure 4**). The most significant trait for PC1 was the total fresh mass (**Table 1**). The  
298 accessions at the lower end of PC1 are those whose growth was most affected by salinity but  
299 were still able to retain high levels of K in the leaf, while at the higher end, there are the  
300 accessions with higher levels of plant growth, leaf area, leaf number, and stem and root  
301 length.

302 PC2 accounted for an additional 15% of the total variability among seedling traits and  
303 appeared to be related to the ion content and some growth traits (**Table 1 and Figure 4**). The  
304 accessions with succulent leaves and higher accumulation of Na in the leaf were located at  
305 the lower end of PC2, while those with increased leaf number and K retention in the leaf  
306 were located at the higher end of PC2. The PC2 also divided the root Na concentration and  
307 root length, where those accessions with high Na concentration in the root had the shortest  
308 root.

309 PC3 accounted for 11.7% of the total variability among salinity tolerance-related traits. It was  
310 significantly associated with total fresh mass but had a stronger association with leaf traits,  
311 such as elongation factor (length/width) and Na concentration (**Table 1**). This could suggest  
312 that Na concentration in the leaf is independent of the other traits.

313 PC4 accounted for an additional 10% of the total variability and is significantly associated  
314 with Na and K accumulation in the leaf and root, but also, with leaf number and area, and  
315 root length (**Table 1**).

316 Overall, the PCA indicates that in this experiment, the primary traits that varied and  
317 correlated with each other were total fresh mass, ion content and some leaf traits, such as  
318 elongation factor.

319 **Cluster analysis suggests that salinity tolerance at the seedling stage is best defined by**  
320 **the ability of the plant to maintain growth under salt stress conditions**

321 Cluster analysis is a suitable method to study large datasets, involving multiple variables. It  
322 allows the grouping of accessions with similar traits and the recognition of hidden patterns or  
323 trends in the data. To analyse how the different accessions grouped by traits related to salinity  
324 tolerance and to see if any of the traits predominantly explain the overall variation.

325 The K-means cluster analysis (MacQueen, 1967) of the surviving 38 accessions of *S.*  
326 *cheesmaniae* and 24 accessions of *S. galapagense* after treatment, was used to classify  
327 accessions into different clusters (K), where the accessions within the same cluster are as  
328 similar as possible, while accessions from different clusters are as dissimilar as possible. The  
329 number of clusters K=2 was chosen with the aim of separating the most tolerant accessions  
330 from the least tolerant while considering some salinity-tolerance related traits. A total of 11  
331 non-redundant traits were selected, based on the strong correlation of these 11 traits with  
332 other traits measured but not among each other (**Figure 3**).

333 Considering the values of the selected traits, the Euclidean distance between each accession  
334 and the cluster mean was calculated to assign the accession to the nearest cluster. A new  
335 mean value of each cluster was calculated after an accession was assigned to it and every  
336 accession was checked again to see if they were closer to a different cluster. These steps were  
337 iteratively repeated until convergence was achieved.

338 Bar plots were used to visualize the distribution of the accessions by cluster for each specific  
339 trait, a similar visualization strategy is commonly used when plotting Q-matrices and

340 identifying K clusters in population structure studies (Pritchard *et al.*, 2000). The accessions  
341 were arranged in descending order and the bars are colored by cluster (Figure S6). By  
342 visualizing bar plots for all traits, it was easy to identify that the plant fresh mass was  
343 predominantly defining the clustering by K=2. From this, it was observed that the accessions  
344 of both species were best grouped by their fresh mass production under salt stress relative to  
345 control conditions (Figure S6). Thus, the two clusters divide the accessions of each species of  
346 Galapagos tomato into those with high tolerance and low tolerance to salinity, in terms of  
347 growth maintenance (**Figure 5**). Cluster 1 included accessions with higher fresh mass  
348 production during salt stress relative to control, indicative of their ability to better maintain  
349 growth under salt stress. Cluster 1 of *S. cheesmaniae* had 23 members (**Figure 5A**) and  
350 cluster 1 of *S. galapagense* had 14 members (**Figure 5B**).

351 **Natural variation exists across Galapagos tomato accessions in terms of their salinity  
352 tolerance mechanisms**

353 Phenotypic data were also analyzed using a hierarchical clustering approach (**Figure 6**),  
354 which was found to be more complex, but more informative, than the K-means clustering  
355 method. A heat map paired with the dendrogram obtained by hierarchical clustering, provide  
356 a way to visualize and simplify large datasets. This method is routinely used for gene  
357 expression data analysis (Eisen *et al.*, 1998) and metabolomics (Tikunov *et al.*, 2005). More  
358 recently, it has proven useful also to analyse genotypes and traits interactions (Chen *et al.*,  
359 2014; Julkowska *et al.*, 2016; Clark, 2016; Awlia *et al.*, 2016).

360 The heat map divided the accessions into two clusters, also defined by fresh mass in salt-  
361 treated plants relative to control plants. Moreover, the heat map showed clustering of  
362 accessions based on other traits within the main mass-related clusters (i.e. K and Na  
363 concentrations in both leaf and root differed greatly between groups of accessions, despite

364 them having similar plant fresh mass). This indicates a pronounced natural variation in  
365 salinity tolerance mechanisms within the Galapagos tomato collection.

366 In *S. cheesmaniae*, the hierarchical clustering method (**Figure 6A**) separated the accessions  
367 into two main clusters, which display contrasting values of fresh mass, leaf area, stem length,  
368 leaf number, root length, leaf elongation, leaf Na and K concentration and green pixel count.  
369 Within the cluster of accessions with high relative fresh mass, five different clusters could be  
370 distinguished, that differed in leaf succulence, root K concentrations, root length, leaf Na<sup>+</sup>  
371 concentration and leaf elongation (**Table 2**).

372 The *S. galapagense* accessions also separated into two clusters (**Figure 6B**), based on their  
373 relative fresh mass, leaf area, root Na, root K, leaf K, and green pixel count. The cluster with  
374 high relative fresh mass was divided into two clusters differing in leaf succulence, leaf Na  
375 concentration and root length (**Table 2**).

