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1 **A simple high efficiency and low cost *in vitro* growth system for phenotypic**

2 **characterization and seed propagation of *Arabidopsis thaliana***

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16 **ABSTRACT**

17 ***Background***

18 Arabidopsis research relies extensively on the use of *in vitro* growth for phenotypic analysis
19 of the seedlings and characterization of plant responses to intrinsic and extrinsic cues. For this
20 purpose, stress-free optimal growth conditions should be set up and used as a reference
21 especially in studies aimed at characterizing the plant responses to abiotic and biotic stresses.
22 Currently used standard *in vitro* protocols for growth and characterization of *Arabidopsis*
23 *thaliana* plants often suffer from sub-optimal composition due to an excessively high
24 nutritional content which represents a stress per se and an experimental bias.

25 ***Results***

26 We describe a simple protocol for *in vitro* growth of Arabidopsis plants in which the
27 phenotypic analysis is based on an optimized and nutritionally balanced culture medium. We
28 show that the protocol is robustly applicable for growth of several Arabidopsis mutants,
29 including mutants lacking the root system. This protocol enables rapid high scale seed
30 production *in vitro* avoiding soil usage while saving space and time. The optimized *in vitro*
31 protocol aims at: 1) making *in vitro* growth as close as possible to natural soil conditions by
32 optimizing nutrient balance in the medium; 2) simplifying phenotypic and molecular
33 investigation of individual plants by standardizing all steps of plant growth; 3) enabling seeds
34 formation also in genotypes with severe defect in the root system; 4) minimizing the amount
35 of waste and space for plant growth by avoiding soil usage.

36 ***Conclusions***

37 Here we report an optimized protocol for optimal growth of *Arabidopsis thaliana* plants to
38 avoid biases in phenotypic observation of abiotic/biotic stress experiments. The protocol also
39 enables the completion of the whole life cycle *in vitro* within 40-45 days and a satisfactory

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40 seed set for further propagation with no need for facilities for plant growth in soil and seed
41 sterilisation.

42 ***Keywords***

43 Nutritional stress, Reproductive development, Seeds formation *in vitro,* Root-less mutants

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44 **Introduction**

45 Over the last decade, Arabidopsis has become a widely adopted model system in plant
46 biology for a variety of reasons. These include a short life cycle, its fully annotated genome,
47 the simplicity of the root system, the availability of natural diversity sets, large numbers of
48 mutants and easy procedure of genetic transformation [1].

49 Current research largely relies on the extensive use of *in vitro* growth of seedlings for
50 phenotypic analysis and characterization of plants' responses to intrinsic and extrinsic cues.
51 Physiological and molecular biology experiments, aimed at unravelling plants' responses to
52 abiotic and biotic stresses, in principle require starting plant material that has been grown in a
53 nutritionally stress-free environment before and during analysis. Hence, growth conditions
54 should as be as close as possible to the optimal. In the majority of cases researchers use
55 culture media based on Murashige and Skoog composition [2], which has been originally
56 designed for callus growth and is characterized by a specific nutrient balance with high
57 nitrogen contents, a high N:P ratio (60:1.25) and with chloride as a major anion. These ratios
58 and absolute contents do not fit with nutrient content in plant tissues and nutrient's ratios
59 which are widely known to be optimal for plant nutrition [3]. In many cases such a medium
60 itself may induce stress-associated phenotypes [4]. A hallmark of this phenotype is the time-
61 dependent change in the Arabidopsis meristem size that reaches its maximum at 5 days after
62 germination while root growth almost stops at day 8 [5]. This impact of medium composition
63 on plant growth and phenotypic behavior is also exampled by Arabidopsis growth on the
64 more balanced modified Hoagland medium where the root meristem size increases up to day
65 12, and the root's growth continues till 3 weeks [6, 7]. These effects are particularly relevant
66 considering that one of the central aims of adopting *in vitro* culture of seedlings is the
67 investigation of the root system architecture (RAS). Root architecture is notoriously
68 dependent on nutrient availability [8, 9]. For these reasons, it is essential that for adequate

