AN EXPERIMENTAL SETUP FOR ASSESSING LIGHT AND MINERAL NUTRITION EFFECTS ON *ARABIDOPSIS THALIANA* HEYNH. PHENOTYPE

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Abstract

Being able to quantify the phenotype is very important, especially in relationship with the genetic background or the environment. Our research article tested an experimental setup for its ability to analyse the influence of light quality and nitrogen availability on the phenotype. For this purpose, *Arabidopsis thaliana* plants were grown in specially designed hydroponic setups placed in custom made light boxes and were analysed using imaging and image analysis techniques. Parameters for growth (projected rosette area, absolute growth rate, relative growth rate) and parameters for general morphology of the plant (compactness, stockiness) were assessed. Plants grown in red light achieved higher projected rosette area, but were more sensitive to changes in nitrogen concentration, while plants grown in blue light developed a smaller surface area, but were less sensitive to changes in nitrogen concentration. Compactness and stockiness were strongly influenced by light quality, having higher values for plants grown in blue light. Nitrogen concentration did not influenced compactness or stockiness parameters. Overall, the experimental setup and the methodology presented were robust and precise enough to produce good quality data and to allow the identification of both obvious and not so obvious effects of environmental factors on plant phenotype.

Key words: light, nitrogen availability, image analysis, phenotype

Sometimes, a big issue for plant scientists is to conceive appropriate experimental setups to help them tackle important research questions or hypothesis which are many times complex and difficult to address. In this background, our article contributes with an example of experimental setup used to assess light and mineral nutrition effects on *Arabidopsis thaliana* phenotype.

Plants differ a lot in appearance, even if we compare plants from the same species or varieties (Pérez-Pérez J.M. *et al*, 2002). This happens because the external aspect of plants is composed from a huge amount of traits, which are the expression of the genome, guided by the environmental conditions. These sums of traits are commonly referred to as phenotypes, and the biological research area that describes and measures the phenotypes is called phenomics. After all the advancement of the last couple of centuries in the field of plant genomics, it became clear that the development of phenomics as a research field should follow, in order to better understand how genes and the environment are shaping the final appearance.

The classical way to assess a phenotype involved using callipers or measuring fresh and dry weights of the plant at the end of the vegetative stage, to receive insight in the plant's aerial biomass. All this methods are either destructive, stressful for the plant or low resolution in terms of assessment over time (Dhondt S. *et al*, 2013). The solution came when non-destructive imaging of the plants started to be employed. Over the past decade, several research groups started to build custom-made plant phenotyping platforms equipped with imaging systems (Granier C. *et al*, 2006; Walter A. *et al*, 2007; Jansen M. *et al*, 2009; Arvidsson S. *et al*, 2011; Skirycz A. *et al*, 2011; Tisné S. *et al*, 2013; Apelt F. *et al*, 2015). Using image analysis methods, growth and other parameters can be quantified in a non-invasive manner.

Image analysis is the process of extracting relevant information from the raw data obtained using sensors. When the objective is to assess morphological traits, the most useful sensor is a camera. There are different examples of setups using cameras, some of them being even able to quantify information in three dimensions (Bours R. *et al*, 2012; Nagel K.A. *et al*, 2012; Apelt F. *et al*, 2015), but the most simple way, especially if the plants analysed have a planar development like

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Arabidopsis thaliana, is to take two-dimensional pictures from above using a single camera (Schmundt D. *et al*, 1998; Leister D. *et al*, 1999; Boyes D.C. *et al*, 2001; Granier C. *et al*, 2006; Arvidsson S. *et al*, 2011; Dhondt S. *et al*, 2014). Specific information is then extracted from the images using specialized image analysis software.