376 The phenotypic data collected were integrated into a Shiny App: Isla\_Tomate,  
377 [https://mmjulkowska.shinyapps.io/La\\_isla\\_de\\_tomato/](https://mmjulkowska.shinyapps.io/La_isla_de_tomato/), allowing interactive clustering of the  
378 data. The identified clusters can be validated using the Isla\_Tomate App, by grouping the  
379 accessions into clusters, based on the chosen trait and examining the significant differences  
380 between the clusters based on each trait. Significance was calculated using Tukey pair-wise  
381 comparison with p-value < 0.05. The data for both species showed that clustering by plant  
382 fresh mass forms two significant groups (Figure S7), which we can divide into the two  
383 groups of high and low salinity-tolerance accessions.

384 The dendograms presented in **Figure 6** represent how similar individual accessions react to  
385 salt, based on the selected traits. When this grouping of the accessions was compared to their  
386 geographical origin or genetic distance between them (Pailles *et al.*, 2017), no correlation

387 was observed. This suggests that geographical origin and evolutionary history do not  
388 influence salinity tolerance mechanisms.

389 The two types of trait association analyses (PCA and clustering analyses) indicated similar  
390 trait influences determining differences between accessions and their responses to salinity.

391

## 392 **Discussion**

393 Wild relatives of modern crops surely possess useful traits that can potentially improve plant  
394 performance under salt stress. Hence, there is a need for characterizing and screening the  
395 available germplasm. However, their wild nature and great natural variation in many traits  
396 make them difficult to study quantitatively with conventional methods. The specific  
397 mechanisms through which Galapagos tomatoes are tolerant to salinity are not known.  
398 Identification of salinity tolerance mechanisms in Galapagos tomatoes will facilitate the  
399 improvement of salinity tolerance in current tomato elite varieties.

400 In this study, most available accessions of wild tomatoes endemic to the Galapagos Islands  
401 were screened for salinity tolerance traits. The first objective was to develop an efficient  
402 screening method that allowed the quantitative comparison of salt stress responses of  
403 different wild tomato seedlings. For this, a commercial ebb-and-flow supported hydroponics  
404 system was used. The use of hydroponics for experimental screenings allows better control of  
405 the growth media, stress exposure and experimental reproducibility (Genc et al. 2007, Munns  
406 et al. 2010). In this case, the hydroponics system facilitated an even delivery of NaCl to the  
407 plants at the root level. The plastic-beads substrate conserved moisture during drainage,  
408 supported the roots, and protected them from breaking and allowing uncontrolled  $\text{Na}^+$  influx  
409 into the root system (Miller, 1987). An opaque substrate was preferred to simulate the light  
410 blocking properties of soil and to limit algal growth. The system's flexibility allowed

411 treatment at the same developmental stage, independent of the growth rate of each of the  
412 different genotypes. This is necessary for large-scale experiments with a large number of  
413 genotypes from different species that have widely different rates of growth.

414 The second objective was to determine the most informative traits indicating salinity  
415 tolerance in Galapagos tomato seedlings while highlighting the most tolerant accessions and  
416 their potential underlying mechanisms for salinity tolerance. It is widely known that salt  
417 stress affects many aspects of plant growth, such as biomass production, yield,  
418 photosynthesis and leaf metabolites (Chinnusamy *et al.*, 2006; Munns and Tester, 2008;  
419 Munns and Gillham, 2015; Negrão *et al.*, 2017). Hence, there are many traits that could be  
420 recorded and analyzed to accurately assess the salinity tolerance of a plant. In this study, it  
421 was found that many of the traits were highly and significantly correlated, so the salinity  
422 tolerance could be assessed by focusing attention on only a few representative traits. From  
423 the highly-correlated leaf-related traits, leaf area was chosen as it was previously reported to  
424 be an important trait for salinity tolerance in tomato (Cuartero and Fernández-Muñoz, 1998;  
425 Dogan *et al.*, 2010). From the highly-correlated plant growth-related traits, the total fresh  
426 mass was chosen since it describes the increase in biomass and water retention at the whole  
427 plant level. Other traits, which did not correlate as strongly with the leaf- or growth-related  
428 traits, such as, root and stem length, leaf number, leaf elongation, leaf succulence, and Na and  
429 K concentrations in root and leaf, were included in further analyses. Leaf K was the only trait  
430 which negatively correlated with multiple traits. In general, the two Galapagos tomato  
431 species, *S. cheesmaniae* and *S. galapagense*, did not differentiate in PCA. This indicated high  
432 phenotypic variability within both species that was greater than differences between the two  
433 species, which are clear at the genetic level (Pailles *et al.*, 2017).

434 The PCA revealed that the selected traits contributing most to describe the variation across  
435 accessions were total fresh mass, leaf area, leaf succulence, and leaf K concentration. Plants

436 with greater fresh mass and leaf area shared a tendency to have more leaves, longer stems and  
437 roots, and higher root K concentration, whereas leaf succulence, leaf elongation, K and Na  
438 concentrations in leaf, and root Na concentration tendencies differed greatly. The presence of  
439 similar tendencies in the two species suggests that salinity-tolerance related traits are  
440 conserved across these two species.

441 Total plant fresh mass, which is a measure of growth maintenance during salt stress, was the  
442 trait driving most of the variation across accessions. Growth maintenance has been widely  
443 acknowledged to be a good estimate of salinity tolerance (Genc *et al.*, 2007; Negrão *et al.*,  
444 2017), especially at the seedling stage, since it is not possible in young plants to measure any  
445 commercially relevant traits such as yield. Thus, the genotypic variability for salinity  
446 tolerance was assessed in this study, based on the maintenance of growth under saline  
447 conditions relative to control conditions. This assumption was further confirmed by K-means  
448 clustering analysis, where the plant fresh mass of salt-treated plants relative to control plants  
449 was identified as the main trait driving the accessions clustering. The accessions were  
450 categorized into two groups with high or low salinity tolerance, based on the relative fresh  
451 mass. Within these groups, it was tested whether Galapagos tomato accessions showed co-  
452 varying groups of tolerance traits that are consistent across accessions, to categorize them as  
453 traits of influence in salinity tolerance. However, remarkable trait diversity was found within  
454 the highly-tolerant group, which suggests the presence of different mechanisms of salinity  
455 tolerance between accessions within the species, consistent with previous reports by Rush  
456 and Epstein (1981) and Cuartero *et al.* (1992).