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69 interpretation of physiological and molecular results, especially when the adaptive strategies
70 to changing environments are the focus of research, a nutrient balance as close as possible to
71 the one existing in soil should be adopted for *in vitro* culture media.
72 Hydroponic culture, as an alternative for soil culture, represents a reliable procedure in terms
73 of space and reproducibility. Hydroponics itself also requires a completely different optimal
74 medium composition in order to avoid representing a stress per se [4].
75 In addition, in the commonly used protocols after initial *in vitro* growth and characterization,
76 mutant or wild type plants are typically transferred to soil pots for further phenotypic
77 evaluation and for propagation (seed production).
78 This procedure requires soil and is costly in terms of time and greenhouse space, while it
79 generates a large amount of waste. Although the Arabidopsis time span per generation is
80 relatively short, the completion of its life cycle from-seed-to-seed in soil requires around 6 to
81 8 weeks under optimal conditions in greenhouse [10]. Greenhouse conditions require
82 significant energy inputs for plant care including watering, nutritional supplementation and
83 protection from pests and fungi with the use of chemicals or through biocontrol. Seeds arising
84 from greenhouse grown plants require further sterilization, a process that is not always
85 successful and may give rise to contaminations and loss of (sometimes precious) material.
86 Using average laboratory conditions, the full growth cycle of Arabidopsis plants usually takes
87 up to 10 weeks and requires labor and space for successful growth and propagation.
88 Considering these limitations, we have sought for a suitable, simple, rapid procedure to be
89 carried out entirely *in vitro*, based on a culture system with nutrient composition optimized for
90 plant growth and characterization. This system minimizes nutritional stress, by being more
91 closely similar to natural conditions than the commonly used MS medium, and resembles
92 growth of plants in greenhouse in terms of seed set and seed maturation. A procedure aimed at
93 obtaining seed-set entirely *in vitro* and at shortening the seed-to-seed generation time in

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94 Arabidopsis, has been previously described [11]. This procedure was based on the
95 germination of mature seeds in picloram and benzyladenine in the first generation and of
96 immature seeds in the following generations, thus allowing the authors to obtain up to 12
97 generations annually [11]. However, the amount of seeds generated by using this procedure
98 appeared limited and the method was shown to be successfully applicable only to some
99 Arabidopsis ecotypes. In addition, immature seeds, while accelerating the propagation speed,
100 do not allow making precise quantitative characterization of each generation and introduce a
101 significant bias in experiments aimed at evaluating phenotypes related to all aspects of plant
102 biology.
103 The method we present here allows completing the whole *Arabidopsis* life cycle within 40-45
104 days, starting from mature seeds while ensuring phenotypic observation of plants in the
105 absence of external or additional perturbations (e.g. hormone treatments or use of immature
106 seeds) and a satisfactory seed set for further investigations.
107

108 **Methods**

109 ***Plant material***

110 *Arabidopsis thaliana* (L.) Heyhn lines Col-0, auxin response mutants *tir1* [12], *tir1xafb1,2,3*
111 [13], *plt1,2,3* [14], *miao* [15]).

112 ***Reagents***

113 1. TK1 medium, for *in vitro* growth of seedlings (Table 1). Stock solutions were made by the
114 following way: x2000 Micro salts stock. Dissolve in the following order: 25 mg
115 $Na_2MoO_4 \cdot 2H_2O$, 800 mg $H_3BO_3$, 1800 mg $MnCl_2 \cdot 4H_2O$, 20 mg $CuSO_4 \cdot 5H_2O$, 20 mg
116 $CoCl_2 \cdot 6H_2O$, 180 mg $ZnSO_4 \cdot 7H_2O$, 80 mg NaJ in a final volume of 100ml $ddH_2O$. 400x
117 Fe-chelate stock: Dissolve 372.2 mg $Na_2EDTA \cdot 2H_2O$ and 278.5 mg $FeSO_4 \cdot 7H_2O$ in
118 separate glass beakers under continuous stirring at 70°C in 20 ml $ddH_2O$ each. Mix the

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119 solutions and prolong heating till the solution gets a gold-yellow color. Adjust volume to
120 50 ml.
121 Crucial: AM (Arabidopsis medium corresponding to ½ MS, Murashige and Skoog medium)
122 medium has an N:P:K ratio of 60:1.25:20 which is sub-optimal for plant growth ([2]). A
123 more balanced medium in terms of nutrient ratio (TK1 medium) (see text for explanations)
124 is suggested for *in vitro* growth of plants, especially in studies focusing on plant nutrition
125 and stress response. Hoagland's (Hg) medium [16] (Table 1) supplemented with 2%
126 sucrose for *in vitro* seed propagation. Crucial: 2% sucrose is essential for high recovery of
127 seeds.
128 2. Seed sterilisation solution (20% sodium hypochlorite Roth, cat. no. 9062.3).
129 3. Murashige and Skoog (MS) medium (Duchefa, cat. No. M0245.0050).
130 4. Sucrose (Roth, cat. no. 9097.1).
131 5. Peptone ex casein (Roth cat. No 8986.1)
132 6. Sterile de-ionised water.
133 ***Materials***
134 1. Square 12 cm Petri plates.
135 2. Glass tubes (Scott Duran, 18 cm) with cotton tops. Crucial: cotton top is essential for
136 proper aeration and seed drying. When a plastic top is used, humidity remains high and
137 seeds do not dry.
138 3. Forceps, Scalpels (sterile).
139 4. Miracloth (Calbiochem Catalog No. 475855).
140 5. Tops: (Rotilabor, Kultur stopfen CTE0.1).
141 ***Procedure***
142 Overview of procedure steps and time required for each step.
143 1. Seeds sterilization (can be omitted if *in vitro* formed seeds were used); approximately 50

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144 min -1 h.

