In Vitro Photoautotrophic Arabidopsis Culture (PAC) Manual

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Table of contents

1

In-vitro photoautotrophic culture of *Arabidopsis thaliana*

PAC summary

Photoautotrophic culture can assure more normal physiology of plants in vitro. *Arabidopsis thaliana* can be grown in-vitro on sugar-free media if the appropriate conditions can be met. These conditions include:

Ventilation of vessel. CO_2 is the primary limiting factor of photosynthesis *in vitro* (followed by light intensity and presence of sugar). CO_2 can be supplied into the vessel by enhancing natural ventilation (diffusion).

Sufficient light intensity. A minimum of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux is necessary. This light intensity must be measured with **a quantum sensor** and should be measured at the plant level.

Photoperiod. *Arabidopsis thaliana* is a quantitative long-day plant. Short day conditions of 8 to 10 hours of light can prevent the plant from bolting at young vegetative growth stage. Long-day conditions of 16 hours will assure bolting and flowering. Photoperiod needs to be selected based on the purpose of experiment, but photoautotrophic plants *in vitro* tend to bolt earlier than greenhouse grown plants or photomixotrophic plants grown in sugar containing media. Therefore, photoperiod control is very critical in PAC.

2

In-vitro photoautotrophic culture of *Arabidopsis thaliana*

PAC summary cont…

Temperature. A light period temperature of 22-23 °C is ideal and the dark period temperature can be lower than that (i.e., 18-21 °C). However, lowering night temperature lowers 24-h average temperature and thus slows the overall growth.

Nutrient solution. In this manual, a general purpose hydroponic nutrient solution was considered. The nutrient formulation is listed in table 1. However, other commonly used nutrient solutions, such as Hoagland and Arnon (1938), Lloyd and McCown WPM (1981) or half-strength of Murashige and Skoog (1962) can be used if preferred.

Substrates.
Porous substrate (containing pores filled with air even when saturated with water) generally results in the best plant growth. However, agar can be used if it is more preferable for the experiment. Depending on the substrate used, the nutrient solution needs to be adjusted for pH so that the pH is in an acceptable range throughout the growth period.

3

Tips on culture vessels and ventilation

Culture vessel. A clear culture vessel is necessary to allow maximum light transmission. The culture vessel must be vented with microporous filter(s) to allow sufficient gas exchange to allow photosynthesis to occur. The preferred vessels are GA-7 vessels (Figure 1) with two Milli-seal filters (pore size 0.45 μm, covering two 8 mm holes) which meet these criteria. The vented Microbox™ culture vessel with green (color code for extra gas exchange) filter is also acceptable (Figure 2). Both are autoclavable and reusable for several times.

Figure 1. GA-7 vessel with microporous filters. Filters are available as seen here.

Figure 2. Microbox vessel with microporous filter.

4

More on culture vessels and ventilation

Figure 3

Figure 4

Millipore sells a pack of 375 Milli-seal adhesive filter disks (Figure 3). The company does not recommend autoclaving (as a small number of filters may become detached under certain conditions). But generally this product has been observed to work without failure even after many autoclave cycles.
http://www.millipore.com/catalogue/item/fwms01800

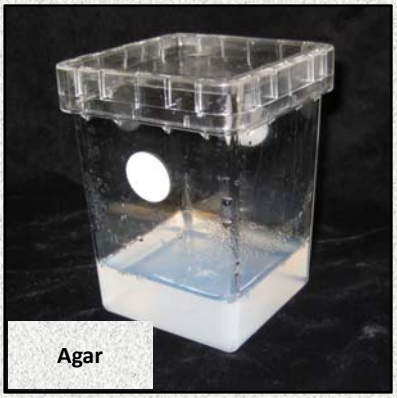
Figure 4. The Microbox is a clear polypropylene box, equipped with a hermetically closing polypropylene cover. The cover is made of crystal-clear PP plastic and provided with a PP filter and can be autoclaved several times.
http://www.combiness.com/Combiness.htm

5

Tips on substrate (supporting material)

Substrate. Various substrates can be used for photoautotrophic culture (Figure 5). In general, substrates with good air porosity and stable pH properties promote plant growth through enhanced root activity and nutrient uptake. However if no solid, porous, pH stable substrates are available, agar can be used. Different substrates require different preparation (see later section).

Figure 5.