457 Maintenance of growth, defined by an increase in mass, is one of the most important  
458 mechanisms contributing to salinity tolerance. According to Munns and Termaat (1986), leaf  
459 growth is more affected by salinity than root growth. In this study, leaf area was also  
460 observed to be an important trait contributing to plant mass. However, the physiological

461 mechanisms underlying leaf growth inhibition under salt stress are not fully understood  
462 (Neves-Piestun and Bernstein, 2001). The current results showed a strong and significant  
463 correlation between leaf area and leaf elongation, followed by leaf area and leaf number.  
464 These found correlations could provide insights into the mechanisms by which salt stress  
465 affects leaf growth.

466 K concentration was found to be generally lower in all the high-tolerance accessions when  
467 compared to the low-tolerance accessions, especially in leaves. However, one group of *S.*  
468 *cheesmaniae* tolerant accessions showed higher levels of potassium in the root and leaf  
469 samples. Percy et al. (2016) reported that reduction in K<sup>+</sup> efflux in halophytes is linked to  
470 reduced H<sup>+</sup> efflux, which saves energy, allowing more resources to be redirected for plant  
471 growth. Therefore, the ability to maintain K<sup>+</sup> uptake and a high K<sup>+</sup>:Na<sup>+</sup> ratio under salt stress  
472 can be an important mechanism of salinity tolerance (Chen *et al.*, 2007; Shabala and Cuin,  
473 2008). K<sup>+</sup> deficiency in plants can impair photosynthesis (Cakmak, 2005), as well as many  
474 other aspects of cellular function, such as protein synthesis (Flowers and Dalmond, 1992).  
475 The four most salt tolerant accessions of *S. cheesmaniae* and the two most salt tolerant  
476 accessions of *S. galapagense* showed high K in their roots, which could indicate that they are  
477 good at maintaining K uptake under salt stress. In some accessions, higher K in the roots  
478 seems to go hand in hand with low K in the leaves, which could be explained by higher K<sup>+</sup>  
479 re-translocation, useful to assist NO<sub>3</sub><sup>-</sup> uptake and distribution (Taleisnik and Grunberg 1994),  
480 or lower K<sup>+</sup> translocation from roots to shoot. In addition, bigger leaves appeared to have a  
481 lower K concentration compared to smaller leaves, which could be due to a dilution effect,  
482 e.g. having a similar amount of K to that of the smaller leaves but more water content (Jarrell  
483 and Beverly, 1981).

484 Increase in leaf succulence (measured as water per unit leaf area), a strategy to reduce salt  
485 concentrations in photosynthetic tissues (Han *et al.*, 2013), is another known mechanism of

486 salinity tolerance in some plants, including tomato (Cuartero and Fernández-Muñoz, 1998).  
487 The hierarchical clustering of accessions and traits showed that both *S. cheesmaniae* and *S.*  
488 *galapagense* accessions each formed a cluster of accessions with increased leaf succulence  
489 and low leaf Na concentrations. This might be caused by the succulence increasing cell size,  
490 thereby diluting the salt without increasing the leaf (Munns *et al.*, 2016).

491 The accumulation of Na<sup>+</sup> relative to biomass can also be an indicator of salinity tolerance.  
492 However, Na<sup>+</sup> is toxic when it accumulates in the cell cytosol, resulting in ionic  
493 disequilibrium (Hanin *et al.*, 2016). Additionally, Na<sup>+</sup> reduces the availability of K<sup>+</sup> binding  
494 sites for important metabolic processes in the cytoplasm (Wei *et al.*, 2017). For the plant to  
495 protect itself when exposed to salt stress, it has to either limit the entry of Na<sup>+</sup> through the  
496 roots, or to control Na<sup>+</sup> concentration and distribution once inside (Tester and Davenport,  
497 2003; Hanin *et al.*, 2016). The Na<sup>+</sup> that enters the root cells is extruded from the cytoplasm  
498 into the apoplastic space and compartmentalized into the vacuole (Maggio *et al.*, 2007). This  
499 process is called tissue tolerance (Munns *et al.*, 2016). One cluster from each species  
500 included tolerant accessions with high Na concentration in their leaves, which suggests  
501 significant levels of tissue tolerance (Munns *et al.* 2016). The *S. cheesmaniae* cluster  
502 appeared to respond to high Na accumulation in the leaves by growing more leaves, while the  
503 *S. galapagense* cluster appeared to increase leaf succulence.

504 In conclusion, the seedling screen study allowed characterization of the responses of 67  
505 Galapagos tomato accessions to salt stress. Individual accessions were classified based on the  
506 phenotypic traits contributing to salinity tolerance. Interestingly, it was observed that  
507 individual salt tolerant accessions from the Galapagos Islands use different mechanisms to  
508 maintain their growth at the seedling stage under saline conditions. The different  
509 combinations of characteristics found across all the studied accessions, while maintaining a  
510 good relative fresh mass, indicate that the Galapagos tomatoes are naturally diverse and have

511 different mechanisms to tolerate high salinity. In terms of growth maintenance under stress,  
512 the accessions LA0317, LA1449 and LA1403 displayed exceptional salinity tolerance at the  
513 seedling stage. However, to assess which mechanisms are the most effective for salinity  
514 tolerance, the tolerant accessions should be studied further at later growth stages, such as the  
515 reproductive stage, to evaluate the effect of salinity on yield. Additionally, trials to evaluate  
516 their performance under field conditions are recommended. Dissecting the genetic basis of  
517 salinity tolerance mechanisms through a genetic characterization and/or transcriptomic  
518 approach, together with our results, would facilitate the selection of useful accessions as  
519 genetic sources for breeding salinity tolerance traits into commercial tomatoes.

