A High-Throughput Physiological Functional Phenotyping System for Time- and Cost-Effective Screening of Potential Biostimulants

Running title: Physiological-phenotyping of biostimulants under drought

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ABSTRACT

The improvement of crop productivity under abiotic stress is one of the biggest challenges faced by the agricultural scientific community. Despite extensive research, the research-to-commercial transfer rate of abiotic stress-resistant crops remains very low. This is mainly due to the complexity of genotype $\times$ environment interactions and in particular, the ability to quantify the dynamic plant physiological response profile to a dynamic environment.

Most existing phenotyping facilities collect information using robotics and automated image acquisition and analysis. However, their ability to directly measure the physiological properties of the whole plant is limited. We demonstrate a high-throughput functional phenotyping system (HFPS) that enables comparing plants' dynamic responses to different ambient conditions in dynamic environments due to its direct and simultaneous measurement of yield-related physiological traits of plants under several treatments. The system is designed as one-to-one (1:1) plant–[sensors+controller] units, i.e., each individual plant has its own personalized sensor, controller and irrigation valves that enable (i) monitoring water-relation kinetics of each plant–environment response throughout the plant's life cycle with high spatiotemporal resolution, (ii) a truly randomized experimental design due to multiple independent treatment scenarios for every plant, and (iii) reduction of artificial ambient perturbations due to the immobility of the plants or other objects. In addition, we propose two new resilience-quantifying-related traits that can also be phenotyped using the HFPS: transpiration recovery rate and night water reabsorption.

We use the HFPS to screen the effects of two commercial biostimulants (a seaweed extract—ICL-SW, and a metabolite formula—ICL-NewFo1) on *Capsicum annuum* under different irrigation regimes. Biostimulants are considered an alternative approach to improving crop productivity. However, their complex mode of action necessitates cost-effective pre-field phenotyping. The combination of two types of treatment (biostimulants and drought) enabled us to evaluate the precision and resolution of the system in investigating the effect of biostimulants on drought tolerance. We analyze and discuss plant behavior at different stages, and assess the penalty and trade-off between productivity and survivability. In this test case, we suggest a protocol for the screening of biostimulants' physiological mechanisms of action.

INTRODUCTION

To meet the food-security demands of an increasing global population, crop yields must double by 2050 (Ray et al., 2013). Despite an increase in crop productivity in the last few decades, the increased rate is not expected to match the demand, mainly due to the negative effects of climate change (abiotic environmental stresses such as drought, temperature extremes and flooding) and degrading soil quality. In fact, commercially grown crops are expected to achieve, on average, only about 50% of their potential yield under field conditions (Hatfield and Walthall, 2015; Foyer et al., 2016). In the last three decades, vast research had been invested in improving plant responses to various stresses. Nevertheless, the bench-to-field transfer rate (ratio of patents to marketed commercial seeds) of abiotic stress-resistant crops is very low, due to the high complexity of dynamic plant–environment interactions (Graff et al., 2013, Dalal et al., 2017).

Physiological phenotyping for crop improvement

The major gap between the successful breeding and yield improvement results from the unpredictable outcome of the complex genotype $\times$ environment interactions (Miflin 2000; Moshelion and Altman 2015). To date, the major obstacle to bridging this gap has been the lack of an efficient method for identifying and quantifying yield-related traits at early stages of plant growth across vast numbers of plants/genes (Moshelion and Altman 2015, Negin and Moshelion 2017). Another potential bottleneck is the genotype–phenotype gap. The availability of new molecular tools has enhanced the efficiency of classical breeding and crop improvement (Spindel et al., 2015; Bhat et al., 2016; Collard and Mackill 2008; Gosa et al., 2018). To achieve meaningful results in drought tolerance, molecular approaches to crop improvement must be linked to suitable phenotyping protocols at all stages, such as the screening of germplasm collections, mutant libraries, mapping populations, transgenic lines and breeding materials, and the design of OMICs and quantitative trait locus experiments (Salekdeh et al., 2009). Thus, to improve crops and to meet the challenges ahead, the genotypic view and emphasis on genomics need to be balanced by a phenocentric approach with an emphasis on phenomics, to minimize the genotype–phenotype gap (Miflin 2000). The development of a high-resolution, high-throughput diagnostic screening platform for the study of whole-plant physiological performance that serves for phenotypic screening might bridge this gap (Moshelion and Altman 2015).

Indeed, the number of phenotyping facilities has increased dramatically in the last decade. Most of these facilities collect information using robotics and automated image acquisition and analysis (White et al., 2012; Kumar et al., 2015; Ghanem et al., 2015; Fischer et al., 2014; Fiorani and Schurr 2013; Gosa et al., 2018). Nevertheless, the quest for more detailed and in-depth phenotyping of the dynamic genotype $\times$ environment interactions and plant stress responses (in particular during drought) has put the capability of the existing methods into question (Ghanem et al., 2015; Li et al., 2014; Halperin et al., 2017; Rahaman et al., 2015; reviewed by Gosa et al., 2018). Herein, we demonstrate a high-throughput functional phenotyping system (HFPS) composed of gravimetric systems that enable us to compare plants' dynamic responses to different ambient conditions in dynamic environments, due to its direct and simultaneous measurement of the yield-related physiological traits of all plants under several treatments.

[3]

Phenotyping for biostimulants in drought response

126
127 Apart from the traditional strategies to improve crop productivity under an uncertain
128 environment and abiotic stress, an alternative approach is evolving. This approach
129 considers the use of organic molecules, externally applied to the plant at low
130 concentrations, to stimulate many aspects of growth and development, pathogen
131 defense, stress tolerance and reproductive development. These organic molecules,
132 collectively termed biostimulants, have become more and more common in the global
133 market in the last two and a half decades (reviewed by Yakhin et al.,
134 2017). Biostimulants have been defined in many different ways. In the scientific
135 literature, the term biostimulant was first defined by Kauffman et al. (2007) in a peer-
136 reviewed paper, with modifications: "biostimulants are materials, other than
137 fertilizers, that promote plant growth when applied in low quantities" (reviewed by du
138 Jardin, 2015). However, the definition of biostimulants adopted by the European
139 Biostimulants Industry Council specifies that these materials should not function by
140 virtue of the presence of essential mineral elements, known plant hormones or
141 disease-suppressive molecules (Brown and Saa 2015). Recently, biostimulants were
142 defined by Yakhin et al. (2017) as "a formulated product of biological origin that
143 improves plant productivity as a consequence of the novel, or emergent properties of
144 the complex of constituents, and not as a sole consequence of the presence of known
145 essential plant nutrients, plant growth regulators, or plant protective compounds."
146 However, due to their complex composition and diversity, biostimulants are classified
147 differently by different research groups. Many categorize biostimulants based on the
148 natural raw materials used, the origin of their active ingredients and modes of action,
149 inclusion or exclusion of microorganisms, and/or mode of action of the biostimulant
150 (Ikrina and Kolbin 2004; Basak 2008; Du Jardin 2012; Bulgari et al. 2015; Yakhin et
151 al., 2017).