Agar

Rockwool
1 inch cubes

6

Tips on seed germination

Medium for germination:
Seeds are best germinated on a standard germination media, such as ½ strength MS media containing 1% sucrose (10 $g \cdot L^{-1}$), using standard seed preparation, disinfestation and seeding protocols.
Photoperiod:
Note that seeds should be germinated under the same short-day photoperiod that the plants will be grown under, as long-day photoperiod during germination to early vegetative stage seems to induce flowering.

(left) When seeds were germinated under long day (16 hours photoperiod) for 2 weeks (to two true leaf stage) and transferred to short day, nearly 100% bolting was observed after 2 weeks in PAC.

(right) When seeds were germinated under short day (10 hours photoperiod) for 2 weeks and transferred to short day, no bolting was observed even after 3 weeks in PAC.

7

Tips on transplanting seedlings

Plant size. Seedlings need at least two fully expanded true leaves (Figure 6) before transplanting to photoautotrophic conditions. This can be achieved after 10-14 days from seeding. Transplanting after four fully expanded leaves (Figure 7) is more challenging to do because the roots are longer.

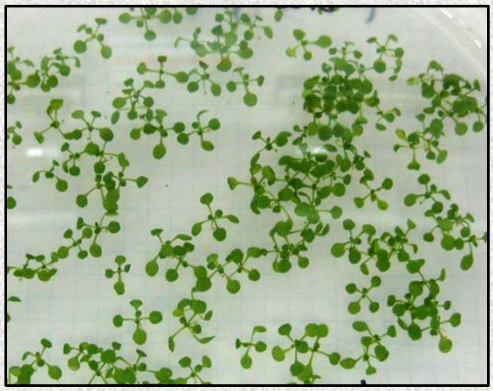


Figure 6. Example of seedlings with 2 cotyledons plus 2 true leaves, ready to transplant from germination plate containing 1% sucrose.

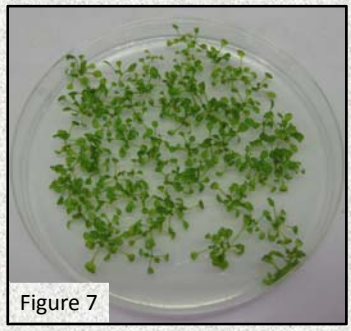


Figure 7

8

Tips on transplanting

Transplanting:
Plants should be transplanted into the vented vessel, using sterile technique and forceps. A hole should be made in the substrate to accept the seedling roots, and the hole can be made simply with the sterile forceps (Figure 8). Rockwool cubes already have holes.
Seedlings should be carefully removed, one at a time, from the germination media and planted into the substrate (Figures 9 a,b). Plant 4-6 plants per GA-7 vessel, evenly distributed in the vessel.

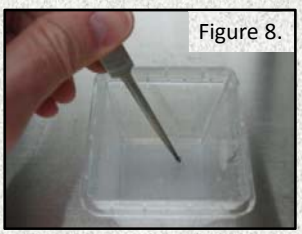


Figure 8.


Figure 9a.

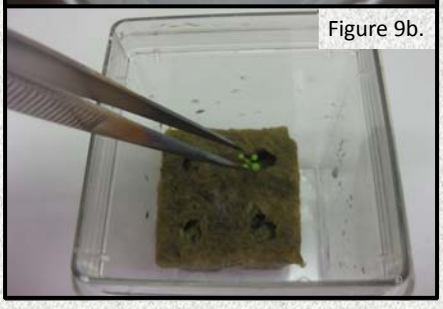
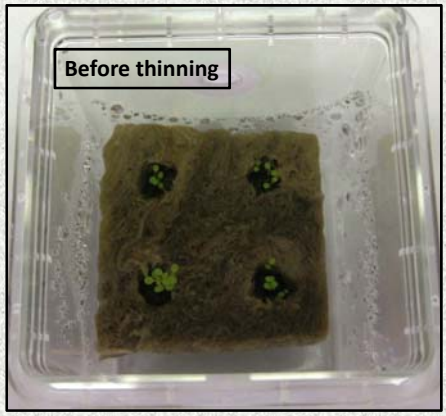
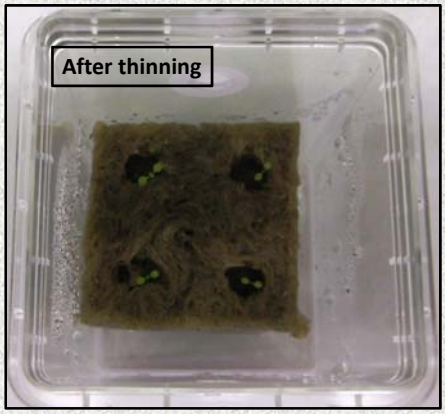


Figure 9b.