145 2. Plant growth on TK1 medium, for phenotypical and genetic characterization;
146 approximately 17-22 days.

147 3. Transfer of plants to Hoagland medium in glass tubes; approximately 30 min.

148 4. Seed formation in Hoagland medium in glass tubes; approximately 7-10 days.

149 5. Seed drying and collection; approximately 7-10 days.

150 ***Procedure details***

151 ***Plant growth and characterization***

152 ***Step 1. Seed sterilization and cold treatment***

153 Place seeds in a Miracloth (Millipore, catalog number 475855) bag and transfer to 20%
154 NaOCl solution, containing 0,05% Tween 20. Apply short-time vacuum for 1 minute and
155 incubate seeds for additional 7 minutes. Wash seeds in sterile distilled water 3 times, 5
156 minutes each, and transfer to 40 mm diameter Petri plates.

157 **Comments**: This step can be omitted if seeds were generated in sterile *in vitro* conditions.

158 After sterilization, seeds are transferred to 12 cm square Petri plates containing TK1 nutrient
159 medium in 1% agar (Carl Roth, Art. Nr.5210.2). Dishes are kept (with covers) at room
160 temperature for 6-8 hours to allow imbibition, then they are moved to 4°C in the dark for 14-
161 16 hours for stratification, following standard procedures.

162 **Comments:** The 6-8 hours imbibition at room temperature is a crucial step for much more
163 effective synchronization of seeds germination.

164 ***Step 2. Seedling growth***

165 Transfer plates to a growth room with 16/8 h day/night cycle, 24°C, with a light intensity of
166 80-100 µmol/m$^2$/sec (or continuous light, depending on experimental strategy) for further
167 growth. Seedlings are grown on the agar surface, as in standard AM based protocols. Roots
168 can be collected for immuno-localization analyses at this stage, from 5-10 days old plants as

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169 described in [17]. Seedlings are kept in plates until plants transit to bolting/flowering. In wild
170 type plants this usually takes 15-18 days starting from seed soaking.

171 ***Step 3. Transfer of plants to tubes with Hoagland medium for in vitro seed propagation and***
172 ***maturation***

173 As soon as plants start bolting, the shoots can be transferred to tubes with Hoagland medium.
174 The root system can be cut with a scalpel and can be collected for molecular analyses. The
175 rosette with the flowering stem is transferred to tubes containing Hoagland medium
176 supplemented with 0,8% agar in a sterile bench. We recommend to add in each tube not more
177 than 2-2.5 ml of medium. The tubes are then moved back to the growth room with a 16/8 h
178 day/night photoperiod. Seed formation and maturation occur within the next 2-3 weeks.
179 During this time the medium in the tubes undergoes slight drying.

180 **Comments**: Successful seed formation can occur only if tubes are covered with cotton top
181 and accompanied by plants drying. In the case of plastic top, water evaporation is prevented
182 thus causing high humidity inside tubes, that prevents efficient seed formation and drying.
183 The tops are prepared from cheesecloth (or paper towel) and cotton and can be re-used several
184 times. Tops can be prepared from double layers of towel or cheesecloth filled with cotton and
185 finally closed with a rope (Figure 1 and 3 A and B). Alternatively, tops can be ordered
186 (Rotilabo®-Kulturstopfen CTE 0.1).

187 We recommend to autoclave tubes and medium separately and add 2-2.5 ml of the medium to
188 the tubes just before usage since medium dries after prolonged storage even at 4°C.

189 If a low amount of the medium remains in the tubes while the siliques and seeds are still
190 green, we recommend to add 1 ml of fresh liquid Hoagland’s medium to the tube and extend
191 cultivation for 1-2 more weeks.