152
153 Biostimulants are used in all stages of agriculture, namely, in seed treatments, during
154 plant growth, and postharvest. They are applied both as foliar sprays and through the
155 soil. Biostimulants may function in various ways, as comprehensively summarized by
156 Yakhin et al. (2017). Their mechanism of action may comprise activation of nitrogen
157 metabolism or phosphorus release from soils, generic stimulation of soil microbial
158 activity, or stimulation of root growth and enhanced plant establishment. They
159 stimulate plant growth by enhancing plant metabolism, stimulating germination,
160 improving photosynthesis, and/or increasing the absorption of nutrients from the soil,
161 thus increasing plant productivity. Studies have shown a clear protective role of a
162 diverse number of biostimulants against abiotic stress, as reviewed by Van Oosten et
163 al. (2017). Nevertheless, and despite the extensive literature suggesting that
164 biostimulants decrease the effects of abiotic stress (and in particular drought stress),
165 information regarding their physiological mechanisms of action is limited. The large
166 number of potential candidate biostimulants and the need to elucidate their particular
167 modes of action, optimal concentrations, and types of application, create a substantial
168 bottleneck in the research and development of new biostimulant products. High-
169 throughput phenotyping technologies have been successfully employed in some
170 aspects of plant breeding (Araus and Cairns, 2014; Tardieu et al., 2017), but their
171 application to assess plant biostimulant action has been limited (Petrozza et al., 2014;
172 reviewed by Rouphael et al., 2018), despite the potential benefits of using these
173 technologies in biostimulant product screening (Rouphael et al., 2018).
174

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175 In this study, we tested the effectiveness of physiological phenotyping for
176 understanding the physiological 'mode of action' of biostimulant activity on the
177 whole plant's drought response. We tested the impact of biostimulants on several
178 quantitative yield-related physiological traits: transpiration rate, growth rate, and
179 water-use efficiency (WUE).
180
181 **MATERIALS AND METHODS**
182
183
184 **Plant Material**
185 The seeds of pepper (*Capsicum annuum* var. Rita) were obtained from Zeraim
186 Gedera-Syngenta, Israel. For germination, the seeds were sown in a tray with 10-mL
187 cones filled with commercial growing medium (Matza Gan, Shaham, Givat-Ada,
188 Israel), composed of (w/w) 55% peat, 20% tuff and 25% puffed coconut coir fiber.
189 The trays were well irrigated and kept in the same greenhouse (on side-tables) where
190 the experiment was performed. When the seedlings were 4 weeks old, the growing
191 medium was carefully washed off (to avoid root damage) the seedling roots and the
192 seedlings were immediately transferred to 4-L pots filled with 20/30 sand (Negev
193 Industrial Minerals Ltd., Israel). The numbers 20/30 refer to the upper and lower size
194 of the mesh screen through which the sand was passed (20 = 20 squares across one
195 linear inch of screen), resulting in a sand particle size of between 0.595 and 0.841
196 mm. The volumetric water content (VWC) of the freely drained substrate, noted as
197 pot capacity, was ~24% (for details, see Experimental Setup section).
198
199 **The Physiological Phenotyping Platform**
200 The experiment was conducted in June–July 2018 in a commercial-like greenhouse
201 located at the Faculty of Agriculture, Food and Environment in Rehovot, Israel. The
202 greenhouse temperature was controlled using fans that blow air through a moist
203 mattress, keeping it below 38°C. The temperature and relative humidity (RH) were
204 21–38°C and 30–80%, respectively. The plants were grown under natural light
205 (midday maximum of 1300 μmol$\square$s^{-1} m^{-2}), representative values for natural
206 conditions during the summer in the central part of Israel, including Rehovot. The
207 temperature, RH, photosynthetically active radiation, barometric pressure and vapor
208 pressure deficit in the greenhouse were continuously monitored by Plantarray
209 meteorological station (Plant-Ditech Ltd., Israel).
210
211 The functional phenotyping system Plantarray 3.0 platform (Plant-Ditech) was used to
212 monitor the plants' performance during the entire experimental period by controlling
213 the schedule and quantity of irrigation. This platform (Figure 1A and Supplementary
214 Figure 1), which enables performing high-throughput physiological functional
215 phenotyping, includes 72 units of highly sensitive, temperature-compensated load
216 cells that are used as weighing lysimeters. Each unit is connected to its personalized
217 controller, which collects the data and controls the irrigation to each plant separately.
218 An independent controller for each pot enables tight feedback irrigation, based on the
219 plant's transpiration rate. Each controller unit is connected to its neighbor for serial
220 data collection and loading to a server. A pot with a single plant is placed on each
221 load cell (for more details, see Experimental Setup section). The data were analyzed
222 by SPAC-analytics (Plant-Ditech), a designated online web-based software that

223 enables viewing and analyzing the real-time data collected from the Plantarray
224 system.
225
226 **Nutrition and Treatments**
227 The composition of the nutrients supplied to the plants by the irrigation system
228 (fertigation) is provided in Table 1. Two different commercial biostimulants were
229 used: seaweed extract (ICL-SW) and a metabolite extract formula (ICL-NewFo1)
230 (both supplied and produced by ICL Specialty Fertilizers, Holland). The biostimulants
231 were prepared in two different containers that were placed on two additional load
232 cells to precisely track their application. The biostimulants were provided to the plants
233 together with the nutrients via the controlled irrigation system (Supplementary Figure
234 1). The biostimulant concentration and dosage were as per the manufacturer's
235 instructions: ICL-NewFo1 (3.53 mg/L) was provided daily and ICL-SW (0.133 mg/L)
236 once a week.
237
238 The experiment lasted 36 days and included two treatments: (i) ample irrigation that
239 aimed to provide non-stressed conditions for the plants throughout the experiment
240 (termed well-irrigated plants), (ii) controlled drought (days 13–30) preceded by a
241 period of ample irrigation, noted as pretreatment (days 1–12), and followed by
242 resumption of ample irrigation (recovery period) (see Figure 2B and Experimental
243 Setup section for details). The treatments included ICL-SW, ICL-NewFo1 or no
244 biostimulants (control). Overall, we had six different experimental groups: three with
245 ample irrigation (control–well irrigated, ICL-SW–well irrigated and ICL-NewFo1–
246 well irrigated) and three groups subjected to drought (control–drought, ICL-SW–
247 drought, and ICL-NewFo1–drought). Each of these groups consisted of 8–12
248 repetitions (plants) that were arranged in a randomized fashion on the array to ensure
249 uniform exposure of all groups, thereby overcoming the inherent variations in ambient
250 conditions (Figure 1B).
251
252 **Experimental Setup**
253 The experimental setup was generally similar to Halperin et al. (2017) with some
254 modifications. Briefly, before the start of the experiment, all load-cell units were
255 calibrated for reading accuracy and drift level under constant load weights (1 kg and 5
256 kg). Sand was used as the growing substrate because (i) it is an inert substance (sand
257 is free of any nutrients, helping to precisely understand the effect of any chemical
258 applied externally through irrigation), (ii) it is easily washed off the roots (helping to
259 study the roots after the completion of the experiment), and (iii) pot capacity is
260 reached rapidly with a repeatable pattern after each irrigation (at the end of free
261 drainage), helping to study the plants' short-term resilience trait, noted as "night water
262 reabsorption" (see Measurement of Quantitative Physiological Traits section for
263 details). The sand in all of the pots was washed thoroughly several times prior to
264 transfer of the seedlings. Each pot was placed into a Plantarray plastic drainage
265 container on a lysimeter. The container fit the pot size to prevent evaporation. The
266 container has orifices on its side walls at different heights to enable different water
267 levels after drainage of excess water following irrigation. Evaporation from the sand
268 surface was prevented by a plastic cover with a circle cut out at its center through
269 which the plants grew.
270
271 Each pot was irrigated by multi-outlet dripper assemblies (Netafim, Israel) that were
272 pushed into the soil to ensure that the medium in the pot was uniformly wetted at the

273   end of the free drainage period following each irrigation event. Irrigations were
274   programmed to run during the night in four consecutive pulses. A 2-h interval was
275   maintained between the first irrigation pulse and the last three. This irrigation regime
276   enabled determining the plants' night water reabsorption, one of the traits indicating
277   plant resilience (see Measurement of Quantitative Physiological Traits section). The
278   amount of water left in the drainage containers underneath the pots at the end of the
279   irrigation events was intended to provide water to the well-irrigated plants beyond the
280   water volume at pot capacity. The associated monotonic weight decrease throughout
281   the day hours was essential for the calculation of the different physiological traits by
282   the data-analysis algorithms.