520

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## Tables

**Table 1: Contributions of plant traits to the four main PCA axes (eigenvalue > 1), obtained from a matrix of 11 traits × 64 Galapagos and commercial tomato accessions.**

**Values are ranked in order of magnitude in PC1. Traits significantly correlated to each PCA axis ( $\alpha = 0.05$ ) are indicated in bold. All the traits represent the trait value under salt stress relative to control conditions.**

Traits in salt stress relative to control conditions	PC1 (33.8%)	PC2 (15%)	PC3 (11.7%)	PC4 (9.69%)
Plant fresh mass	<b>18.1</b>	1.63	3.83	0.69
Leaf area	<b>15.8</b>	0.09	1.40	4.24
Green pixels	<b>12.7</b>	<b>5.43</b>	<b>12.7</b>	2.36
Stem length	<b>10.4</b>	0.42	0.92	<b>6.06</b>
Leaf K <sup>+</sup>	<b>9.76</b>	<b>12.5</b>	<b>6.83</b>	<b>8.04</b>
Root K <sup>+</sup>	<b>8.12</b>	0.39	0.54	<b>21.9</b>
Leaf number	<b>8.08</b>	<b>4.47</b>	<b>8.66</b>	<b>12.0</b>
Leaf elongation	<b>7.77</b>	0.45	<b>10.0</b>	<b>23.3</b>
Root Na <sup>+</sup>	<b>5.72</b>	<b>12.8</b>	<b>4.39</b>	<b>8.92</b>
Root length	<b>2.65</b>	<b>15.4</b>	<b>8.19</b>	<b>8.38</b>
Leaf succulence	0.50	<b>35.5</b>	<b>5.44</b>	0.01
Leaf Na <sup>+</sup>	0.34	<b>10.8</b>	<b>37.0</b>	3.98

**Table 2: General description of the main mechanisms identified in each of the clusters.**

Species	Highly tolerant accessions cluster	High maintenance of particular feature in saline conditions:
<i>S. cheesmaniae</i>	1	Leaf water
	2	Root K <sup>+</sup>
	3	Root length
	4	Leaf Na <sup>+</sup> exclusion
	5	Leaf elongation and Na <sup>+</sup> compartmentalization
<i>S. galapagense</i>	1	Leaf water
	2	Leaf Na <sup>+</sup> exclusion and root length

## Figure Legends

**Figure 1: Screening system description.** A. Workflow from germination to final harvest: one seed on an agar plug, two agar plugs per pot, pots filled with plastic beads. After germination and thinning to only one plant per pot, they were moved to grow in hydroponics. After 10 days of salt stress, the plants were harvested to record the effects of salinity on their physiology. Recorded traits are listed. B. Supported hydroponics system for screening plants for salinity tolerance at the seedling stage. Diagram adapted from Kruger and Doyle (2016). Pots are held on top of the grow tray. A 100 L reservoir tank with nutrient or saline solution is located under the grow tray and a pump is used to feed the solution up to the grow tray. The pump is controlled by a timer, programmed for 15 min ON/OFF intervals. Solution floods the grow tray for 15 minutes and drains back into the reservoir tank when the pump turns off for another 15 min.

**Figure 2: Salinity tolerance across the studied accessions**, measured as the difference in dry mass between the start and end of the treatment of plants grown in saline conditions relative to plants grown in control conditions. Different colours represent the different species, *S. cheesmaniae* (coral), *S. galapagense* (green), *S. lycopersicum* (blue).

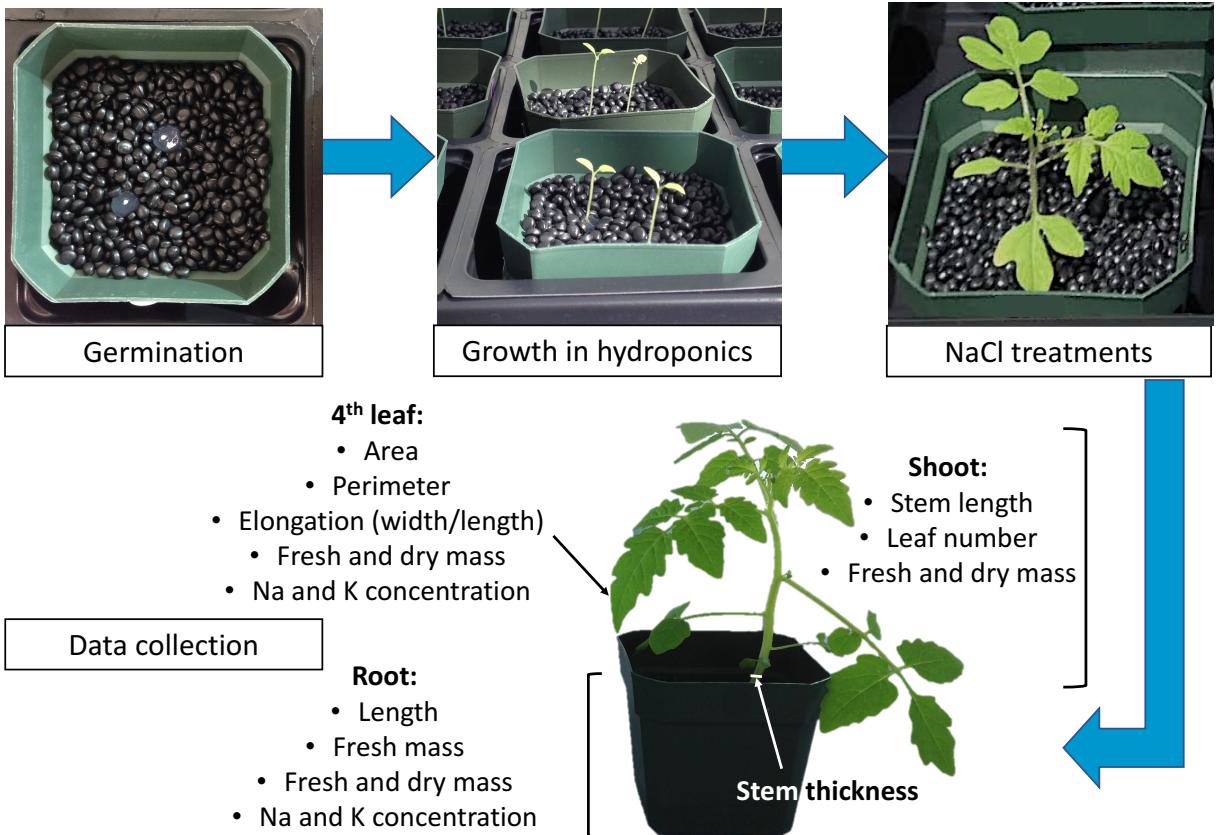
**Figure 3: Pearson correlation matrix of the recorded traits in salinity relative to control conditions.** Large circles represent strong correlations and small circles represent weak correlations. The color scale bar indicates the sort of correlation, where 1 denotes completely positive correlation (dark blue) and -1 denotes completely negative correlation (dark red) between two traits. Only significant correlations are shown ( $p$  value $<0.05$ ).