Growth in plants is a complex phenomenon, tightly controlled by developmental programs (Gonzalez N. *et al*, 2012) and strongly affected by environmental factors. From the environmental factors, light and mineral nutrients are probably two of the most important components that facilitate and regulate plant growth and development. Light, besides driving the photosynthesis and being the primary energy source that makes everything possible, acts as well as a signal, controlling gene expression, physiology and developmental programs (Carvalho S.D., Folta K.M., 2014). As a signal, light is perceived by the plants through a series of photoreceptors able to monitor the electromagnetic spectrum from 260 nm (UV-C) to ~730 nm (far-red) (Kami C. *et al*, 2010). Mineral nutrients, on the other hand, are powerful regulators of growth and development too, especially nitrogen which acts both as a nutrient and a signal for plant growth (Crawford N.M., 1995; Scheible W.-R. *et al*, 2004; Rubin G. *et al*, 2009).

In this background, our research was focused on developing a methodology robust enough to help us quantify some effects of light quality and nitrogen availability on mature plant phenotypes and shoot architecture.

MATERIAL AND METHOD

Plant material and the experimental setup

Arabidopsis thaliana ecotype Landsberg erecta (Ler) seeds, obtained from The Nottingham Arabidopsis Stock Centre (NASC), were used. Seeds were washed very well in an Eppendorf tube and left for 3 days with a drop of bidistilled water at 4°C for imbibition and stratification. Using a toothpick, seeds that looked bigger and most viable were placed on some seedholders (one seed per seedholder). Seedholders were made from Eppendorf tube caps with a 2.5 mm hole drilled on top, and filled with a solidified nutrient solution (1/16 Murashige and Skoog (1962) medium + 0.9 % agar (w/v)) providing support for the seeds and nutrition for the plants in the first few days of development (*figure 1 A*). The seedholders were placed in a hydroponic setup (*figure 1 B*) filled with ~500 ml nutrient solution. There were 4 hydroponic setups in total, representing 4 experimental variants, each variant containing 21 plants.

In the first day seeds were sown, placed in the hydroponic system and covered with cling film to maintain moisture for germination. In the fourth and fifth day after sowing (4 & 5 DAS) all roots of the germinated plants were gently fixed in the agar using the tip of a toothpick. At 6 DAS the cling film was perforated, starting the acclimation of the plants to growth room humidity. At 7 DAS the cling film was removed completely and the nutrient solution was replaced with fresh one in all four setups. At 8 DAS measurements for the phenotype begun and lasted until 15 DAS. At 11 DAS the nutrient solution was refreshed again.

Throughout the entire process the experimental variants were placed in custom made light boxes able to provide specific illumination conditions using light emitting diodes (LEDs) (OSRAM Golden Dragon, Osram Opto-Semiconductors GmbH, Germany). The light boxes were made from extruded polystyrene covered with aluminium foil for uniform illumination.

Growth conditions and experimental variation of light and nitrogen content

The experiment took place in a growth room at 24°C and 60% humidity. The photoperiod was set at 16 h light with 8 h dark, simulating long day conditions.

After sowing, all four experimental variants were placed under light. Two variants (V1 and V2) were placed under blue light (455 nm) and the other two variants (V3 and V4) were placed under red light (660 nm). The irradiance was set at 130 $\mu moli \cdot m^{-2} \cdot s^{-1}$ for all experimental variants.

Regarding the nutrient solution, all four variants were supplied with ¼ strength Murashige and Skoog (MS) medium until 11 DAS. At 11 DAS two experimental variants (V2 and V4) received ¼ strength MS medium as usual and the other two variants (V1 grown in blue light and V3 grown in red light) received modified ¼ strength MS medium (Nitrogen was decreased from 15 mM to 0.5 mM diluting both NH_4NO_3 and KNO_3 solutions 30 times; KCl was added to compensate for the lower potassium ions concentration).

Phenotype analysis

To analyse the phenotype was used a non-invasive technique based on imaging and image analysis. A commercially available imaging and image analysis system (Scanalyzer PL, LemnaTec, Wuerselen, Germany, http://www.lemnatec.com) was employed for data collection. The software setup for the phenotyping system was similar to that previously described by Arvidsson S. *et al* (2011).