284   The drought treatment started on day 13 and ended when the plants' daily
285   transpiration had reached ~80 mL per day. To prevent rapid depletion of the water in
286   the sandy soil in the pots, we conducted gradual deficit irrigation that reduces the
287   irrigation levels every day to 80% of the previous day's transpiration, for each plant
288   separately and independently (using Plantarray's automated feedback irrigation
289   system; Figure 2B).

291   **Measurement of Quantitative Physiological Traits**
292   The following water-relations kinetics and quantitative physiological traits of the
293   plants were determined simultaneously, following Halperin et al.'s (2017) protocols
294   and equations implemented in the SPAC-analytics software: daily transpiration,
295   transpiration rate, normalized transpiration (E), transpiration rate vs. calculated VWC
296   using a piecewise linear fit, and WUE. Cumulative daily transpiration was calculated
297   as the sum of daily transpiration for all 36 days of the experiment. The VWC in the
298   sand medium was calculated by a mass balance between the system weight at pot
299   capacity when free drainage ceases and its concurrent weight.

301   The estimated plant weight at the beginning of the experiment was calculated as the
302   difference between the total system weight and the sum of the tare weight of pot +
303   drainage container, weight of soil at pot capacity, and weight of water in the drainage
304   container at the end of the free drainage. The plant weight at the end of a growth
305   period (calculated plant weight) was calculated as the sum of the initial plant weight
306   and the multiplication of the cumulative transpiration during the period by the WUE.
307   The latter, determined as the ratio between the daily weight gain and the daily
308   transpiration during that day, was calculated automatically on a daily basis by the
309   SPAC-analytics software. Note that the WUE approached a constant value during the
310   pretreatment period.

312   The plant's recovery from drought was described by the rate at which the plant gained
313   weight following resumption of irrigation (recovery stage). The physiological trait
314   representing the plant's transpiration recovery from drought was determined as the
315   ratio between the slope of the daily transpiration increase during the recovery phase
316   (recovery slope) and the slope of the daily transpiration decrease during the drought
317   period (stress degree). The slopes were calculated using a linear regression.

319   The night water reabsorption trait was determined as the difference in system weight
320   between the end of the last and first irrigations of a given irrigation event (i.e. single
321   night), representing the water absorbed by the plant during the very short period when
322   transpiration is practically negligible. This calculation is based on the fact that the

323   drainage of surplus water in sand is rapid and pot capacity is reached prior to the
324   subsequent irrigation (Supplementary Figure 2). We considered the plants' short-term
325   water reabsorption capability during the recovery stage to be an additional
326   physiological trait representing the plant's resilience to drought. Note that the water
327   reabsorption by the plant during the night hours was normalized to its weight.
328
329   The recovery stage lasted 6 days, after which the experiment was stopped. As pepper
330   is an indeterminate plant, it did not reach its full yield capacity. Consequently, the
331   experiment was terminated at this stage as the treatment conducted to that point had a
332   direct effect on the existing fruit. The shoots and fruit were harvested from ~10-week-
333   old plants, irrespective of their size, in the early morning hours. The fresh shoot
334   weight was calculated by the system as the difference in actual gravimetric weight
335   between the day of shoot harvest at 0400 h (at the end of the last irrigation) and the
336   following day at the same time. The fruit were collected from the harvested shoot and
337   counted. The fruit and shoots (without fruit) were weighed when a constant weight
338   had been reached during drying in a hot air oven at 60°C. The roots were collected
339   from the pots, washed thoroughly to remove the sand particles, and dried in a hot air
340   oven at 60°C until no further reduction in weight was measured, and finally weighed.
341   The total dry plant weight is the sum of dry shoot weight, dry root weight and dry
342   fruit weight.
343
344   **Statistical Analysis**
345   Means were compared using analysis of variance (ANOVA) and Student's *t*-test
346   (noted in the figure legends) in JMP Pro 14 software.
347
348   **RESULTS**
349
350   A randomized experimental design was performed to quantitatively compare the
351   impacts of two biostimulants (seaweed extract ICL-SW and metabolite formula ICL-
352   NewFo1) on the plant's key physiological traits. The effects of the two biostimulants
353   were compared to controls (no biostimulant) under two irrigation scenarios: (i) well
354   irrigated, and (ii) drought stress starting with a well-irrigated period, then a controlled
355   drought phase and a successive recovery period (Figure 2B).
356
357   **Biostimulants Affect Plant Water Loss**
358   Daily transpiration increased gradually for all six groups during the well-irrigated
359   period (pretreatment; Figure 3A). Conversely, daily transpiration and VWC in the pot
360   gradually decreased throughout the drought period that started on day 13 of the
361   experiment (Figures 3A and B, respectively). Daily transpiration and VWC and
362   increased sharply upon irrigation resumption on day 31 of the experiment (recovery
363   period) (Figures 3A and B, respectively). The physiological drought point (defined as
364   the soil VWC value that begins to limit transpiration rate [critical VWC, $\theta_{critical}$ (θc)])
365   was determined for the plants subjected to drought (Figure 3C). A $\theta c = 0.15$ was
366   obtained for the control and two biostimulant treatments, but due to the different
367   pattern of VWC decrease in the ICL-SW-treated plants compared to the other two
368   groups (Figure 3B), they reached θc on different days. The θc for the control and ICL-
369   NewFo1-treated plants was reached on day 22.5, and on day 21 for the ICL-SW-
370   treated plants (Figure 3B,C). The impact of drought on the daily transpiration rate
371   pattern of the treated and untreated plants relative to that of the three well-irrigated
372   groups is illustrated in Figure 3D for days 27–29, revealing that the ICL-SW-treated