More tips on planting materials

If the seed lot being used has strong germination performance, it is possible to eliminate germination on sucrose containing media and directly sow disinfested seed into the PAC vessel on sugar-free media. Sowing seeds singly is challenging so thinning is usually necessary after germination. Seeded vessels can be placed immediately under high light.





10

Tips on media preparation

Media stock preparation:

Hydroponic nutrient solution stock is prepared as a 100X concentrated stock solution. It is prepared in 2 different stock solutions, A and B, as described in the formulation in table 1. Separate stock solutions prevent precipitation by keeping reactive compounds (namely Ca^{2+}, PO_4^- and SO_4^-) separate. It is not necessary but if preferable, 3 different stock solutions can be prepared: macronutrient stock A, macronutrient (Ca^{2+}) stock B and a separate micronutrient stock C.

Media preparation:

With 100X stock solution, nutrient solution used in vessels is prepared by adding 10 ml of each stock solution to make up a final volume of 1 liter. Sucrose is NOT added to the nutrient solution for PAC.

Media pH can be adjusted by adding NaOH or HCl. The pH should be adjusted so that the media pH remains in an acceptable range throughout the culture period. For the hydroponic nutrient solution describe in table 1, a starting pH of 5.8 is usually acceptable. It is recommended that prior to culturing with critical plant material, the media to be used is prepared, dispensed with the substrate to be used and autoclaved to determine the effect of the substrate and autoclaving on the media pH. Once an acceptable starting pH is determined for your particular media and substrate, test your system prior to use with non-critical plant material.

11

Table 1. Recommended general greenhouse hydroponic nutrient solution (modified after Jensen, unpublished). Molar concentration and full strength media concentration is provided along with 100X concentration stock recipe.

Component	Molar	Full strength	100X stock
Stock solution A			
KNO_3	1.93 mM	195.0 mg L^{-1}	19.5 g L^{-1}
KH_2PO4	1.52 mM	206.5 mg L^{-1}	20.6 g L^{-1}
$MgSO_4$-7H2O	2.47 mM	608.6 mg L^{-1}	60.9 g L^{-1}
K_2SO_4	2.75 mM	478.9 mg L^{-1}	47.9 g L^{-1}
NH_4NO_3	2.71 mM	217.1 mg L^{-1}	21.7 g L^{-1}
$Na_2B_4O_7$-10H_2O	30 µM	3.0 mg L^{-1}	300 g L^{-1}
$MnSO_4$-H_2O	10 µM	1.69 mg L^{-1}	169 g L^{-1}
$ZnSO_4$-7H_2O	5.0 µM	1.45 mg L^{-1}	145 g L^{-1}
$CuSO_4$-5H_2O	0.8 µM	0.195 mg L^{-1}	19.5 g L^{-1}
Na_2MoO_4-2H_2O	0.5 µM	0.125 mg L^{-1}	12.5 g L^{-1}
Stock solution B			
$Ca(NO_3)_2$-4H_2O	3.12 mM	736.5 mg L^{-1}	73.6 g L^{-1}
$CaCl_2$	1.87 mM	275.1 mg L^{-1}	27.5 g L^{-1}
Fe Sprint 330	0.036 mM	20.0 mg L^{-1}	2.0 g L^{-1}
Or instead of Sprint 330			
Fe(III)EDTA (MW 385.06)	0.036 mM	13.9 mg L^{-1}	3.31 g L^{-1}

12

Table 2. Comparison of elemental composition of different nutrient solutions. Values are in $mg \cdot L^{-1}$.

Element	GH hydroponic	Hoaglands	WPM	1/2x MS
NO_3-N	152	196	136	276
NH_4-N	38	14	70	144
P	47	31	39	19.5
K	350	235	493	392
Mg	60	49	36	18
Ca	200	160	120	60
S	167	64	230	24
Cl	133	0.0	46	106
B	0.34	0.5	1.1	0.54
Mn	0.55	0.5	7.25	2.75
Fe	2.0	1.01	5.6	2.8
Zn	0.33	0.05	1.96	0.98
Cu	0.05	0.02	0.06	0.005
Co	0.0	0.0	0.0	0.005
Mo	0.05	0.01	0.1	0.05
I	0.0	0.0	0.0	0.32

13

Tips on media preparation with Rockwool cubes

For Rockwool cubes.