The measurements started at 8 DAS and lasted for 8 days. The plants were measured once a day at ~2 hours (+/- 15 minutes) after lights turned on. From 21 plants per experimental variant, only 15 plants were taken into account, the rest being discarded as outliers (due to poor germination or improper growth).

The analysed parameters are grouped in two categories: one category that quantifies aspects of growth (projected rosette area (PRA), absolute growth rate (AGR), and relative growth rate (RGR)) and one category that quantifies aspects of morphology (Compactness and Stockiness). Vanhaeren H. *et al* (2015) offer a detailed description of the parameters and how they are calculated.

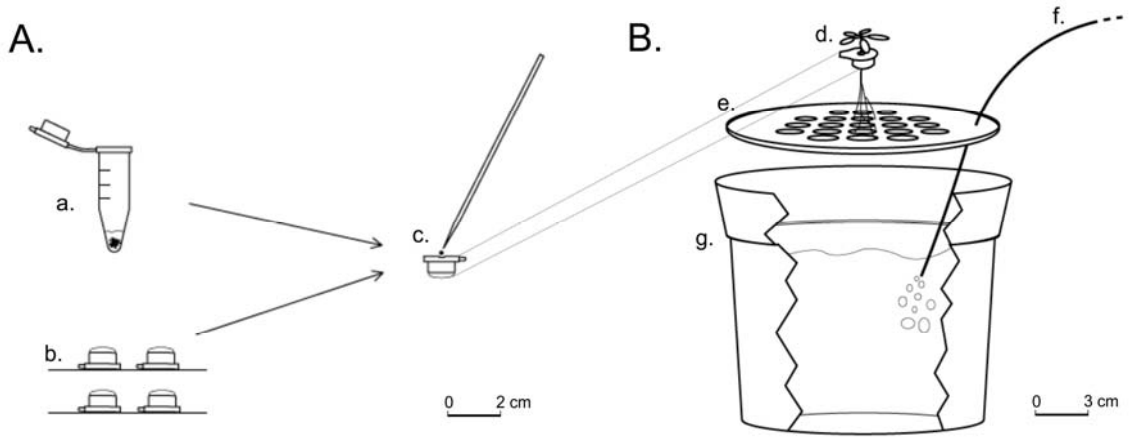

Figure 1 **The graphical representation of sowing Arabidopsis seeds (A) and the hydroponic system in which seedholders are placed (B). The elements present in the figure are: a – Eppendorf tube with seeds, b – perforated Eppendorf caps filled with agar, c – placing the seed using a toothpick, d – perforated Eppendorf cap with plant, e – support for the Eppendorf caps, f – aeration tube, g – container holding the nutrient solution.**

RESULTS AND DISCUSSIONS

The experimental setup

The Landsberg erecta ecotype germinated fast (after day one signs of germination could be seen) and uniform. The process of prior stratification and picking the seeds that looked bigger and most viable really helped with uniform germination and even growth.

We found out that placing just one seed per seedholder, instead of placing a couple and trimming them later, is a better choice because avoids tangling of the seedlings and results in less manipulation of the plants in the early stages when they are small and sensitive. However, some gentle manipulation is required at the fourth and fifth day after sowing in order to fix the plants firmly in agar and to give direction to roots that tend to grow on the surface of the agar. The seedholders made from perforated Eppendorf tube caps are an excellent choice because they can hold enough agar to confer plants stability and nutrition in the first stages of development, being as well really easy to move plants around. For example, Conn S.J. *et al* (2013) described a procedure in which seeds were germinated on Eppendorf caps in bulk and then moved to some other hydroponic setup. The seedholders and all the elements that come in contact with the growth medium should be of black or dark colour as stated by Conn S.J. *et al* (2013), in order to limit illumination of the solution and avoid algae growth. Blue light caused algal development where agar or liquid was in contact with it.

