

Determination of plant resistance to carbamate herbicidal compounds inhibiting cell division and early growth by seed and plantlets bioassays

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Herbicide-resistant plants can be generated by either traditional breeding procedures or genetic engineering. Analyses of plant responses to a newly developed herbicide or the tolerance level of a newly developed plant line to a given herbicide are based on various bioassays. Here, we describe several methods for quantitative measurements of plants' responses to propham application, as a model herbicide of the carbamate family. Dose–response assays include seed germination and analyses of shoot and root elongation on paper. To better reflect the natural interaction between the plant, the soil and the herbicide, a protocol for germination and root elongation on sand is described. Finally, a more sensitive bioassay is based on plant growth on agar medium. The described protocols are simple, reproducible and can be easily adopted for a variety of plant species and for various herbicides. Plants' response to a given herbicide can be determined within a few weeks.

INTRODUCTION

It is predicted that by the year 2025, the world population will have reached 8 billion, resulting in a substantial increase in food demand. The ability to satisfy this demand will rely on the genetic improvement of plant crops toward increased yield potential¹, as well as tolerance to pests and herbicides. The term herbicide resistance is used to describe the ability, trait or quality of a population of plants within a species or large taxon, or of a plant cell in culture, to withstand a particular herbicide at a dosage that is substantially greater than the dosage the wild type of that plant is able to withstand, with a near-normal life cycle^{2–4}.

Several different mechanisms enable plants to avoid the effects of the herbicides they encounter^{4,5}. These mechanisms can be grouped into three categories: the first, “enhanced detoxification” or “metabolic resistance”, includes the cases in which the herbicide is enzymatically detoxified before reaching the target organelle or site^{6–10}. The second, “sequestration” or “compartmentation”, includes those mechanisms that eliminate the herbicide molecule from its target site^{11–16}. Compartmentation of the herbicide or its degradation products is performed before they reach the site of action: for example, immobilization of a lipophilic herbicide molecule by partitioning into lipid-rich glands or oil bodies^{14–16}. The third is “altered target-site resistance,” which includes those mechanisms that render the specific site of herbicide action resistant to the chemical. This mechanism can act via alteration or mutation of the different target-site protein structures. An alternative mechanism might involve overproduction of the endogenous plant molecule's herbicide target site, allowing enough additional protein to remain to complete normal function and growth¹⁷. Several of these mechanisms can work in concert to produce whole-plant resistance.

Traditionally, herbicide-resistant crops have been generated by breeding under selective pressure or crossing with herbicide-resistant species, including the pre-existing lack of susceptibility, and

selection of resistant variants from a diverse species. Nowadays, crop germplasm improvement also includes selection and incorporation of desirable herbicide-resistance qualities by genetic manipulations based on previously described mechanisms^{18–21}. Herbicide tolerance is the most widely planted transgenic crop trait. Globally, about 75% of the genetically modified crops are engineered for herbicide tolerance²².

The family of carbamate herbicides is made up of highly volatile compounds, which act as selective preplanting, pre-emergence and post-emergence herbicides. A typical representative of this herbicide family is propham (1-methyl phenylcarbamate, C₁₀H₁₃NO₂). It effectively controls many annual grasses and certain broadleaf weeds. It inhibits root and epicotyl growth, normal cell division, protein and amylase synthesis, proteolytic activity of isolated chloroplasts, and it affects mRNA activity²³. The carbamates act mainly through inhibition of cell division in the roots and coleoptiles, and they are readily translocated acropetally; therefore, soil surface applications must be performed in the weeds' root zone.

A novel approach to conferring resistance to carbamate herbicides was based on the expression of catalytic antibody in transgenic plants²⁴. In that study, we developed a new carbamate herbicide, one that can be catalytically destroyed by the aldolase antibody 38C2. By separately expressing the light chain and half of the heavy chain (Fab) of the catalytic antibody in the endoplasmic reticulum of two different plant lines of *Arabidopsis thaliana*, and cross-pollinating these two transgenic plants to produce an F1 hybrid, we demonstrated that the catalytic antibody is active *in planta* and confers resistance to the developed carbamate herbicide.