**Figure 4: PCA biplot of 62 Galapagos tomato accessions and 2 commercial tomato varieties, based on the variance in 11 salt-stress related physiological traits, explained by two principal component axes.** The two components explained 33.8% and 15% of the variance, respectively. Arrows denote the strength of the trait influence on the first two PCs. The transparency of the arrows indicates the contribution to the variance in the dataset, ranging from 5% (lightest) to 12.5% (darkest). The direction and length of the arrows indicate how each trait contributes to the first two components in the PCA. Aligned vectors indicate a strong positive correlation between the two traits. Vectors at right angles/opposites indicate no correlation/negative correlation, respectively. This analysis shows that all growth-related traits are correlated and places the individual accessions where the corresponding. The first component shows that leaf K concentration is negatively correlated with the other representative traits. The second component shows leaf K concentration is negatively correlated with the other representative traits. Individual accessions are placed on the ordination plane. Different colours and symbols represent the different species, *S. cheesmaniae* ( $\diamond$  - coral), *S. galapagense* ( $\blacktriangleright$  - green), *S. lycopersicum* ( $\square$  - blue).

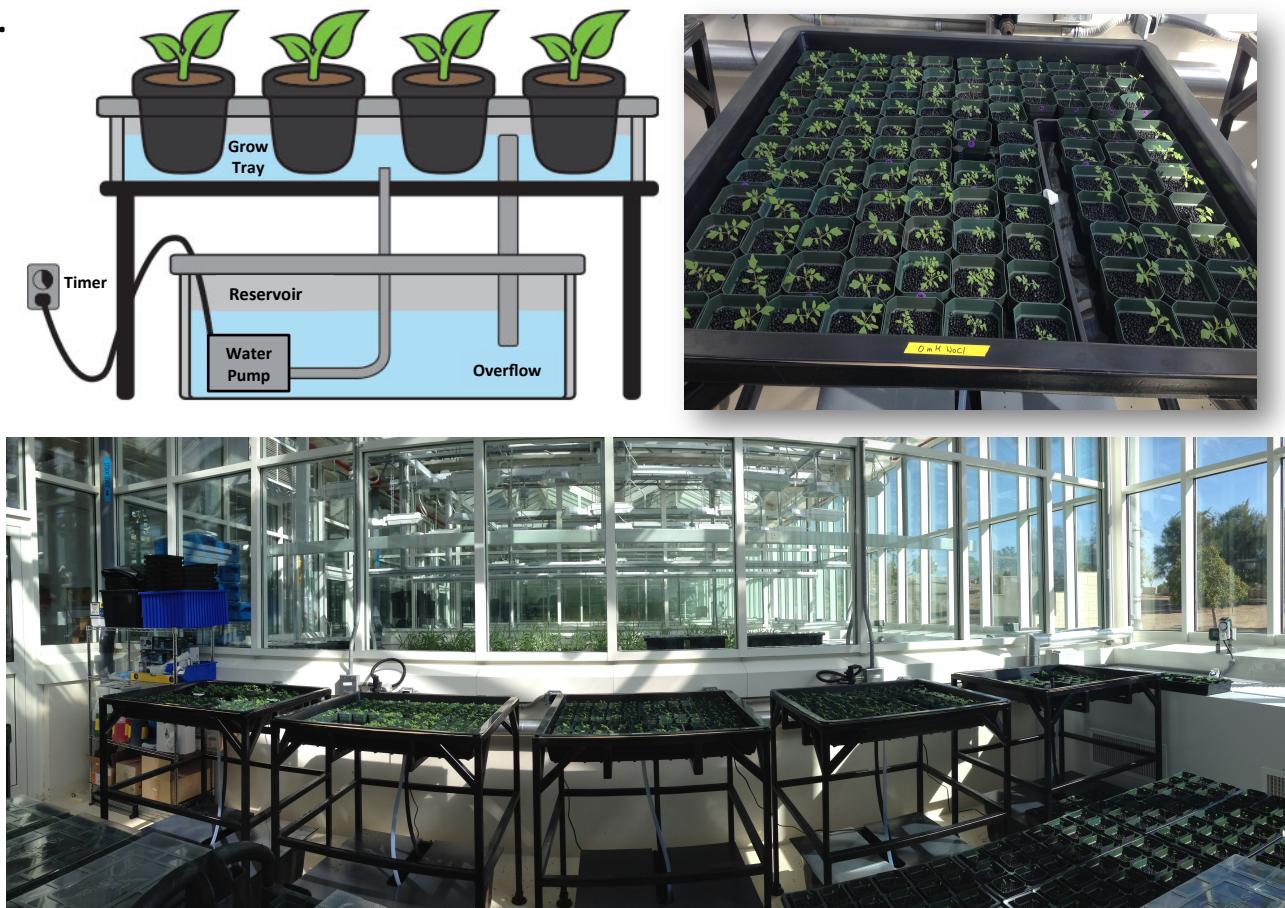
**Figure 5: K-means clustering (K=2) of each one of the Galapagos tomato species.** A barplot for each trait was plotted with the accessions in descending order, different colors represent different cluster assignment. Cluster number was chosen based on the most informative grouping. A. *S. cheesmaniae* within-cluster sum of squares by cluster: 217.6 and 136.9 respectively (between\_SS / total\_SS = 20.2%). B. *S. galapagense* within-cluster sum of squares by cluster: 115.9 and 94.1 respectively (between\_SS / total\_SS = 23.9%). Both species showed a clean cluster separation when accessions were arranged in descending order by the total plant fresh mass in salt stress relative to control conditions.