Using the 16 h light – 8 h dark photoperiod speeds up the life cycle of Arabidopsis plants (Karlsson B.H. *et al*, 1993; Martinez-Zapater J.M. *et al*, 1994), resulting in shorter and more stable experiments. Plants were analysed until first evidences of bolting started to show up and this happened around 15 DAS. Dhondt S. *et al* (2014) who used the same photoperiod observed bolting appearing around 19 DAS, but they used a different ecotype (Columbia-0) and some growing conditions were as well different (21°C temperature and 60 $\mu moli \cdot m^{-2} \cdot s^{-1}$ irradiance). During the analysed vegetative period, plants developed only six leaves which is very good for top imaging because reduced leaf superposition, giving more accurate results.

Even though other researchers (Dhondt S. *et al*, 2014) reported using a concentration of ½ strength Murashige and Skoog medium, we found it to be too concentrated for normal plant growth and used instead ¼ strength. In order to achieve deprivation, the nitrogen concentration for the modified MS medium was similar with that reported by Martin T. *et al* (2002).

Light quality and nitrogen availability effects on phenotype

The variants grown in red light resulted in a larger projected rosette area than those grown in blue light for the most part of the experiment

(*figure 2* a). Only in the last two days of the measurements, second experimental variant (V2) increased its projected rosette area becoming the largest at the end of the experiment. The absolute growth rate graph points as well to a difference in growth between experimental variants grown in blue light and those grown in red light (*figure 2 b*). The difference in growth between red and blue started to be visible only after day 8, which is a good indication that 8 DAS is a convenient time to start the measurements.