Tolerance of a new plant variety, produced by either traditional breeding procedures or genetic engineering, is usually examined by various bioassay methods. These methods are also applicable for analyses of the toxicity of a newly designed herbicide. As tolerance or sensitivity levels are quantitative characteristics, dose–response



assays are the most common procedures. Application of increasing concentrations of the desired herbicide can be performed by “over the top” or post-emergence spray of the mature plant (or small seedlings) or by its incorporation into the soil or the growth medium. Selection of the appropriate assay depends on the molecular interaction between the herbicide and the plant tissue, namely the mechanism enabling plant tolerance or sensitivity to the specific compound.

Bioassays performed at the mature plant level require relatively long growing periods and large experimental areas, and are therefore more costly and less efficient than those performed at early stages of plant development. Influence of the herbicide on a basic cellular process (e.g., cell division, protein and/or nucleic acid synthesis) enables the use of a given bioassay at the seed or seedling level. In this case, tests are performed on a small scale, usually in the laboratory.

Seed germination tests provide a standard protocol to examine the influence of a herbicide on the viability of plant tissue. In this test, water is supplied by absorption through a medium or substratum, such as sand or absorbent paper. Percent germination

is one of the accepted indexes for the ability of seeds to germinate in medium containing herbicide. More detailed parameters for the quantitative influence of the herbicide on plant performance are those that determine seed quality or vigor. Quality tests, such as root elongation rate or analyses of shoot growth, better differentiate between the responses of the seedlings to different herbicide concentrations or between sensitivities of plant lines, with approximately the same germination percentage, to a defined herbicide concentration.

The protocols described here provide quantitative criteria for seed germination and seedling quality (vigor) in the presence of a given herbicide interfering with cell division and early growth. These bioassays include detailed analyses of seedling growth and therefore enable quantitative scores for various herbicides or for various plant lines. The protocols are advantageous in that they are simple to use, do not require expensive equipment and can be easily adopted for a variety of plant species and for various herbicides. Several of them have been reviewed previously, for example, “seed germination assay,”²⁵ “sand assay”²⁶ and “germination on agar”²⁷.

MATERIALS

REAGENTS

- *A. thaliana* seeds (ecotype Landsberg erecta [Ler])
- *Sorghum bicolor* seeds
- *Solanum tuberosum* L. cv. Desired sterile plantlets
- *Medicago truncatula* seeds (or seeds of any other desired species)
- Coarse quartz sand, 0.8–1.5 mm particle size (Negev Industrial Minerals Ltd, Omer Industrial Park)
- MS medium (see REAGENT SETUP)
- Propham pestanal (Isopropyl phenyl carbamate; Sigma)
- Ethanol 70% (v/v)
- Methanol 100%
- Sterile ddH₂O

EQUIPMENT

- Growth chambers with an adjustable light/dark cycle and temperatures of 23–25 °C
- Refrigerator (4 °C)
- Oven 50–60 °C
- Laminar-flow hood
- Petri dishes: 140 × 20 mm
- Petri dishes: 90 × 15 mm
- Test tubes (25 × 95 mm) and translucent cap
- Whatman paper d1 (for sterilizing *A. thaliana* seeds and for germination in Petri dishes)
- Seed germination paper (Anchor Paper, Anchor Company)
- Thin transparent plastic bags (A4)
- Flexible metal strips, 2-cm wide
- A box: 48 × 31.5 × 31.5 cm
- Thin tweezers

REAGENT SETUP

MS medium Autoclave MS medium (4.4 g Murashige & Skoog salts, 30 g sucrose, 8 g agar per liter; pH 5.8) and cool to approximately 50 °C. Add the

appropriate herbicidal solution (see PROCEDURE), mix well and pour into Petri dishes (140 × 20 mm).

Herbicide solutions The presented protocols refer to herbicides of the carbamate family. Propham serves as a model herbicide for the described assays. To perform dose–response experiments, propham solutions should be made to final concentrations in the range of 0–100 p.p.m.

Herbicide solutions for the “germination on paper” assay Dissolve thoroughly 1, 5, 10, 50 and 100 mg propham powder in 10 ml methanol. Add the various solutions to 990 ml sterilized water to form final concentrations of 1, 5, 10, 50 and 100 p.p.m., respectively. The control (0 p.p.m. propham) is made by adding 10 ml of methanol to 990 ml of sterilized water (1%).