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373 plants experienced a significantly lower midday (between 1200 and 1400 h)
374 transpiration rate under drought but reached a significantly higher transpiration rate
375 under full irrigation (Figure 3E). Under ample irrigation, the ICL-NewFo1-treated
376 plants had a significantly higher transpiration rate than the control plants, and a
377 similar reduction in transpiration rate under drought (Figure 3E).
378
379 **Biostimulants Enhance Biomass and WUE**
380 Transpiration was normalized to biomass by using the calculated plant weight for the
381 entire experimental period (36 days) for all six groups (Figure 4A). The rate of plant
382 weight gain during the well-irrigated period (pretreatment) was similar for all six
383 groups, and decreased during the drought period for the three drought-stressed groups.
384 The rate of weight gain for the latter groups began to increase again during the
385 recovery period (Figure 4A). Nevertheless, the higher rate of weight gain for the ICL-
386 SW-treated plants during this latter period resulted in significantly higher dry shoot
387 biomass than for controls at the end of the experiment, probably due to the cumulative
388 effect of this trend (Figure 4B). The correlation between shoot dry biomass and
389 cumulative daily transpiration, which is, in fact, the dry-weight-related WUE, was
390 relatively high ($R^2 > 0.8$) for both the well-irrigated and water-deprived plants (Figure
391 4C). Plant transpiration normalized to plant weight, E (Figure 4D), was low for
392 biostimulant-treated plants under both well-irrigated and drought conditions compared
393 to its value for untreated controls. Here again, the ICL-SW-treated plants showed
394 significantly lowest midday E under drought (in accordance with the transpiration
395 rate, Figure 3E). The higher measured transpiration rates (Figure 3E) and higher dry
396 shoot biomass (Figure 4B) for the biostimulant-treated plants compared to the
397 controls under ample irrigation indicate an improvement in fresh weight-related
398 WUE. However, this improvement (increase of ~18% for ICL-SW-treated and ~14%
399 for ICL-NewFo1-treated plants) was not significant (*P*-value for ICL-SW was 0.067
400 and for ICL-NewFo1, 0.16; Figure 4E).
401
402 **Biostimulant Effect on Plant Resilience**
403 The two considered traits for an estimation of the plants' recovery from drought stress,
404 i.e., resilience, were: (i) whole-plant transpiration recovery: the rate at which the daily
405 transpiration increases following irrigation resumption was compared to the rate at
406 which the daily transpiration decreases during the drought period. For the sake of
407 simplicity, both rates were determined as the linear regression of the respective data
408 points (Figure 5A), showing that ICL-SW reduced plant resilience compared to
409 control plants (Figure 5B); (ii) the night water reabsorption (namely, regaining the
410 water that was lost during the day; see Supplementary Figure 2) for the pretreatment
411 and recovery periods, depicted in Figures 5C,D and 5E,F, respectively. The night
412 water reabsorption during the pretreatment period was significantly higher for the
413 biostimulant-treated plants compared to the control, with the highest values for the
414 ICL-NewFo1-treated plants (Figure 5C). The drought stress reduced night water
415 reabsorption capability during recovery for all three groups. Nevertheless, compared
416 to the control, the biostimulants improved the reabsorption capability during recovery,
417 with significantly highest capability for ICL-NewFo1-treated plants (Figure 5E). A
418 similar trend was observed when the night water reabsorption was normalized to the
419 plant weight, with significantly highest reabsorption capability in ICL-NewFo1-
420 treated plants compared to the control (Figures 5D,F).
421
422 **Biostimulant Effect on Fruit Number**

423   As pepper plants are indeterminate, we decided to terminate the experiment shortly
424   after recovery, despite the fact that full fruit weight potential had not been reached.
425   Nevertheless, at this stage, fruit set in all groups was assumed to reflect the treatment,
426   as seen in the distribution of the three different fruit sizes (small, medium and
427   commercial) (Supplementary Figure 3). For the well-irrigated plants, 33% of the
428   control fruit reached a commercial size, compared to only 19% of ICL-SW-treated
429   and 14% of ICL-NewFo1-treated plants' fruit. The total number of fruit was counted
430   for all six groups and correlated to cumulative daily transpiration (Figure 6). ICL-SW
431   significantly enhanced the total fruit number under ample irrigation (Student's t-test);
432   however, the ICL-SW-treated plants were significantly affected by the drought
433   relative to their well-irrigated condition (Figure 6A). As similar results were observed
434   for the transpiration rate of these treated plants (Figure 3E), we calculated the
435   correlation between total fruit number and cumulative daily transpiration. The
436   correlation for well-irrigated plants was slightly better ($R^2 = 0.5$) than that for plants
437   subjected to drought (Figure 6B).
438
439   **DISCUSSION**
440
441   **Advantages of the HFPS in Pre-Field Screening for Promising Candidates and**
442   **Effective Treatments**
443   Most high-throughput phenotyping facilities are based on remote sensing or imagers
444   (Araus et al., 2018), and are expected to show improved temporal phenotypic
445   resolution. However, their effective spatial resolution is still relatively limited to
446   morphological and indirect physiological traits. In addition, measurements are not
447   taken simultaneously; given the fact that the plant response to a dynamic environment
448   is dynamic, simultaneous measurements are needed for comparative analyses. Thus,
449   the selection of candidates and treatments for testing remains a challenge. High-
450   throughput phenotyping platforms in greenhouses have the advantage of
451   characterizing individual pot-grown plants without the constraints imposed by
452   overlapping canopies from neighboring plants or variable climatic conditions that can
453   hamper data-acquisition accuracy (Fernandez et al., 2017). Although an effective
454   approach would be to screen biostimulants for their mode of action from "field to
455   greenhouse", the "greenhouse-to-field" approach is not only time- and cost-effective
456   but also narrows the number of products to be tested later under field conditions
457   (Rouphael et al., 2018). On the other hand, the accuracy of controlled growth
458   environments in targeting genetically complex traits is questionable, as phenotypes
459   from spaced pots and controlled conditions are poorly correlated with phenotypes in
460   field environments, where plants compete with their neighbors (Nelissen et al., 2014;
461   Poorter et al., 2016; Fernandez et al., 2017; Fischer et al., 2018; Rebetzke et al.,
462   2018). We suggest that to better correlate a plant's response to its environment, it is
463   important to phenotype under conditions that are as similar as possible to those in the
464   field. Thus, an efficient pre-field phenotype-screening experiment should offer the
465   possibility to predict yield penalties in response to environmental adversity in the
466   early stages of plant growth. Choice of the appropriate phenotyping method is one of
467   the key components in pre-field screens (phenotyping) for complex traits under
468   abiotic stress conditions (reviewed by Negin, 2017). This improves the chances of the
469   selected candidates performing well under field conditions. The following principles,
470   tested in this study, may contribute to this goal.
471

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472   (i) Conducting experiments under semi-controlled conditions that are typical of
473   farmers' growing facilities (see Figure 2A). The spaces between the pots were kept to
474   a minimum to mimic commercial growth conditions.

475

476   (ii) Using a truly randomized experimental design to mimic the biological variability,
477   as well as the spatial and temporal variability in ambient conditions in the growth
478   facility. Here, we used a randomized experimental design with one-to-one (1:1) plant–
479   [sensors+controller] units which enabled running an independent feedback irrigation
480   scenario for every plant (Figure 1, Supplementary Figure 1). Each controller was
481   associated with a dual-valve system that allowed creating a specific combination of
482   irrigation scenarios independently for each plant. Moreover, it overcame many of the
483   experimental artifacts that could result from the "pot effect" (Gosa et al., 2018) by
484   using controlled-deficit irrigation that reduced the irrigation levels every day to 80%
485   of the previous day's transpiration (for each plant separately and based on its
486   individual performance), preventing rapid reduction in pot soil water content. This
487   created a relatively homogeneous drought scenario for all plants (Figure 2).

488

489   (iii) Conducting comparative and continuous measurements for all plants' water-
490   relations kinetics (direct physiological traits) in response to the three-phase scenario
491   (control–drought–recovery). This experimental approach offered several advantages
492   in interpreting the plant's interactions with the environment as it compared each
493   plant's profile to its own profile in the different phases (Figure 3A) as well as to all
494   other plants' profiles in the experiment, simultaneously. Moreover, clarity of the stress
495   conditions, providing the ability to repeat the exact stress scenario in other
496   experiments, is also important when studying a desired stress-related trait. The trait in
497   question might respond differently in plants showing different types of drought
498   tolerance under different drought conditions (Negin and Moshelion, 2017). Therefore,
499   for better resolution of the drought response in pot experiments, the severity and
500   strategy of the drought stress must be well defined. To achieve a quantitative and
501   cooperative response of the plants to a combination of biostimulants and drought
502   treatment, we divided the experiment into three phases: before drought (pretreatment),
503   during drought which was defined by the physiological drought point (θc), and
504   recovery immediately after drought (resilience).