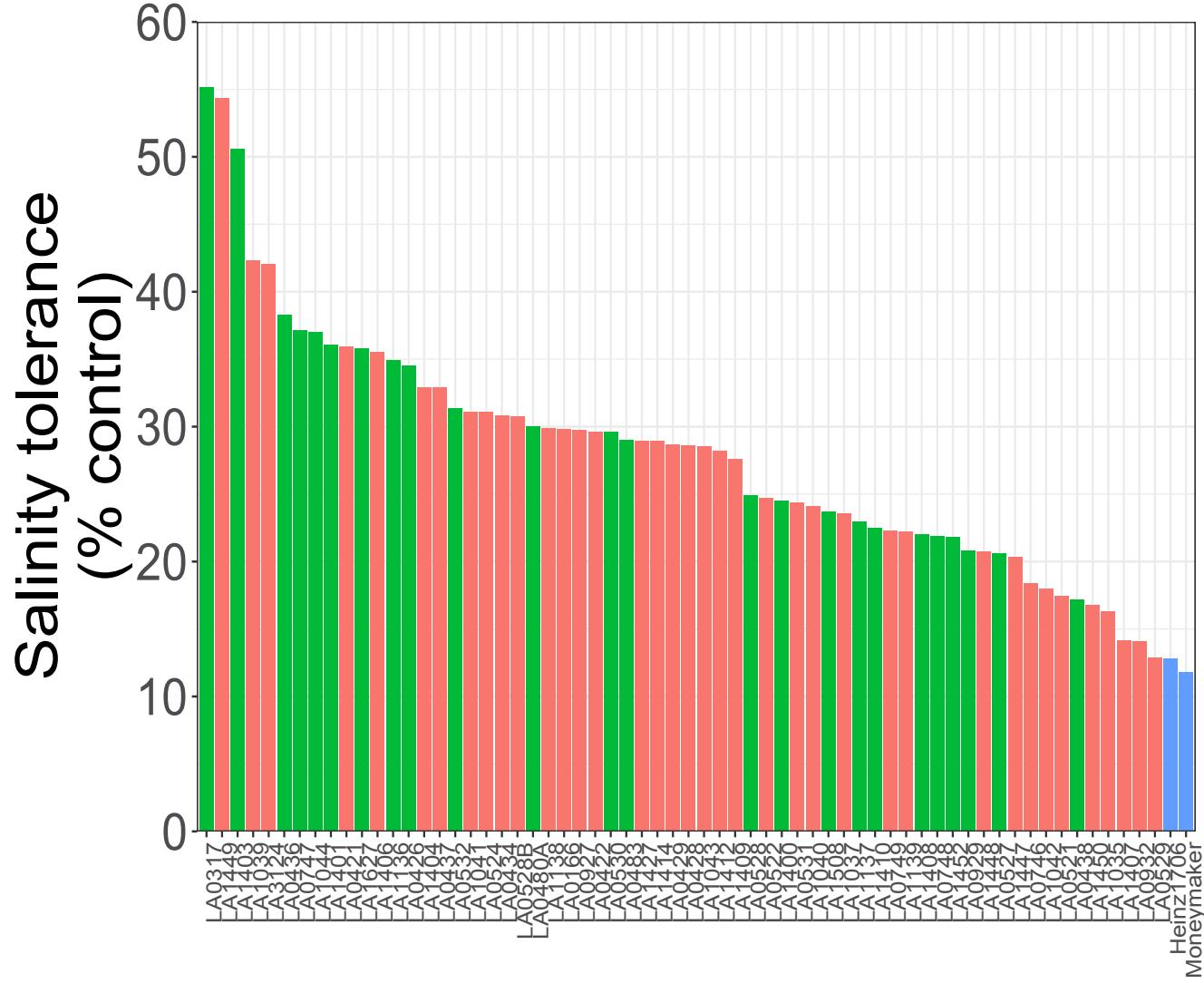
**Figure 6: Hierarchical clustering and heat map of all accessions and important salinity-tolerance related traits, divided by species.** Each column represents a trait and each row represents an accession. Accessions with large fresh mass, in salt stress relative to control conditions, clustered together. However, they differed in other traits. Further clustering by the similarity in traits is indicated in the figure, showing diversity in salt stress responses among Galapagos tomato accessions. A. *S. cheesmaniae* accessions divide into two main clusters. The accessions in the upper cluster share high values of total fresh mass. This cluster is further divided into 5 other clusters (indicated in the figure) with a distinctive trait that differentiates each cluster from other clusters (**Table 2**). B. *S. galapagense* accessions divide into two main clusters. The accessions in the lower cluster share high values of total fresh mass. This cluster is further divided into 2 other clusters (indicated in the figure) with a distinctive trait that differentiates each cluster from other clusters (**Table 2**).

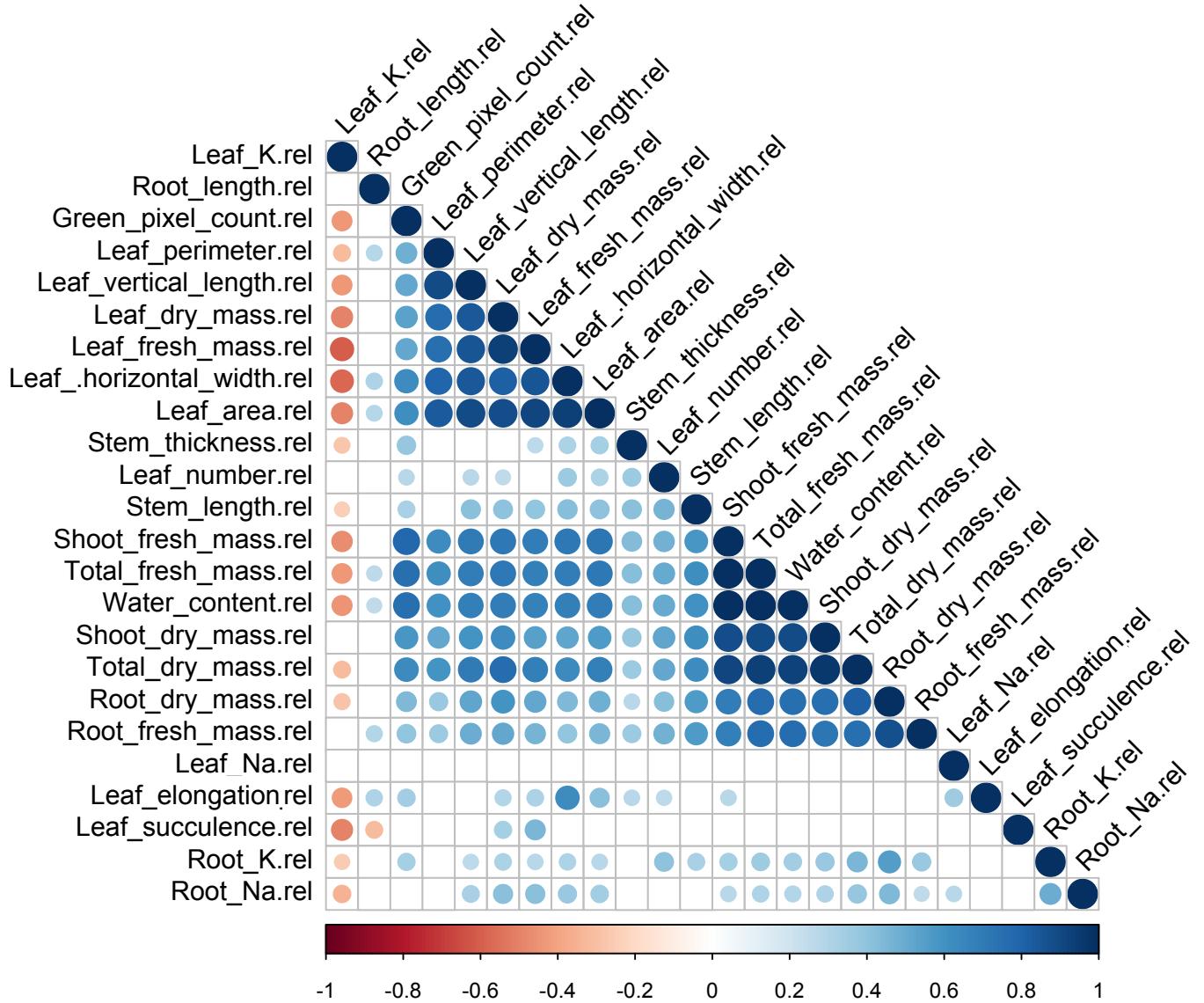
A.