Herbicide solutions for “germination on agar” assay Prepare the 10 ml propham solutions as described above (1, 5, 10, 50 and 100 mg propham in 10 ml methanol). Add the 10 ml concentrated solutions to 990 ml of sterile MS medium. Mix thoroughly and pour into Petri dishes. **! CAUTION** Appropriate clothing, eye protection and gloves should be used during the preparation of methanol solutions. **▲ CRITICAL** Herbicides of the carbamate family do not dissolve in water. Methanol provides a suitable organic solvent for these herbicides; however, methanol concentrations higher than 1% can be toxic to plants. Therefore, herbicide powder must be dissolved completely in methanol before adjustment to the final concentrations with sterile water. One should make sure that the final solution is homogeneous, with no aggregates.

Sand washing Fill an empty vertical column (18 cm diameter, 50 cm long) with coarse quartz sand. Wash the sand column for 30 min with a strong flow of water, from the top. Keep washing for 1 h more with a weak water flow (drops). Transfer the sand to a large flat pot (5 liters) with a drain hole at the bottom. Shake the pot in order to drain standing water. Dry the sand completely in a 60–80 °C oven for 24 h.

PROCEDURE

Germination and root elongation of seeds

1| Choose the appropriate experimental conditions for the seeds for germination. Most seeds are best germinated on paper (A). Germination in plastic sleeves (B) provides the best information on root elongation, however it is not suitable for very small seeds (e.g., *Arabidopsis*) or very large seeds (e.g., sunflower). Germination on sand (C) better reflects the plant’s response to the herbicide under field condition. This option is suitable for seeds that are larger than the sand particles (e.g., tomato, pepper, cereals, etc.) but is not suitable for large seeds (e.g., peanuts, cotton, etc.) or very small seeds (e.g., *Arabidopsis*). Germination and growth of plantlets on agar (D) is the most sensitive bioassay and also suitable for species that are characterized by vegetative propagation (e.g., potatoes).

PROTOCOL

(A) Standard seed germination assay on paper

- (i) Place two layers of round sterile sheets of Whatman (d1) paper at the bottom of 90 × 15 mm Petri dishes.
- (ii) Apply 5 ml aliquots of the herbicidal solutions (0, 1, 5, 10, 50 and 100 p.p.m.; see REAGENT SETUP) to each dish. Wait until the papers are completely wet.
- (iii) Seed sterilization—place the *Arabidopsis* seeds on Whatman (d1) paper in a Petri dish. Wash the seeds with 70% ethanol and let them dry in a hood for 1–2 h (until complete evaporation of the ethanol and water).
▲ CRITICAL STEP Always sterilize larger amounts of seeds because there is some waste during the sterilization procedure.
- (iv) Place ten sterilized seeds in a line (with tweezers) at the center of the upper third of the Petri dish (**Fig. 1**).
- (v) Place Petri dishes in a cold room (4 °C) in the dark for 3 days for vernalization.
- (vi) Transfer Petri dishes to the germination room (25 °C) under dark conditions for 2–3 days (until radicles protrude from the seed coats). At this stage, plates are tilted face up at a 60° angle.
▲ CRITICAL STEP At this stage, germination percentage can be determined for the various herbicide concentrations. Germination is defined as the stage at which the seed's radicle reaches 1 mm in length. Germination percentage is the percent of germinated seeds on a specific day out of the total number of seeds in the Petri dish. Note that germination percentage is not always a sensitive parameter for herbicidal effect on plant growth. Therefore, a specific herbicide might not reduce germination percentage when tested at “regular” concentrations.
- (vii) To determine shoot and root growth, the Petri dish should be placed in the light (photon flux density of at least 60 μmol m⁻² s⁻¹ and a photoperiod of 16 h) under the conditions described in Step A(vi).
- (viii) Root and shoot lengths can be determined daily for the next 5–7 days or at the end of the experiment when roots' tips reach the edges of the Petri dish. A comparison between the various treatments (herbicide concentrations) can include the average root and/or shoot lengths on a specific date, or root elongation rate, which is determined by the slope of the curve presenting root length versus time.
▲ CRITICAL STEP A drop of water at the bottom of each Petri dish indicates adequate moisture content of the germinating paper. Up to 1 ml of additional distilled water may be added every 2 days to maintain this adequate moisture level. The lids of the dishes are not sealed, to allow gas exchange.