505

506   (iv) High temporal and spatial resolution of the plant–environment interactions. The
507   ultimate trait, yield, is a cumulative trait, measured at the end of the experiment and
508   reflecting the sum of all genetic and ambient parameters affecting the plant
509   throughout the season. This calls for high temporal resolution and continuous
510   measurement of the dynamic plant–environment response. The high-capacity data
511   acquisition (480 measurements per day) of the HFPS enabled tight measurements of
512   the plant's response to the ambient conditions, and also comparing plant performances
513   at different time points during the day (i.e., different ambient conditions), where the
514   differences between the treatments became significant (Figures 3 D,E).

515

516   In this study, we show that the HFPS might be an efficient diagnostic tool for a better
517   understanding of pre-field plant x environment interactions by studying water-related
518   physiological mechanisms under different phases of control–drought–recovery
519   scenarios. In this pursuit, we used biostimulants as a test case due to their reported
520   impact on the plant stress response (Van Oosten et al., 2017). Nevertheless,
521   information on the influence of biostimulants on physiological mechanisms of action

[11]

522   is relatively scarce. Moreover, the use of biostimulants, as with other biotic and
523   abiotic screening studies, is highly complex, and thus identification and
524   characterization of their activity is time-consuming and expensive, as it requires large-
525   scale field experiments. The combination of the two types of treatment (biostimulants
526   and drought) enabled us to evaluate the benefits of the HFPS in investigating the
527   mechanistic effect of biostimulants in drought tolerance.
528
529   **Quantitative Comparison to Understand the Interactions between Key**
530   **Physiological Traits and Their Trade-Offs**
531
532   Both biostimulants increased plant transpiration rate under ample irrigation compared
533   to control plants (Figure 3). However, while the impact of ICL-SW translated to
534   productive mechanisms (faster growth rate and later, higher fruit number), the impact
535   of ICL-NewFo1 translated to survivability mechanisms (lower transpiration rate under
536   drought and faster recovery—i.e., better resilience). Interestingly, the sensitivity of
537   the plants to drought in terms of the critical VWC drought point (θc), at which a
538   further reduction in water content reduces transpiration, remained the same, possibly
539   due to similar root sizes (Supplementary Figure 4). This is because under water-
540   deficit conditions, when water becomes less available to the roots, plants with smaller
541   roots will be limited more quickly (early θc) than plants with larger roots (reviewed
542   by Gosa et al., 2018). Thus θc might be useful in predicting root phenotype.
543   Nevertheless, as soon as the plants were exposed to drought, the two biostimulants
544   induced different response patterns (beyond θc): ICL-NewFo1 treatment resulted in a
545   gradual reduction in transpiration rate, reaching a minimum at a relatively lower
546   VWC than the control and ICL-SW-treated plants (Figure 3C). Again, this type of
547   behavior could explain the better survivability of the ICL-NewFo1-treated plants
548   during the drought period.
549
550   In addition, the functional phenotyping approach revealed good correlations among
551   key agronomical traits within the short study period. For example, our results revealed
552   a high correlation between plant total dry weight and plant weight calculated by the
553   system (Supplementary Figure 5). The fact that the system can calculate the plant
554   biomass throughout the experiment is highly beneficial as it enables a direct
555   measurement of the whole-plant biomass gain, in real time and in a non-destructive
556   manner. In addition, key agronomic traits (such as grain yield) are linearly correlated
557   to water consumption (WUE, reviewed by Gosa et al., 2018). Indeed, throughout the
558   entire experiment, water taken up by the system (representing plant agronomic WUE,
559   slope in Figure 4C) was almost identical to the fresh-weight WUE (taken on the first
560   few days of the experiment; Figure 4E). Namely, ~0.003 g of plant dry weight per 1
561   mL of transpired water vs. ~0.035 g of plant fresh weight per 1 mL of transpired
562   water, respectively, showing a ratio of ~1:10, which is similar to the ratio between the
563   fresh and dry shoot weight (Supplementary Figure 6). This trait is highly beneficial as
564   it enables use of the fresh-weight WUE (determined on the first few days of the
565   experiment), which is calculated for the entire growth period rather than the dry-
566   weight WUE. Interestingly, these results also indicate that WUE is nearly constant
567   throughout the growth period.
568
569   **Phenotyping Resilience**
570   Resilience is one of the key stress-response traits. Nevertheless, the term "resilience"
571   is being used more and more freely, and with popularity comes confusion; thus, it

572 must assume its broadest definition. Resilience is commonly used to represent
573 resistance, or recovery, or both (Hodgson et al., 2015). Plant stress resilience indicates
574 plant survival and productivity after stress. In this study, we introduced two functional
575 traits to quantify resilience: (i) transpiration recovery rate after stress (return of
576 irrigation) and (ii) the plant's ability to reabsorb water at night during recovery from
577 drought. We found that while the biostimulants did not affect the transpiration
578 recovery rate, they did increase the nighttime water reabsorption ability of both the
579 well-irrigated and recovering plants, compared to the non-treated controls (Figures
580 5C,E). This phenomenon can be explained by the positive impact on the fresh
581 biomass (Figure 4A), as normalizing the water reabsorption volume to the plant
582 biomass still resulted in higher values of both biostimulant-treated plants compared to
583 the non-treated control (Figures 5E,F). The difference between the water reabsorption
584 of well-irrigated and recovering plants within the same group (i.e., control, ICL-SW
585 or ICL-NewFo1) indicated drought-inflicted tissue damage, thus the night water
586 reabsorption trait can be used as a tool to estimate tissue damage due to stress.
587
588 **CONCLUSION**
589
590 A comparison of the effects of two biostimulants on drought tolerance using a HFPS
591 revealed known and new relationships between physiological traits. The two studied
592 biostimulants (ICL-SW and ICL-NewFo1) improved the overall transpiration and
593 biomass gain compared to control plants. However, only ICL-SW improved fruit
594 number (Figure 6A) under ample irrigation, which was significantly reduced when the
595 plants were exposed to drought. This might be explained by the shift in resource
596 allocation from the reproductive to non-reproductive or vegetative biomass, for
597 survival. A schematic depiction of the behavior of plants treated with biostimulants is
598 given in Figure 7. The behavior can be explained in terms of risk-taking and non-risk-
599 taking behavior. Under optimal conditions, ICL-SW-treated plants (risk-taking)
600 sustained a longer period of higher transpiration rate and thus a longer period of
601 substantial CO_2 assimilation, resulting in increased productivity (Figure 7A)
602 compared to the ICL-NewFo1-treated plants. This behavior is advantageous only
603 under well-irrigated conditions or during mild stress, but there is a risk of losing water
604 faster during severe stress (Lin et al. 2007; Peng et al. 2007;
605 McDowell et al. 2008; Sade et al. 2009; Moshelion et al., 2015). On the other hand,
606 ICL-NewFo1-treated plants (non-risk-taking) maintain a moderate transpiration rate
607 under optimal conditions, thus not contributing much to their productivity, but
608 resulting in more gradual water loss under drought conditions, thereby reaching the
609 minimal VWC (desiccation) later than the ICL-SW-treated plants, resulting in
610 increased survivability. Thus there is a trade-off between productivity and
611 survivability for the ICL-SW- and ICL-NewFo1-treated plants, respectively, as
612 depicted in Figure 7B (Moshelion et al., 2015).
613
614 We suggest that these two different stimulation approaches should be implemented in
615 different agricultural practices. Thus, the beneficial stimuli of ICL-SW may be
616 implemented in controlled-irrigated crops, while the resilience impact of ICL-
617 NewFo1 can be implemented for non-irrigated crops that are naturally subjected to the
618 uncertainty of the environment. This survivability trait may also be very beneficial for
619 annual crops (e.g., vines, turfs and silviculture) which need to overcome longer stress
620 periods between seasons.
621

[13]

AUTHOR CONTRIBUTIONS

RW and MM conceived the original research plan. AD, NH and IS performed the experiments. RB adapted the algorithms suggested by Halperin et al. (2017) to the calculations performed in this manuscript. AD, RB and MM analyzed the data. AD and MM wrote the manuscript. All authors were involved in reviewing and editing the manuscript.