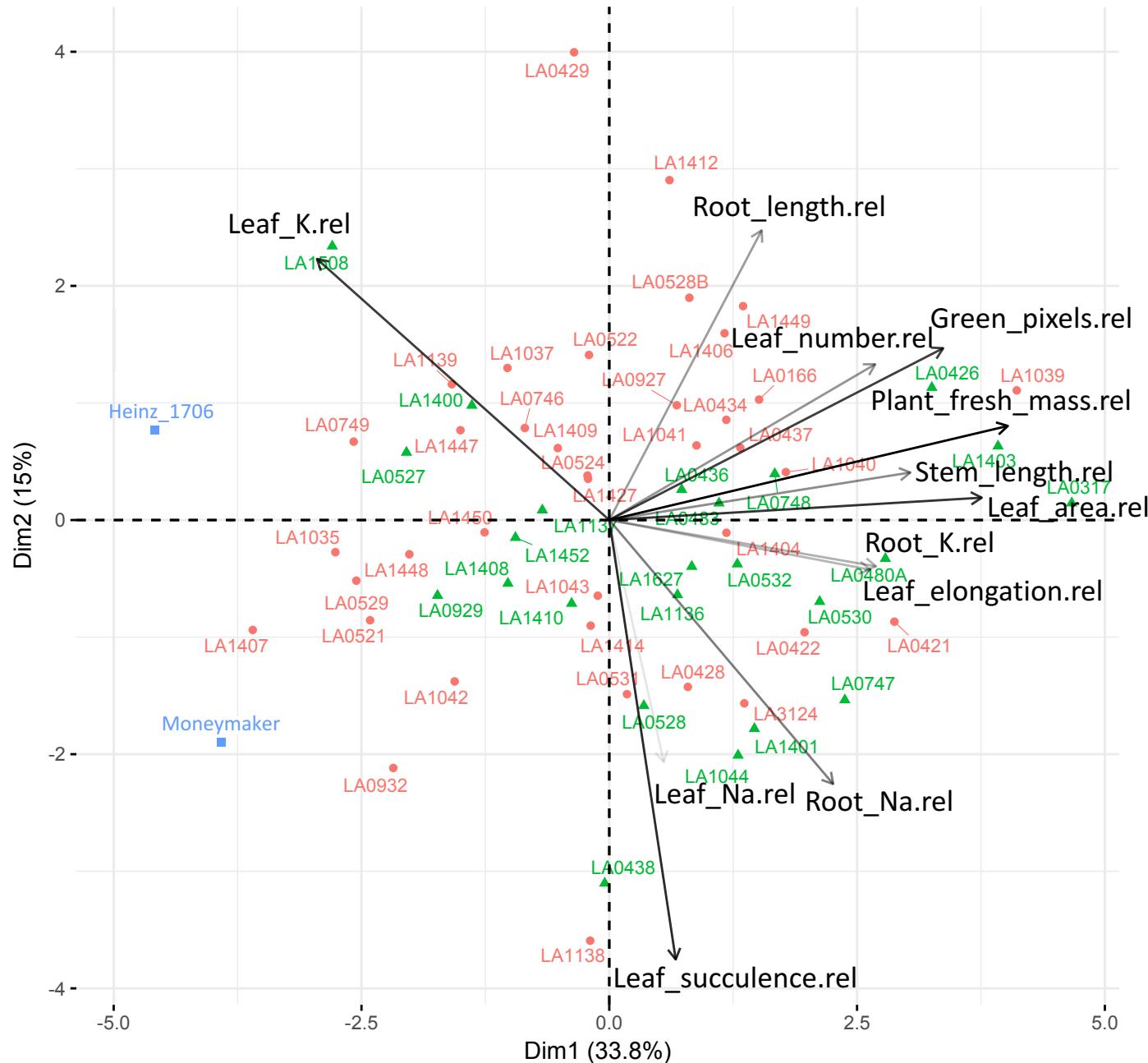


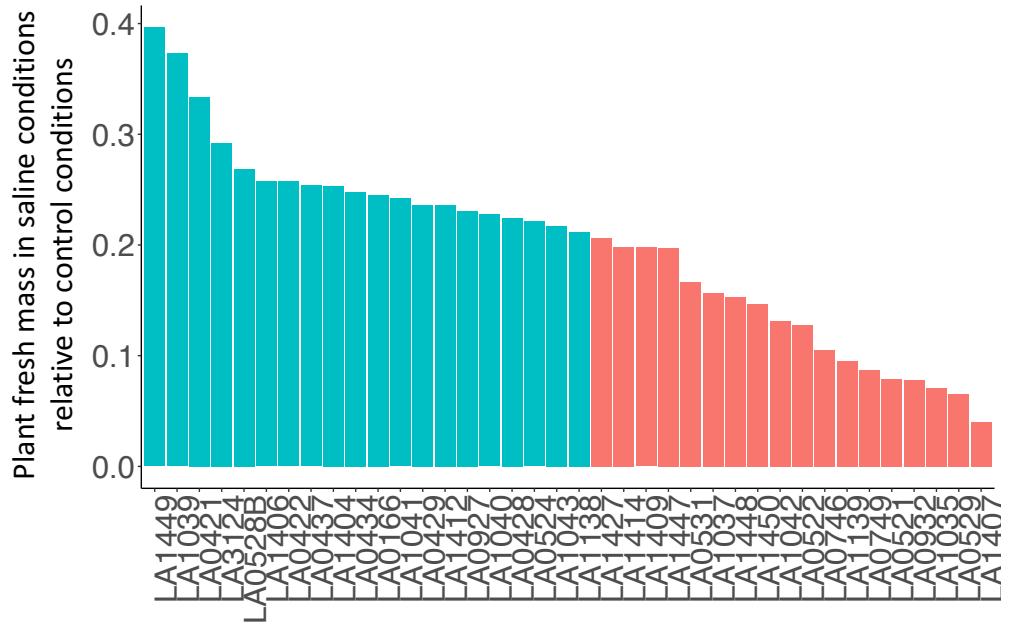