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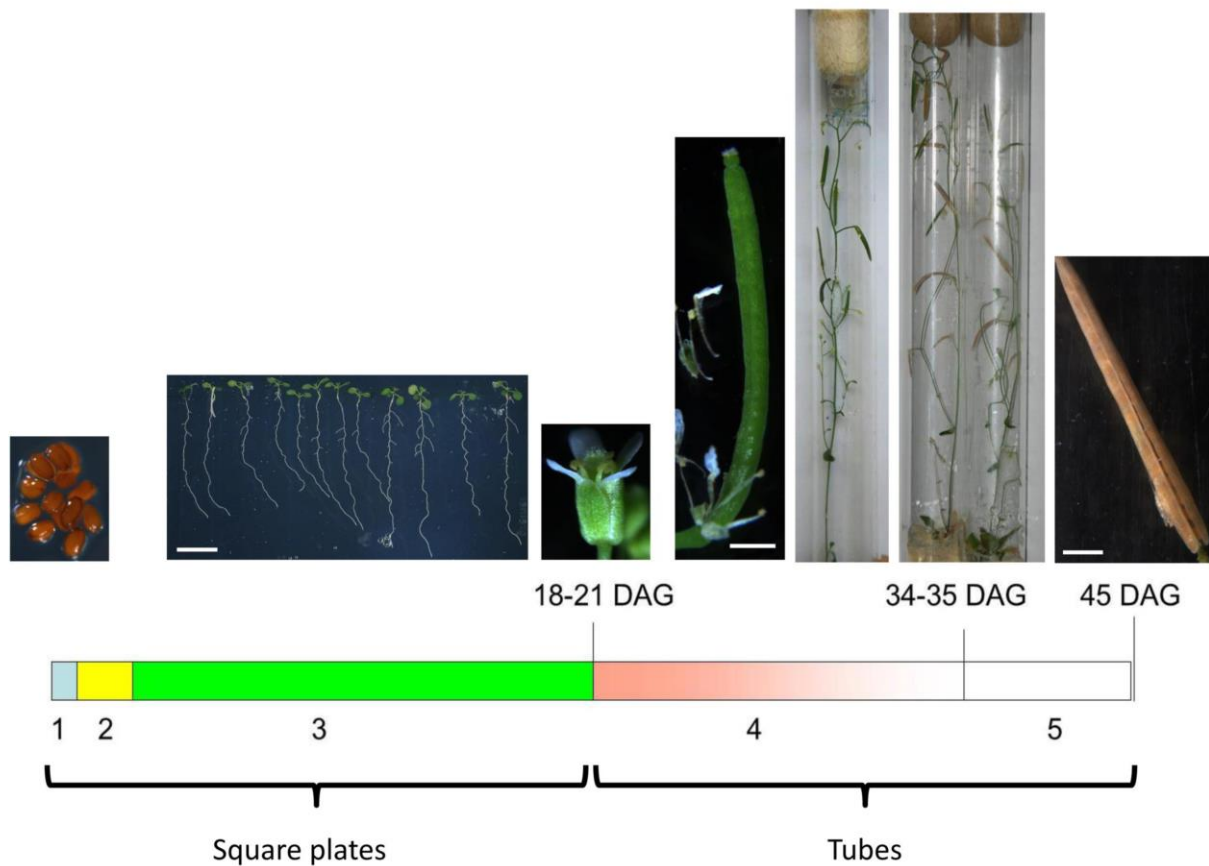
192 ***Step 4. Seed collection***

193 After seeds become completely dry, they can be collected in sterile Petri plates (40 mm
194 diameter). Collect seeds in a sterile bench with sterile forceps directly into sterile plates or
195 tubes. In this case the seed sterilization step can be omitted.

196 A workflow for the whole procedure of *in vitro* growth of Arabidopsis plants for seeds
197 formation is shown in Figure 1.




198

199 **Figure 1.** Workflow of the protocol for *in vitro* seeds formation of Arabidopsis plants.

200 **Results and discussion**

201 ***Effects of media composition on plant growth***

202 Plant phenotyping and molecular analysis is an important task in plant biology. Such analysis
203 should be performed in conditions which are as close as possible to the natural soil conditions
204 because plant phenotypic responses generate through a complex interaction of genotype with