Conflict of Interest Statement: The authors declare that the research was conducted independently and in the absence of any commercial relationships that could be construed as a potential conflict of interest.

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764
765 **FIGURE LEGENDS**
766
767 **FIGURE 1.** Experimental setup. **(A)** View of the randomized experimental setup
768 array consisting of 72 measuring units loaded with *Capsicum annuum*. **(B)** Block
769 diagram of the system. Solid circles – well-irrigated plants; empty circles – plants
770 subjected to the drought-recovery scenario. Green – ICL-SW-treated plants, orange –

[16]

771   ICL-NewFo1-treated plants, blue – control (no biostimulants) plants. Note that all pot
772   surfaces were covered to reduce evaporation, and irrigation was injected into the soil
773   via multi-outlet drippers to ensure even distribution of fertigation and biostimulants
774   (see Supplementary Figure 1).
775

776   **FIGURE 2.** Atmospheric conditions and experimental progress represented as system
777   relative weight throughout the experiment. **(A)** Daily vapor pressure deficit (VPD)
778   and photosynthetically active radiation (PAR) during 36 consecutive days of
779   experiment. **(B)** Raw data showing variation in the weight of the plants (relative to
780   their respective initial weight) over the course of the experiment. Each line represents
781   one plant/pot. During the day, the plant transpires and therefore the system loses
782   weight, seen as a slope in the line curves. The pots were irrigated four times per night
783   (each time to pot capacity), seen as peaks in the line curves. The irrigation was
784   followed by drainage to reach water saturation (nighttime baseline). Note that there is
785   no weight loss during the night. The increase in the nighttime baseline (dashed line)
786   every day results from an increase in plant biomass. During pretreatment, all of the
787   plants were well irrigated; from day 13, half of the plants were exposed to differential
788   drought to reach a similar degree of stress, while the other half continued to be well-
789   irrigated till the end of the experiment. On day 31, the water-deprived plants were
790   recovered and continued to be well-irrigated till the end of the experiment. The three
791   colored lines represent a single plant from each of the three groups: blue line –
792   untreated (with biostimulants) control plants; green line – ICL-SW-treated plants;
793   orange line – ICL-NewFo1-treated plants. Note the different drought-response
794   behaviors of the different plants. The inset figure presents system relative weight
795   change of one plant/pot for two consecutive days.
796

797

798   **FIGURE 3.** Effect of biostimulants on plant transpiration. **(A)** Mean ± SE continuous
799   daily whole-plant transpiration during the entire experimental period (36 days). **(B)**
800   Mean ± SE calculated volumetric water content (VWC) of the water-deprived plants
801   throughout the experiment. **(C)** Piecewise linear fit between transpiration rate and
802   calculated VWC for the plants subjected to drought treatment. **(D)** Mean ± SE diurnal
803   transpiration rate from 0600 to 1900 h during the late drought phase (day 27–29). **(E)**
804   Mean ± SE transpiration rate for days 27–29 from 1200 to 1400 h. Blue bars – no
805   biostimulant control plants; green bars – ICL-SW-treated plants; orange bars – ICL-
806   NewFo1-treated plants. Solid bars – well-irrigated conditions; stippled bars – drought
807   conditions. Groups were compared using ANOVA by Tukey HSD test. Different
808   letters above columns represent significant differences ($P < 0.05$). Each mean ± SE is
809   from at least 8 plants per group.
810

811   **FIGURE 4. (A)** Mean ± SE calculated whole-plant weight during the entire
812   experimental period. **(B)** Mean ± SE shoot dry weight, harvested at the end of the
813   experiment. **(C)** Correlation between shoot dry weight and cumulative daily
814   transpiration. **(D)** Midday mean ± SE E (transpiration rate normalized to plant
815   biomass) for days 27–29 from 1200 to 1400 h. **(E)** Mean ± SE water-use efficiency
816   (WUE). Blue bars – untreated (with biostimulants) control plants; green bars – ICL-
817   SW-treated plants; orange bars – ICL-NewFo1-treated plants. Solid bars – well-
818   irrigated conditions; stippled bars – drought conditions. Groups were compared using
819   ANOVA by Tukey HSD test and Student's t test. Different letters above columns

820 represent significant differences ($P < 0.05$). Each mean ± SE is from at least 8 plants
821 per group.

822

823 **FIGURE 5.** Effect of biostimulants on plant resilience during recovery. **(A)**
824 Mean ± SE continuous total whole-plant daily transpiration of drought-treated plants
825 during the entire experimental period of 36 days. Graph shows days from when stress
826 degree and recovery slope were calculated for analysis. **(B)** Mean ± SE resilience
827 measured as the ratio of the recovery slope (day 31–32) to stress degree (day 18–30).
828 **(C)** Mean ± SE water reabsorption during pretreatment (day 11–14), and **(D)** its
829 mean ± SE normalized to calculated plant weight. **(E)** Mean ± SE water reabsorption
830 during recovery phase (day 33–36), and **(F)** its mean ± SE normalized to calculated
831 plant weight. Blue bars – untreated (with biostimulants) control plants; green bars –
832 ICL-SW-treated plants; orange bars – ICL-NewFo1-treated plants. Solid bars – well-
833 irrigated conditions; stippled bars – drought conditions. Groups were compared using
834 ANOVA by Tukey HSD test and Student's t test. Different letters and asterisk above
835 columns represent significant differences ($P < 0.05$). Each mean ± SE is from at least
836 8 plants per group.

837

838 **FIGURE 6.** Effect of biostimulants on yield. **(A)** Mean ± SE total fruit number per
839 plant. **(B)** Correlation between mean ± SE total fruit number and cumulative daily
840 transpiration. Blue bars – untreated (with biostimulants) control plants; green bars –
841 ICL-SW-treated plants; orange bars – ICL-NewFo1-treated plants. Solid bars – well-
842 irrigated conditions; stippled bars – drought conditions. Groups were compared using
843 ANOVA by Tukey HSD test and Student's t test. Different letters and asterisk above
844 columns represent significant differences ($P < 0.05$). Each mean ± SE is from at least
845 8 plants per group.

846

847 **FIGURE 7.** Schematic model of plant responses to biostimulants (ICL-NewFo1 –
848 orange line, ICL-SW – green line, untreated – blue line) under drought and recovery
849 (modified from Moshelion et al., 2015). **(A)** Plant productivity vs. intensity and
850 duration of stress. Under conditions characterized by an ample water supply, ICL-
851 SW-treated plants have a higher transpiration level than ICL-NewFo1-treated and
852 control plants, and thus higher levels of productivity (e.g., photosynthesis) (Phase I).
853 As mild water stress develops (Phase II), ICL-SW-treated and control plants reduce
854 transpiration steeply with decreasing water availability, limiting productivity. In
855 contrast, ICL-NewFo1-treated plants show a relatively gradual decrease in
856 transpiration and productivity as a trade-off to the decline in leaf water potential and
857 relative water content. Nevertheless, after the initial drought (Phase II), their
858 productivity may still be higher than that of ICL-SW-treated and control plants which
859 have already reached minimal productivity. As drought stress becomes more severe
860 (Phase III), the transpiration values and productivity of ICL-NewFo1-treated plants
861 continue to decline to their minimum. **(B)** Evaluation of recovery from drought is an
862 important step in assessing drought resilience. It reveals the plant's resistance to
863 desiccation and ability to recover its pre-stress productivity, reflecting the extent of
864 the damage caused by severe drought, such as cavitation or leaf/root loss. Both the
865 ICL-SW-treated and control plants recover slowly compared to the ICL-NewFo1-
866 treated plants. ICL-NewFo1 contributes to drought resistance by inducing more
867 gradual water loss and resilience, thus contributing less to plant productivity and more
868 to plant survivability. However, ICL-SW induces relatively faster water loss, and only