Rockwool is designed for the frequent irrigation of greenhouse production. Because Rockwool cubes (Grodan AO25/40) initially contain soluble compounds inhibitory to seedling growth, they must be washed prior to use in culture. The high heat of autoclaving facilitates the removal of the compounds. Submerge the Rockwool cubes in DI water. Autoclave on a 15 minute liquid cycle. Allow to cool to a safe temperature. Drain the water and refill with DI water and allow to stand 10 minutes at room temperature and drain. Refill again with DI water and drain after 10 minutes. This is usually sufficient but may be repeated if desired. Place in a drying oven to dry. Once dry they are ready to use.

A sheet of cubes submerged in DI water in a tray.

2x2 cube sections submerged in DI water in a beaker.

More on media preparation with Rockwool cubes

A GA-7 vessel holds four 1 inch Rockwool cubes (2 x 2). These can be cut out of the sheet of cubes so they remain together for ease of handling (Figure 10). Place the washed and dry, four 1 inch cube sections in the GA-7 and add 75 mL of prepared PAC medium. Put on the cover and autoclave at standard time, temperature and pressure.
For other vessels, add as many cubes as fit and add sufficient media.

Figure 10

15

Tips on media preparation with agar

<u>Agar gelled medium.</u>
Prepare hydroponic nutrient solution and adjust pH to 5.8. Add 0.6% (6 g per liter) of a plant tissue culture agar. Stir until dispersed and then microwave until agar is dissolved. Add about 50-60 mL of nutrient agar solution to the vented GA-7 vessels, cover and autoclave. Allow to cool in a clean hood.
Figure 7 shows nutrient solution with dissolved agar in Pyrex bottle. 50-60 mL dispensed into GA-7 vessel prior to autoclaving.

For other types of culture vessels, add sufficient volume to overcome the water loss due to ventilation and plant growth for that particular vessel and for the expected time in culture.

16

Growth chamber conditions

Once the plants have been planted into the culture vessel, seal the cover of the vessel with a single wrapping of micropore surgical tape (figure 11) or Parafilm™. Place the vessels into a growth chamber with appropriate light intensity (minimum 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$), photoperiod, and temperature (figures 12, 13).

Figure 11.

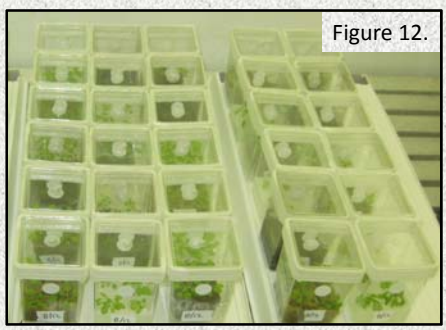


Figure 12.

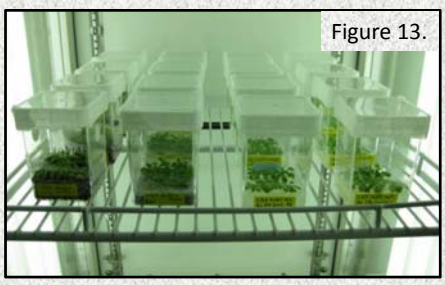


Figure 13.

17

Maintenance

Depending on external environmental conditions, it may be necessary to add sterile distilled water after several weeks of culturing, due to water loss from evaporation. Evidence of this would be lack of visible free liquid (tip the vessel to see if liquid accumulates in a corner of the vessel), or noticeable shrinkage of agar medium. Amount of water loss could be estimated by comparing the vessel weight (after 2-3 weeks) to the initial vessel weight. Change in weight can be attributed to water loss. To add water, work in a clean hood and use sterile technique, remove the tape, remove the cover and add 2-4 mL of sterile distilled water using pipettor with sterile pipette tip. Apply new tape to seal vessel and return to growth chamber.