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205 environmental conditions [7, 8, 9]. Nutrient solutions which reproduce as closely as possible
206 the natural conditions in soils have been described previously and have been intensively used
207 to study plant phenotypic responses to nutrient availability [6, 9]. A sub-optimal composition
208 of the plant culture medium, potentially causing a nutritional stress, may significantly alter
209 plant development and phenotypic characterization, since plant nutrition is a key factor in
210 determining plant development through a complex interaction with genetic cues. Being the
211 organs devoted to cope with nutrient availability, roots are especially sensitive to such
212 conditions so that an alteration in root system architecture (RSA) can be easily caused by
213 improper nutrient availabilities [9]. As an example, it was recently shown [18] that nutrient
214 balance is a key regulator of CLE peptide signaling and, through this, of the whole root
215 architecture. The increasing interest in characterizing the plants' responses to abiotic stresses
216 further poses the need for media allowing reliable analysis in automated phenotypic screening
217 systems specifically aimed at reproducing in the lab nutritional conditions which may be
218 closer to the plant physiological requirements in the field and do not represent a nutritional
219 stress *per se*. This is of particular interest when the genetic factors for response to nutrient
220 availability and homeostasis are sought for. So far the most used nutrient medium for *in vitro*
221 growth of Arabidopsis seedlings is half strength Murashige and Skoog (MS) medium (1962)
222 [2], generally termed AM (Arabidopsis Medium). MS medium was originally designed
223 starting from White's media [19] and optimized for rapid growth of tobacco callus cells in the
224 presence of external supply of hormones [2]. This medium is characterized by a very high
225 nitrogen content, high N:P:K ratio of 60:1.25:20 and contains chloride as a major anion
226 (Table 1). However, the optimal N:P:K ratio for plant growth in soil and in hydroponic culture
227 (5:1:3) is far from that found in the AM medium [3]. Moreover, chloride content should fall
228 within micronutrient levels [20]. In addition, the concentrations of essential micro-nutrients
229 such as Cu and Co are rather low in AM medium. Co, for example, which is an important

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230 inhibitor of ethylene production [21], is frequently overlooked as a medium component,
231 particularly in the case of limited aeration *in vitro*. Nutritional stress may also cause
232 epigenetic changes [22] or alterations in root morphology [23]. Thus a culture medium which
233 strictly follows an optimal nutrient ratio and optimal concentrations of macronutrients and
234 micronutrients would be highly desirable in order to avoid nutritional stress and allow
235 phenotypic evaluations in conditions which are closer to real nutrient availability in the field.
236 To this end, we suggest the adoption of a balanced culture medium (TK1) with an optimal
237 ratio between nutrients (Table 1) for growth, phenotypic analysis and *in vitro* propagation of
238 Arabidopsis, which can be successfully used also for mutants lacking the root system. This
239 medium has been previously optimized for *in vitro* growth of barley [24] and alfalfa [25]. One
240 of the main problems of *in vitro* tissue culture is the formation of precipitates in media after
241 mixing all ions and sucrose [26, 27]; this frequently happens even before autoclaving. This
242 problem can be circumvented by adding a low amount of enzymatically digested casein
243 hydrolysate (Tryptone) (150 mg/l) to the medium. This addition completely prevents
244 precipitations in the medium, while it may serve as a source of amino acids. The total nitrogen
245 contents (12%) and amino acid content (3%) in the Tryptone may have limited impact on the
246 processes studied. In this circumstance we have to mention that an average soil (natural
247 conditions) contains a quite high amount of organic nitrogen/amino acids [28], that are,
248 however, difficult to be standardized.
249 For the evaluation of the effects of composition of media on the root morphology we have
250 compared root growth on AM (½ strength MS) medium and TK1 medium. Root growth
251 proceeded significantly faster on TK1 medium in comparison with AM medium in wt plants
252 (Figure 2). Even more pronounced effects were observed for the auxin response mutants *tir1*
253 [12] and *tir1xafb1,2,3* [13], caused by excess nutrient stress by AM medium (Figure 2A and
254 B). Interestingly, on TK1 medium the root meristem size is increased in both mutants and wt

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(Figure 2D), as well as post-mitotic cell elongation is enhanced (Figure 2 C,D). Similar results were observed for a number of other mutants. We investigated the root growth behavior of well-known mutants defective in cytokinin production (*ipt3ipt5ipt7* [29]) and *miao* ([15] ) mutants on TK1 medium. It was shown that on balanced medium the effects of these mutations on root development were much milder than those observed on ½ MS medium [15], emphasizing the importance of using a nutritionally balanced medium for Arabidopsis growth (Table 2).