[18]

869 increases productivity under optimal conditions while having no effect on the
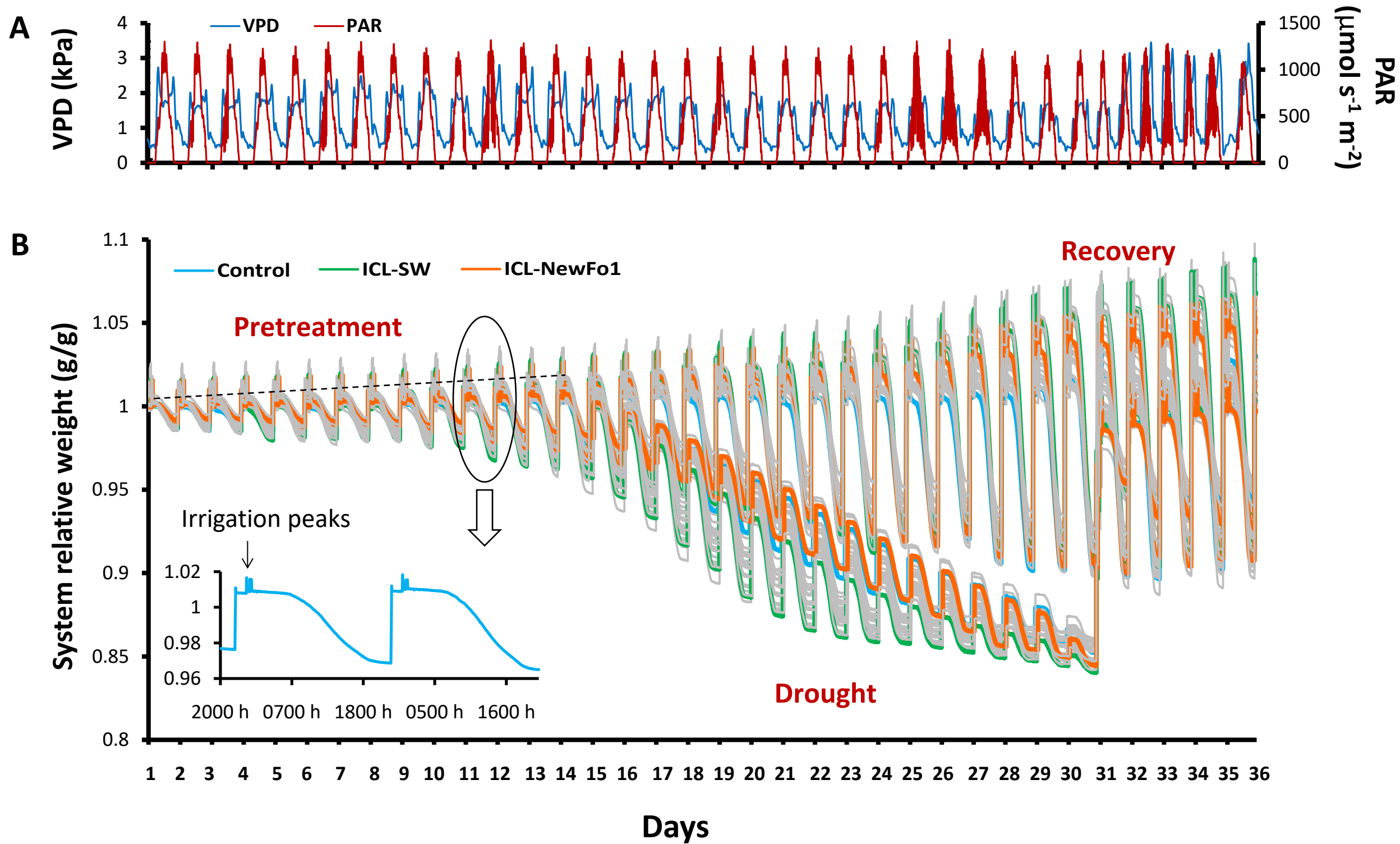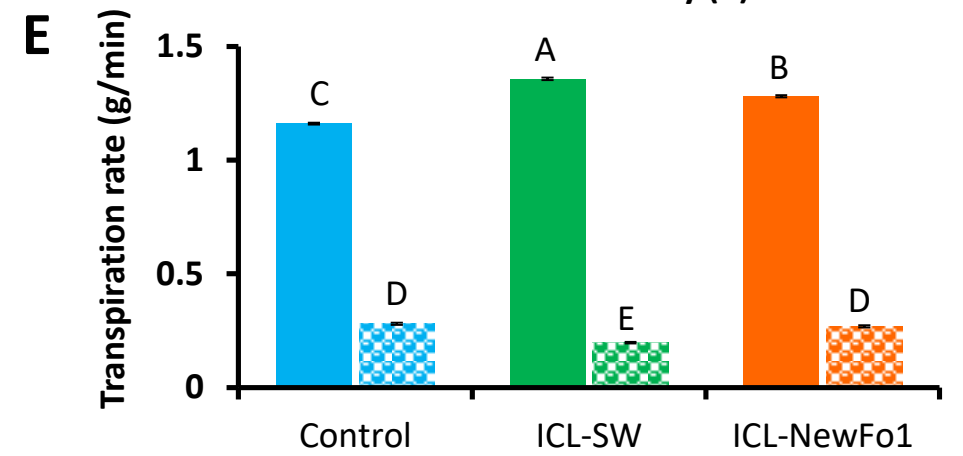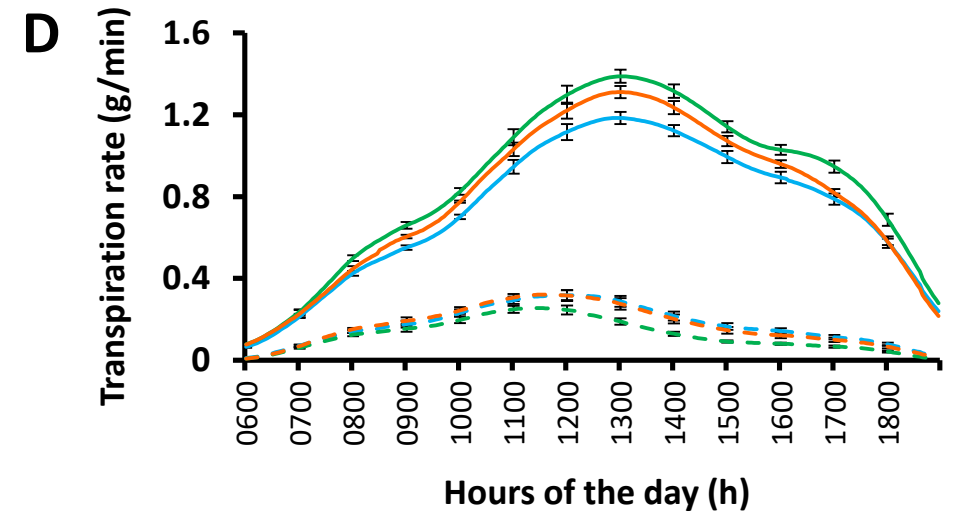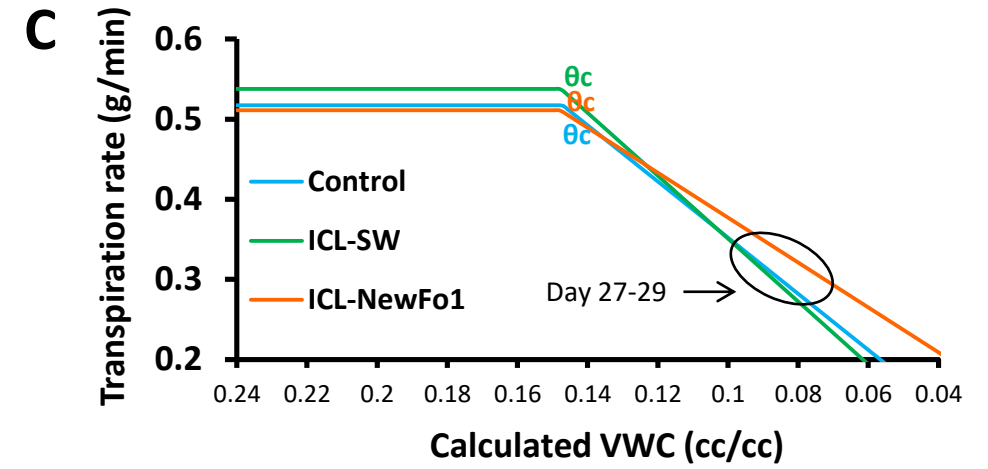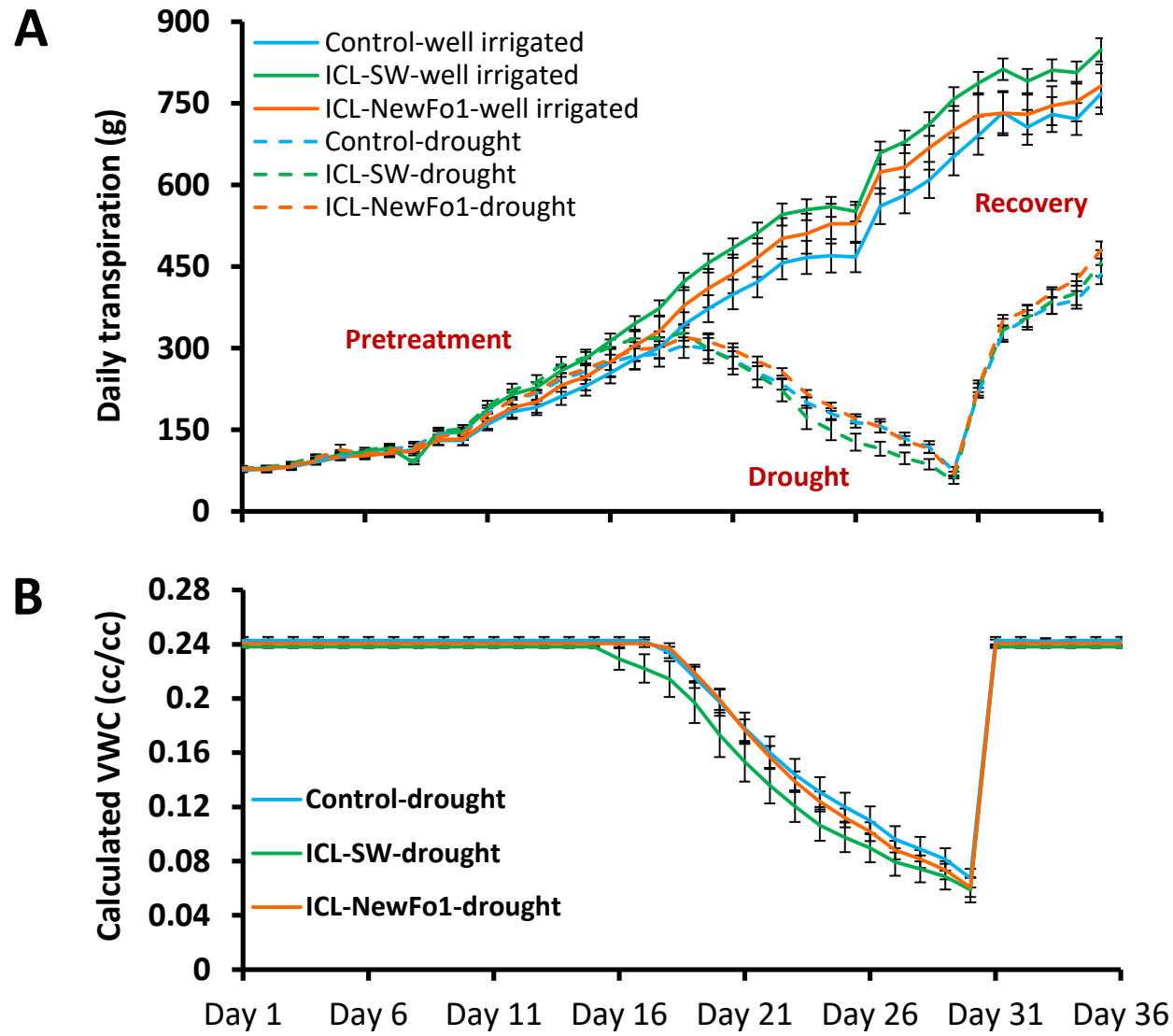870 survivability of the plants under drought.
871
872