? TROUBLESHOOTING

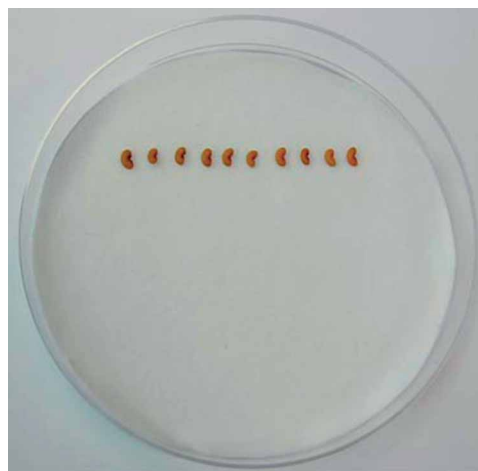


Figure 1 | *M. truncatula* seeds placed on Whatman (d1) paper placed in the center of the upper third part of a Petri dish.

(B) Germination assay in plastic sleeves

- (i) An alternative germination assay is suitable for seeds that are larger than *Arabidopsis* (e.g., *M. truncatula*, *L. esculentum*, etc.). This assay enables detailed analyses of root and shoot growth rate.
- (ii) Sterilize the seeds, referring to Step A(iii) above.
▲ CRITICAL STEP When the assay includes germination of *M. truncatula* seeds, imbibe the seed in a flask of distilled water (stirred) for 24 h before placing the seed on the wet paper.
- (iii) Trim the germination paper to fit A4 plastic bag (19 × 25.5 cm) and insert it into the plastic sleeve, which has been cut in advance: the first cut is made 4 cm

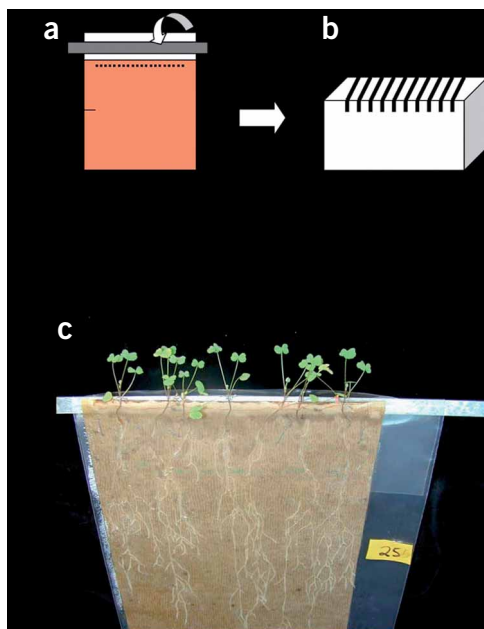


Figure 2 | Preparation of the plastic sleeves containing a germination paper (Anchor Paper). (a) The orange square represents the wet paper, which is placed within a plastic sleeve; the dots indicate the position of the seeds; the broken arrows point to the cut sections; the gray strip represents the 2-cm-wide flexible metal strip around which the upper part of the plastic sleeve (4-cm wide) is folded. (b) The box in which the sleeves are hung vertically. The parallel thick lines represent ten metal strips that are hung in one box. (c) Root system and shoot growth of *M. truncatula* seedlings germinated in the plastic sleeve. The picture was taken 5 weeks after germination.

from the upper side along the edge. The second cut is 3 cm long, 15 cm from the bottom of the paper (Fig. 2a).

- (iv) Add 12 ml of the herbicide solution (see REAGENT SETUP) to each sleeve, via the small hole (3 cm cut) in the plastic sleeve (Fig. 2a). Wait until the paper is completely wet.
- (v) Place 20 sterilized seeds (with tweezers) in a line on the wet paper. The seed line is set 0.5 cm below the long horizontal cut.
- (vi) Fold the edge of the plastic sleeve around a flexible metal strip such that the transverse cut in the plastic sleeve is lying along the metal strip. Attach the fold at both ends, hang the sleeves vertically in a box and cover with black cloth (or plastic), such that germination will take place in the dark (Fig. 2b).

▲ CRITICAL STEP A drop of solution should always be present at the bottom of the sleeve, to ensure adequate wetting.

- (vii) Transfer the box to the growth chamber (25 °C) and monitor germination rate and seedling growth daily (Fig. 2c). A comparison between the various treatments (herbicide concentrations) can include the average root lengths on a specific date, or root elongation rate, which is determined by the slope of the curve presenting root length versus time.