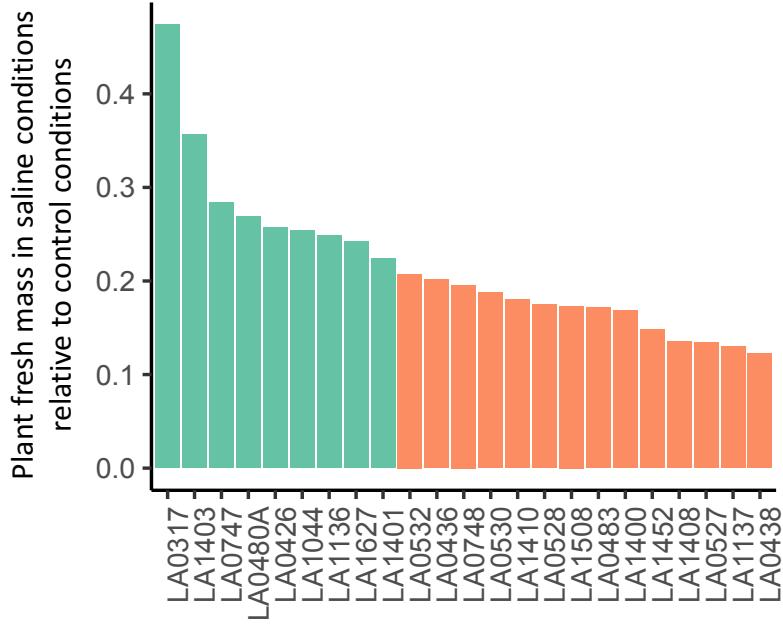
B.

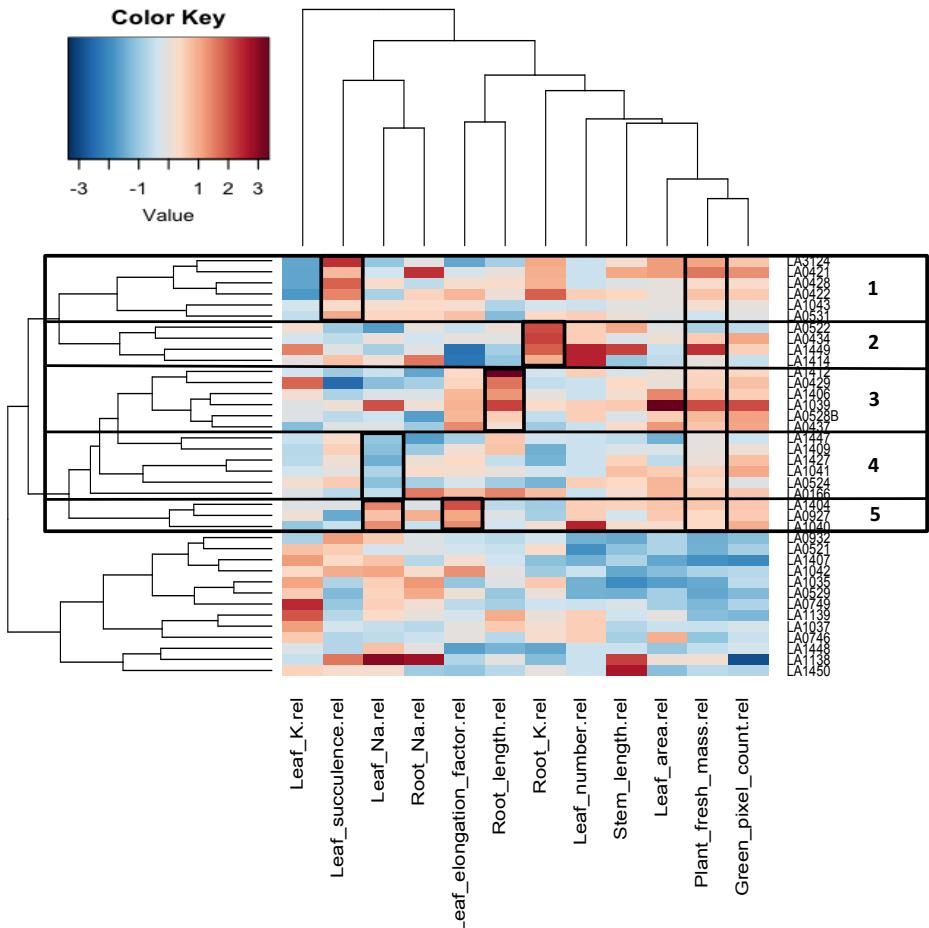








**A.****B.**

**A.****B.**