Figure 1
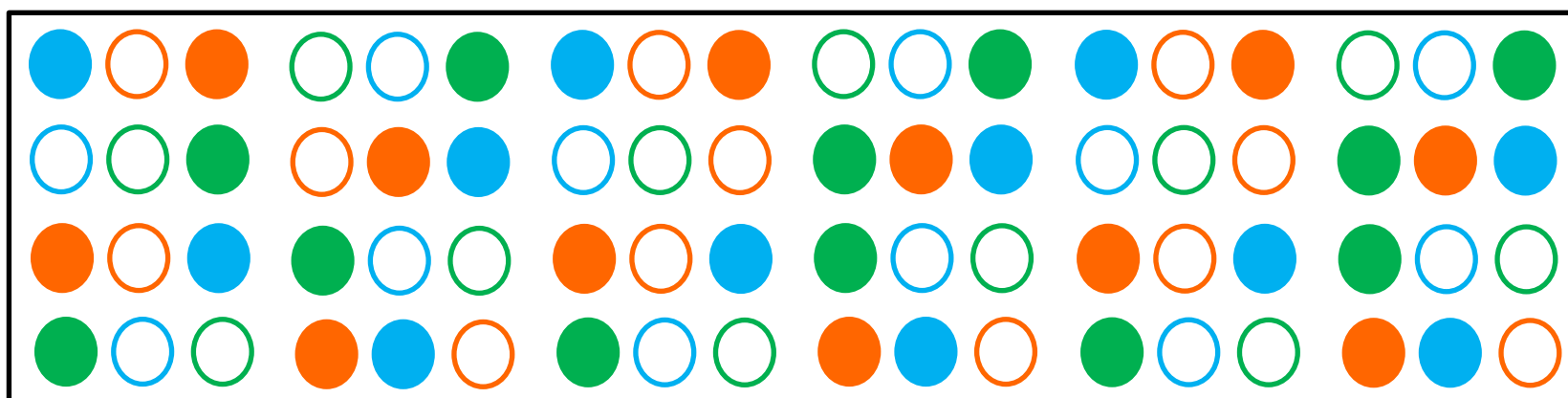
Randomized arrangement of plants

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7