? TROUBLESHOOTING

(C) Germination and root elongation on coarse quartz sand for seeds larger than sand particles

- (i) Germination assay performed on sand better reflects the natural interaction between the herbicide, the soil and the seed, which occurs under field conditions.
 - ▲ CRITICAL STEP** The described protocol is suitable for analyses of seeds that are larger than the sand particles, but is not suitable for large seeds (e.g., peanuts, cotton, etc.) or very small seeds (e.g., *Arabidopsis*).
- (ii) Weigh 140 g of washed and dried coarse quartz sand into a 200 ml beaker or plastic cup.
- (iii) Pour, with a syringe or a cylinder, 14 ml of herbicide solution (see REAGENT SETUP) in each beaker or cup.
- (iv) Cover the beaker or cup with the base of the 9-cm Petri dishes and shake the cup vigorously to thoroughly mix the herbicide solution with sand.
- (v) Pour the mixture into the Petri dish and cover it with the lid. Ensure equal distribution of the mixture in the dish by knocking on the lid.
- (vi) Place the seeds on the sand, 1 cm from the top of each dish. Position the seed such that the radicle will protrude downwards.
- (vii) Seal the dishes tightly with a masking tape, to avoid movement of the sand and roots within the Petri dish, and to avoid fast drying of the sand thus changing the concentration of the herbicide. Mark the top of each plate to maintain vertical orientation.
- (viii) Incubate the plates in the dark at 25 °C tilted in 60° position (face down) for 4–10 days for germination and root elongation. Times may vary for different plant species.

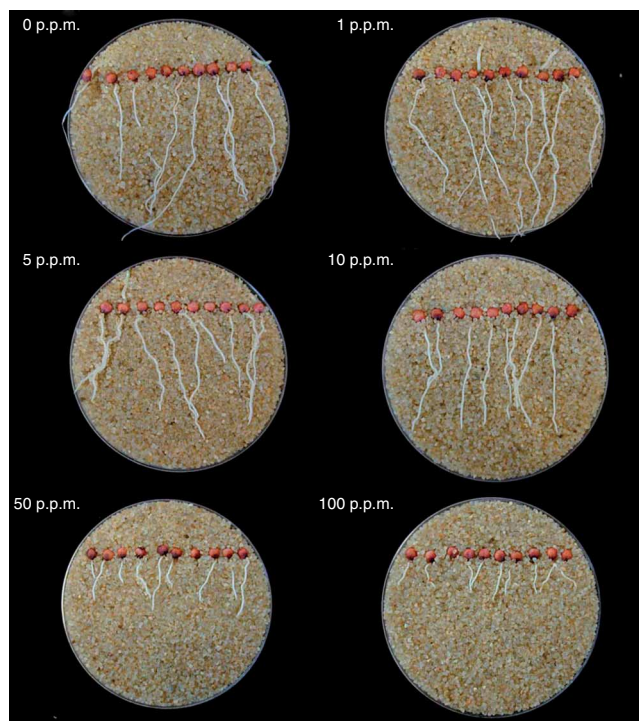


Figure 3 | Influence of propham on germination and root elongation of sorghum seeds using the “germination on sand” bioassay. Propham concentrations (0–100 p.p.m.) are indicated in each picture. Note significant inhibition of root elongation at propham concentrations of 50 and 100 p.p.m. The picture was taken after 5 days of germination.

BOX 1 | ARABIDOPSIS GERMINATION PROCEDURE

1. Sterilize the seed as described in Step 1A(iii)
2. Prepare MS medium with the various herbicide concentrations (0, 5, 10, 50 and 100 p.p.m.), referring to REAGENT SETUP. Using thin tweezers, gently place about 16 sterilized seeds on the MS medium in a line, at the center of the upper third of the Petri dish, leaving space between them. Seal each plate individually
3. Vernalize seeds by placing the Petri dishes (horizontally) in the dark at 4 °C for 3 days
4. Place the plates in a growth chamber at 25 °C. Germinate the seeds in the dark for 3 days
5. Position the Petri dishes vertically, at a 60° angle, and maintain at 25 °C, 12 h light/12 h dark. Measure seedling size after 7–20 days (Fig. 4)

BOX 2 | POTATO CULTURE CONDITIONS

1. Tissue-culture-grown potato plantlets should provide the explants for this bioassay
2. One-node stem segments are taken from the basal nodes (nos. 3–6) of 1- to 2-month-old plantlets
3. Plant each segment in a separate 25 × 95 mm test tube containing 10 ml of MS media with the desired herbicide concentration (see REAGENT SETUP). The segment should be dipped into the agar such that 2–4 mm will be below the agar surface
4. Cover the test tubes with a translucent cap that enables gas exchange with the atmosphere, and hold them in slanted culture tube racks (Sigma). The culture room should be set to a constant 25 °C and a 12 h photoperiod at a light intensity of at least 100 μmol m⁻² s⁻¹
5. Determine shoot and/or root size after 7–14 days

▲ CRITICAL STEP The plates should be positioned at a 60° angle. The orientation is important to enable the elongation of the roots along the lid of the Petri dish. Make sure not to shake or move the dishes, to prevent movement of the germinating seeds.