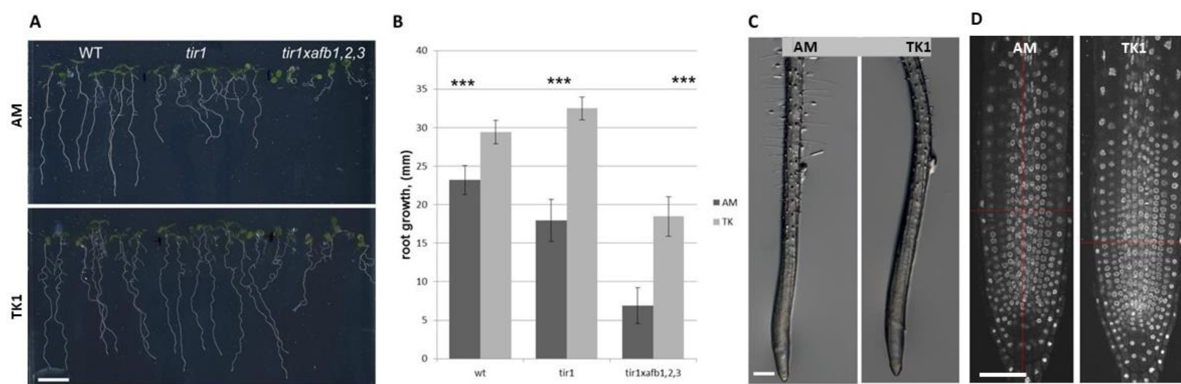


**Figure 2.** Effect of AM and TK1 medium on root development. (A) Plants of wt (Columbia), *tir1* and *tir1xafb1,2,3* mutants were cultured on AM medium for 4 days and were transferred on AM or TK1 medium thereafter. Pictures were taken after 3 additional days of growth. Scale bar - 1 cm; (B) - Quantification of root length (mm) on AM and TK1 medium. Error bar- SD; *** mean significant differences for P ≤0,001. (C) – details of root images. Scale bar - 100 µm. (D) – DAPI stained RAM. Scale bar- 50 µm.

An additional important application of the protocol is that it enables the growth of plants and subsequent generation of seeds from wild type and also from mutants with severe root defects. For optimal seed formation we successfully used Hoagland medium that ensured optimal plant growth and silique formation in comparison to AM (Figure 3). As expected, seed formation requires a relatively high amount of phosphorus and lower nitrogen contents than those present in AM medium, which may prevent normal bolting and growth. Hoagland

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275 medium was developed by Hoagland and Arnon, further revised by Arnon, and has been
276 originally designed for plant growth in hydroponic culture [21]. It provides every nutrient in
277 optimal ratios necessary for seed formation. Presence of carbon and energy sources is crucial
278 for seed formation; therefore addition of 2% sucrose is recommended. Both TK1 and
279 Hoagland medium have much higher biological buffering capacity because they have a lower
280 $NH_4/NO_3$ ratio. This ratio is important because $NH_4$ uptake is much faster compared with $NO_3$
281 uptake. This, in turn, leads to significant acidification of the medium. To avoid such
282 acidification we suggest adding MES.

283

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284 **Figure 3.** Comparison of Hg and AM medium for *in vitro* seeds reproduction. Seedlings were
285 grown for 18 days on TK1 medium, were transferred to tubes and cultured for next 10 days.

286 As far as mutants with impaired root development are concerned, the *plt1,2,3* triple mutant
287 [14], with severe defects in PIN polarity in the roots and in the development of the root
288 system, has been used for this purpose to test the efficacy of the *in vitro* seed formation
289 protocol. *plt1,2,3* (+/-) seeds were germinated and plants with severe defects in root formation
290 were selected. Selected plants (Figure 4 A) were analyzed for defects in establishing PIN1
291 polarization in the root (Figure 4 B, C), and thereby the formation of the auxin gradient, and
292 for a complete lack of the root system and inability to form adventitious roots, to ensure
293 selection of homozygous seedlings. AM and Hoagland medium were compared for their
294 performance in the process of seed formation. Plants containing small excised stems with
295 flowers have been transferred to tubes containing either ½ MS or Hoagland medium
296 (supplemented with 2% sucrose). After 2 weeks in the tubes strong differences were detected
297 in the number and quality of seeds (Figure 4 D, E and F). This procedure allowed recovery of
298 seeds from rootless mutant plants such as the *plt1,2,3*.