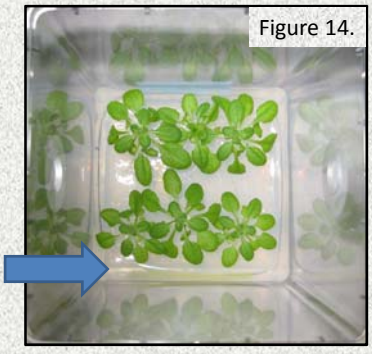
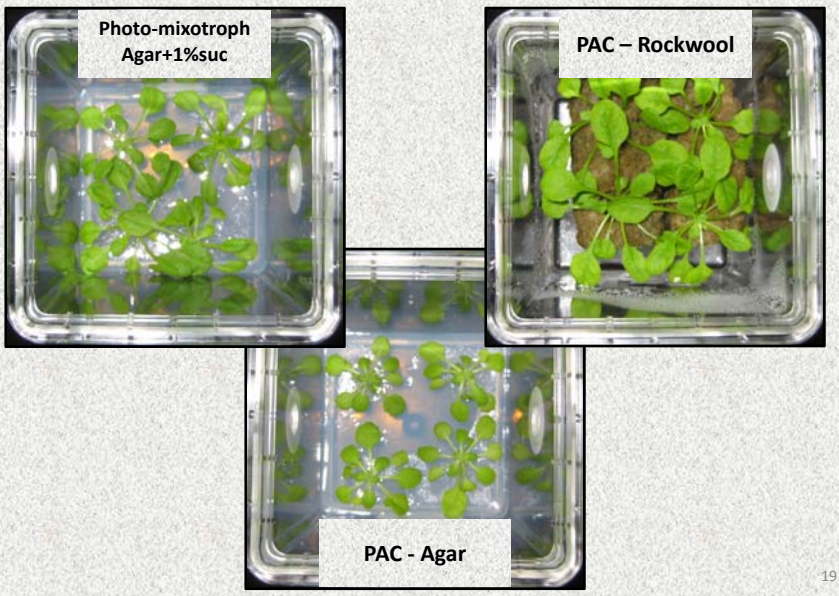


Figure 14.

Figure 14 shows drying agar pulling away from the sides of the vented vessel.

18

Examples of *Arabidopsis thaliana* 'Columbia'.
Plants 30 days after seeding (3 wks in photoautotrophic culture)

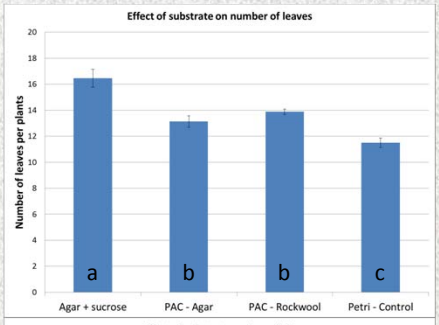


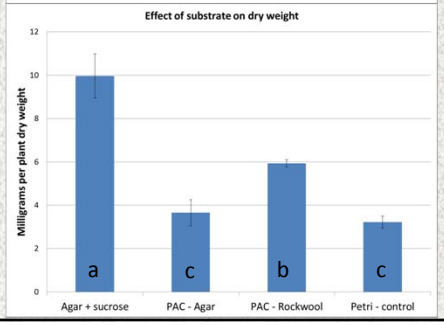
19

Examples of growth of *Arabidopsis thaliana* 'Columbia'.
Plants 30 days after seeding (3 wks in PAC)

PAC plant with hydroponic media

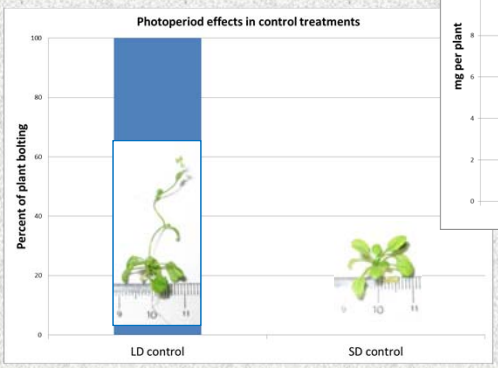
SD control plant with sugar containing MS in petri dish

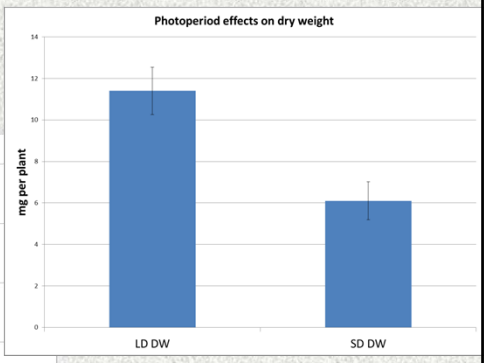




Examples of photoperiod effects in control treatments

Charts at right shows that plants grown under long-day conditions accumulated more dry weight due to greater duration of light, however…





…chart at left shows that 100% of plants under long-day conditions bolted while no plants under short-day did.

LD control plant with sugar containing MS in petri dish – 100% bolting

SD control plant with sugar containing MS in petri dish – 0% bolting

21

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22