- (ix) Root length can be monitored daily (see **Fig. 3** for an example of root length after 5 days of germinating *S. bicolor* seeds). Data analyses can be performed as described in Step 1A(viii).

(D) Germination, root elongation and shoot development on agar

- (i) An alternative protocol for germination and plantlet growth is based on a system in which the plantlets grow on agar.
- (ii) When incorporated to agar medium, the influence of the herbicide on root elongation and plantlets growth is more pronounced than its influence in sand medium. Therefore, the agar protocol provides a more sensitive measure of plant resistance.
- (iii) In **Boxes 1** and **2**, we describe a protocol for seed germination of *Arabidopsis* (**Box 1**) and growth of potato plantlets (**Box 2**), the latter being a representative species characterized by vegetative propagation.
- (iv) Determination of root length in agar may be complicated and, therefore, analyses of plantlets can be based on either arbitrary scores or determination of plantlets weight.
- (v) Fresh weight determination can include the whole plantlet or separate analyses of shoots and roots. The respective tissue can be dried in a 70 °C oven for 48–72 h for dry weight analyses.

● TIMING

Arabidopsis seed vernalization, 3 days

Seed germination and root elongation on paper, 5–7 days

Seed germination and seedling growth in plastic sleeves, 2–4 weeks

Boxes 1 and **2**, germination and plantlets growth on agar, 3–4 weeks

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(viii), 1B(vii), 1C(ix), Box 1 (5) and Box 2 (4)	No herbicidal activity observed	The herbicide is not dissolved properly	Ensure that the herbicide powder is completely dissolved and that the solution is homogeneous, with no aggregates
1A(iii)	Contamination by fungi or bacteria	Poor technique; important to sterilize the media and equipment. Contaminated stock seeds	Ensure that sterilization procedures are adequate. An additional sterilizing treatment can include sterilization of seeds by immersing them in a diluted sodium hypochlorite bleach solution (1:3 bleach:ddH ₂ O) for 10 min followed by three washes with ddH ₂ O. Make sure that no residues of the bleach are left on the seeds

ANTICIPATED RESULTS

The methods described here should provide not only qualitative data, but also quantitative measures of plant tolerance (sensitivity) to a specific herbicide. Root elongation rate under control conditions is in the range of millimeter(s) a day,

resulting in final root lengths of up to 20 cm (in plastic sleeves). When grown on agar or sand media, plantlets weight can reach values of 10–20 mg after 3–4 days. As all bioassays are performed in the laboratory, levels of variations are fairly low and standard errors should not be higher than $\pm 10\%$ of the measured values. Therefore, differences of over 30% in sensitivity to the respective herbicide should be identified.

In addition, these assays enable the quantitative characterization of a newly formed herbicide.

Effective herbicides should inhibit root elongation (and shoot growth) by concentrations in the range of 0.1–100 p.p.m. (Fig. 4).

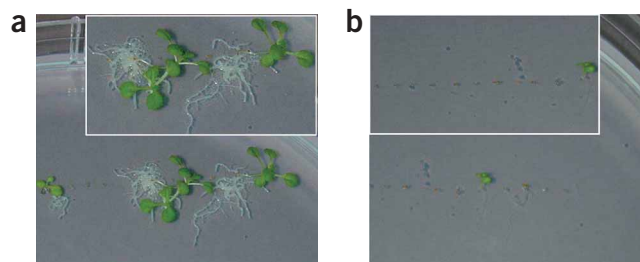


Figure 4 | Influence of Propham on germination of *A. thaliana* seeds and seedlings growth. (a) Plantlets grown on agar containing no propham. (b) Plantlets grown on agar containing 5 p.p.m. Propham. The inserts contain closer look at the germinating plantlets in the respective treatments.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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