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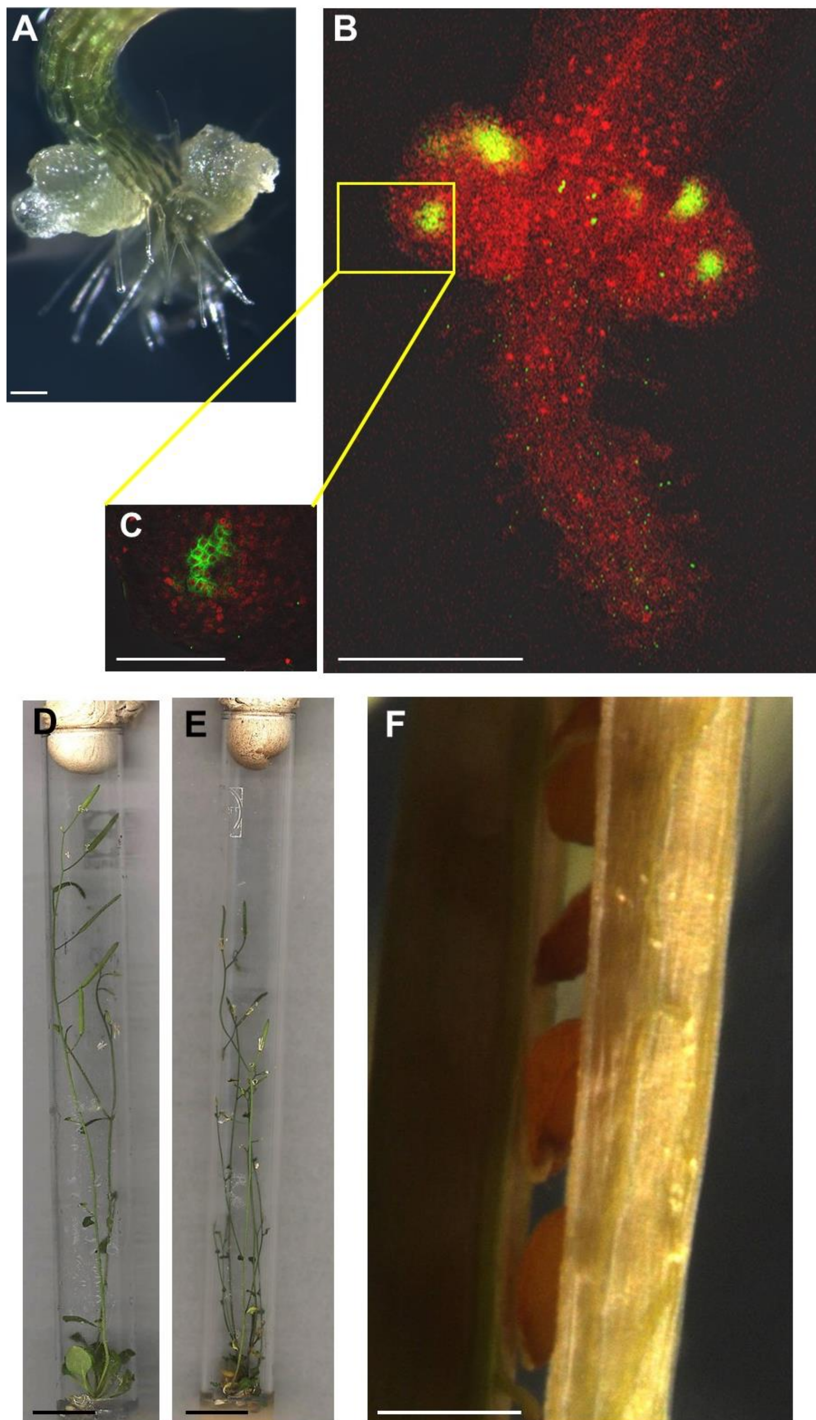


299

300 **Figure 4.** Seeds formation from homozygotic *plt1,2,3* mutant. (A) – 4 days old plt1,2,3
301 homozygotic seedlings. (B, C) – PIN1 immunolocalization (according to the protocol
302 described in [2]) in the selected *plt1,2,3* mutants roots (see text for explanations).

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303 (D) and (E) - wt and *plt1,2,3* mutant plants in tubes after 35 days from seeds soaking. (F)
304 details of a *plt1,2,3* silique at seed ripening stage. Scale bar: A, B- 100 µm; C- 50 µm; D, e –
305 1cm, F – 500 µm.

306 Overall, the application of the *in vitro* growth procedure for seed reproduction described here
307 allows a significant reduction of time and space. The system also has been applied for
308 generation of N15 seeds, which have been used for stable isotope labeling with amino acids in
309 cell culture (SILAC) [30]. In our hands we were able to generate seeds which have more than
310 98% of N15 contents. The advantages of the procedure described here in comparison with
311 previously published procedures are resumed in table 3.

312 ***Conclusions***

313 We report a simple *in vitro* system for the growth, phenotyping and seed reproduction of
314 *Arabidopsis thaliana* plants. The system is based on two steps: 1) seedling cultivation on
315 vertical plates on optimal medium that allows selection and characterization of the plants
316 during the first 3 weeks of growth; 2) Shoot cultivation on modified Hoagland medium during
317 the next 2-3 weeks which allows to generate high amounts of genetically pure and sterile
318 seeds.