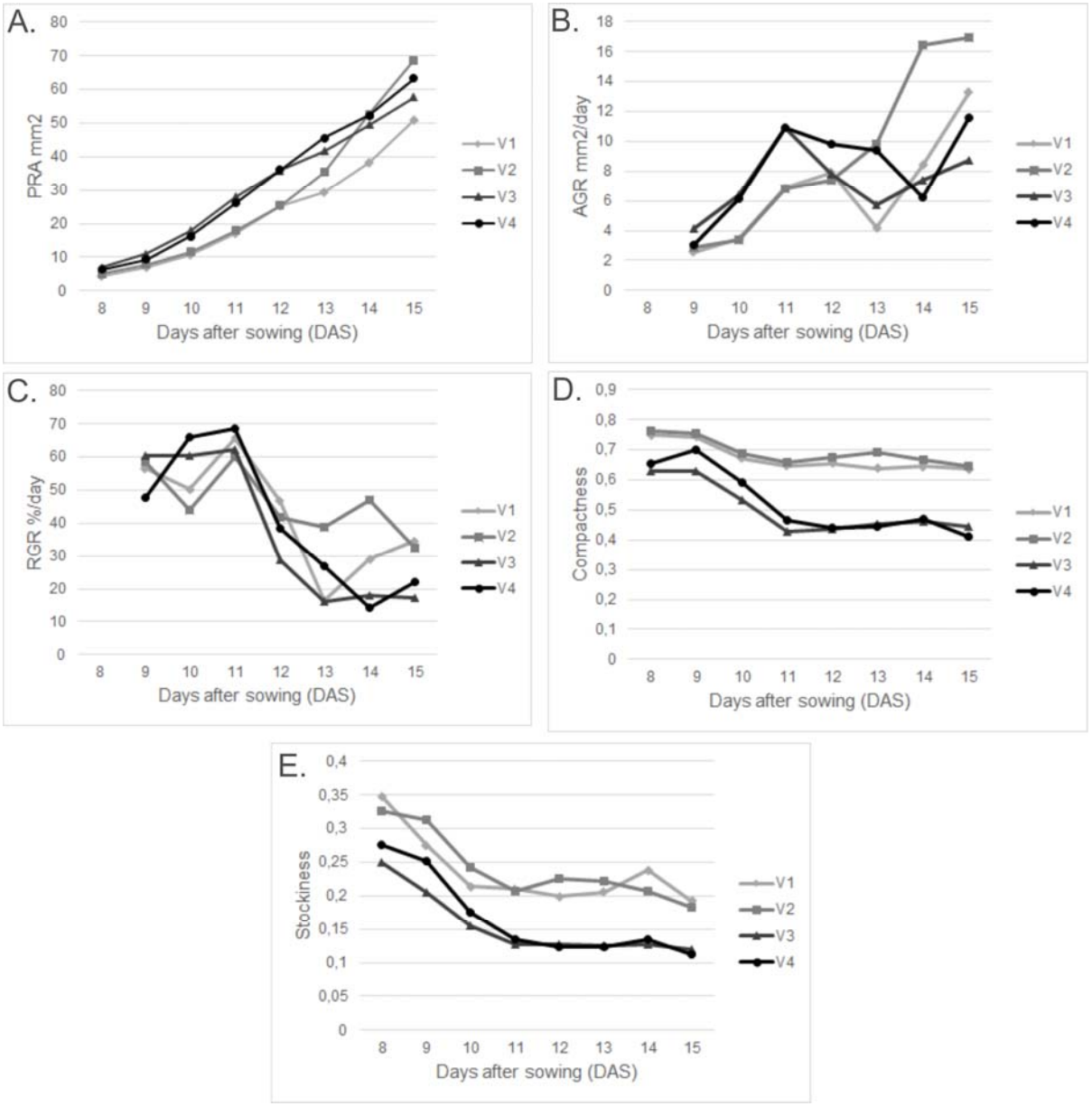

Figure 2 **Graphical representation of phenotype parameters: a – Projected rosette area (PRA), b – Absolute growth rate (AGR), c – Relative growth rate (RGR), d – Compactness, e – Stockiness.**

After day 11 when the nutrient solution is refreshed, growth is slowed down in all experimental variants, this aspect being more visible on relative growth rate graph (*figure 2 c*). The variants which received low nitrogen nutrient solution (V1 and V3), however, exhibited more reduction in growth. An interesting thing is that variants from blue light (V1 and V2) showed less decrease in growth than variants grown in red light (V3 and V4) (*figure 2 b, 2 c*), pointing to the hypothesis that light as a signal can influence how plants react and assimilate mineral nutrients. Plants grown in red light, even though achieve higher projected rosette area, are more sensitive to nitrogen deprivation than plants grown in blue light. Probably this is the explanation to why plant from variant V2 were less affected post 11 DAS

and summed the highest projected rosette area at the end of the experiment.

The morphological parameters (compactness and stockiness) registered a decrease in the first few days of the measurements and became stable afterwards (*figure 2 d, 2 e*). This is normal and it is something encountered by other researchers too (Dhondt S. *et al*, 2014). The difference appears between variants grown in blue light and those grown in red light. Red light promotes larger rosette area but smaller compactness and stockiness than blue light. Interesting to mention is that compactness and stockiness between plants grown in red and blue light, presented already a differentiation at the start of the measurements (8 DAS) (*figure 2 d, 2 e*). This represents another evidence that light starts to influence plant architecture early on. Nitrogen availability does not seem to have an effect on plant compactness or stockiness.

CONCLUSIONS

The experimental setup is robust and precise enough to produce good quality data and to allow the identification of both obvious and not so obvious effects of environmental factors on plant phenotype. In the growth conditions used for this experiment, eight days after sowing is a good time to start the measurements for the growth parameters, but for the morphological parameters might be better to start earlier.

Plants grown in red light tend to have a larger surface area, but appear to be more sensitive to fluctuations of nutrient concentration than plants grown in blue light. However, this requires further investigations. In contrast with nitrogen availability, light spectrum appears to be the only factor that influences compactness and stockiness. Plants grown in red light have higher values of both compactness and stockiness.

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