319 The system enables to shorten the Arabidopsis cycle from seed to seed to about 45 days, while
320 significantly reducing the amount of waste generated from growth in soil pots and obtaining
321 genetically pure lines by completely avoiding cross-pollination. The sterility of all steps of
322 plant growth until seeds drying removes the need for further sterilization steps for the growth
323 of the next generations. In wild type Arabidopsis plants we were able to generate more than
324 300 seeds per plant on average, while in the case of mutants with severe root impairment the
325 amount of seeds was restricted to 100 per tube but still allowed efficient reproduction.

326 **Tables.**

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327 **Table 1.** Composition of different nutrients media (in mg/l) compared in this work for *in vitro*
328 growth of Arabidopsis seedlings (TK1) and seed propagation (Hg). The composition in terms
329 of macro- and micro-nutrients is reported for Hoagland medium (Hg), Arabidopsis medium
330 (AM; half-strength Murashige and Skoog Medium) and for the optimized medium described
331 in this work (TK1).

| Compound | AM (½ MS) | TK1 | Hg |
|---|---|---|---|
| **Macronutrients** | | | |
| $NH_4NO_3$ | 825 | - | - |
| $KNO_3$ | 950 | 900 | 600 |
| $NH_4H2PO_4$ | - | 200 | 125 |
| $MgSO_4x7H_2O$ | 180 | 200 | 300 |
| $KH_2PO_4$ | 68 | - | - |
| $Ca(NO3)_2x4H_2O$ | - | 250 | 980 |
| $CaCl_2x2H_2O$ | 220 | - | - |
| **Fe-Chelate** (2M stock) | 2.5 ml | 2 ml | 2 ml |
| **Micronutrients** | | | |
| $H_3BO_3$ | 3.1 | 4 | 2.86 |
| $MnCl_2 \cdot 4H_2O$ | 8.4 | 9 | 1.81 |
| $ZnSO_4 \cdot 7H_2O$ | 15 | 0.9 | 0.22 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.125 | 0.125 | 0.12 |
| NaI | 0.38 | 0.4 | 0.75 |
| $CuSO_4 \cdot 5H_2O$ | 0.012 | 0.1 | 0.05 |
| $CoCl_2 \cdot 6H_2O$ | 0.012 | 0.1 | |
| **Organic compounds** | | | |
| Bacto-trypthone | - | 150 | 150 |
| Vitamins | ½ B5 vitamines | ½ B5 vitamines | ½ B5 vitamines |
| Sucrose | 10000 | 10000 | 20000 |
| MES | 200 | 200 | 200 |
| pH | 5.6 | 5.6 | 5.6 |

332

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333 **Table 2.** Comparison of the *in vitro* (this article) and in soil seeds generation approaches in
334 Arabidopsis. Asterisk (*) indicates averaged data.

| | Standard growth in soil | ***In vitro* growth** |
|---|---|---|
| **Parameters** | | |
| **Space per plant** | 20 sq.cm | 2 sq.cm |
| **Duration from seeds to seeds** | 60 -70 days | 40-45 days |
| **Amount of waste** | 300 ml (soil) | 3 ml (medium) |
| **Seeds per plant*** | 10000 | 350 |
| **Standardization of growth** | Low | High |
| **Genetic seeds "purity"** | Moderate, isolation of plants by covering is required | 100 % pure from fully isolated plants |
| **Watering, greenhouse care, use of chemicals/pesticides** | Yes | No |
| **Mutants with defects in root development** | Very low yield, not applicable for mutants with severely impaired roots | Applicable to rootless mutants (e.g. *rml1*, *plt1,2,3*). |
| **Generation of plants/seeds containing $N^{15}$ labeling** | Not applicable | Applicable |
| **Seeds sterilization** | Required | Not required. Seeds recovered from *in vitro* grown plants are already sterile |

335

336 **Table 3.** Root length of wt and *miao* mutant seedlings on AM and TK1 medium

| | AM | TK1 |
|---|---|---|
| wt | 20,79±2,11 | 31,72±2,92 |
| *miao* | 4,92±2,49 | 14,41±4,77 |

337

338

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339 **Declarations**

340 ***Ethics approval and consent to participate***

341 Not applicable.

342 ***Consent for publication***

343 Not applicable.

344 ***Availability of data and materials***

345 All data generated or analyzed during this study are included in this published article.

346 ***Competing interests***

347 The authors declare that no competing interests exist.

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352 ***Authors contributions***

353 TP performed the experiments; TP, BR and KP interpreted and discussed the results and wrote
354 the manuscript.

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