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5.4 Preparation and Deposition of Plant Roots for AFM Nanomechanical Measurements

5.4.1 Introduction

In general, cell growth is a mechanical process that balances internal and external stresses allowing or limiting expansion. Plant cells are often compared to “hydraulic machines” due to the similar concept of balanced counterforces between the primary wall stresses and the turgor pressure. Knowledge of the mechanics of plant root cells is essential to understand how plant wall works and, therefore, how plants grow. One method for qualitative and quantitative analysis of mechanical properties is atomic force microscopy (AFM) that measures mechanical properties of living cells or tissues under conditions close to relevant physiological environments (Arnould et al., 2017, Kozlova et al., 2019, Milani et al., 2011, 2014, Peaucelle et al., 2012, 2011, Torode et al., 2018, Yakubov et al., 2016, Zdunek and Kurenda, 2013, Zhao et al., 2005). In our recent study, AFM has been used to investigate properties of root epidermal cells (Balzergue et al., 2017), which are the cells that form the outermost layer of the root. These epidermal cells were studied in situ on living seedlings and were therefore still influenced by the inherent multicellular properties of the root, which is an improvement over studies conducted on isolated living cells.

This chapter explains and illustrates the different steps required to prepare and deposit the seedling, and in particular the root tip, of the plant on a glass slide ready for nanomechanical measurements by AFM.

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5.4.2 Materials

Seedlings are from the *Arabidopsis thaliana* Col^{er105} background hereafter named wild type (WT) (Balzergue et al., 2017). The arabidopsis WT (Col^{er105}), *stop1*⁴⁸, *almt1*³², and *lpr1-2* lines were described previously (Balzergue et al., 2017). To homogenize the germination rate and seedling size, small seeds were screened out with a nylon mesh. During all the setup work, plant preparation is done in a sterile environment to avoid contaminations by bacteria or fungi spores; it is thus performed in a sterile laminar flow hood.

MES-(2-(*N*-morpholino)ethanesulfonic acid (Sigma, M8250) buffer (MES, pH 5.8, 50× concentration). pH is adjusted to 5.8 by adding 10 N of KOH.

SDS (sodium dodecyl sulfate) solution at 0.05% (Sigma, L4390).

Ethanol (#4145872, Carlo Erba, Val de Reuil, France).

Peltier-cooled incubator (IPP 110 +, Memmert, Schwabach, Germany).

FeCl₂: 15 M stock solution (Sigma, 44939).

Petri dish: 4-well plates 125 × 125 mm.

Microtape: Anapore, 9.14 m × 1.25 cm (Euromedis, Neuilly-sous-Clermont, France).

Artificial LED light box: Indoor Led, 45 W, 169 LEDs, 276 × 276 × 14 mm, full spectrum (www.cultureindoor.com).

Glass slides: StarFrost 3 × 1 in. (Knittel Glass, Braunschweig, Germany).

Cover slips: Glass 24 × 32 mm (Biosigma, Cona, Italy).

2 mL Standard Screw Thread Glass Vials, 12 × 32 mm (Thermo Scientific, C4013-2).

Superglue 3: Loctite (Henkel, France).

Silicone adhesive: NuSil MED1-1356 (NuSil Technology LLC, Carpinteria, CA, USA).

Cantilevers: The Pyrex-Nitride Probe (PNP-TR-50, NanoWorld AG, Neuchatel, Switzerland). (cant #2) has 200-µm-long silicon nitride cantilevers and integrated oxide sharpened, pyramidal tips with a height of 3.5 µm (and a 4-µm edge setback), a resonance frequency of 17 kHz, and a 70-nm-thick gold coating.

Growth solution:¹ 20 µL of 50× MES buffer (3.5 mM, pH 5.5–5.8) + 1 mL of MS/10 liquid medium (Balzergue et al., 2017).

MS/10 liquid medium: Sucrose (5 g/1 L) + 20 mL solution 1 (see Table 5.4.1).

¹ To be kept for 1 day max.

Table 5.4.1: Composition of solution 1.

Components	Final concentration
NH ₄ NO ₃	2.1 mM
KNO ₃	1.9 mM
CaCl ₂	0.3 mM
MgSO ₄	0.15 mM
H ₃ BO ₃	10 μM
MnSO ₄	10 μM
KI	0.5 μM
ZnSO ₄	3 μM
Na ₂ MoO ₄	0.1 μM
CoCl ₂	0.01 μM
CuSO ₄	0.01 μM

5.4.3 Sterilization of the Seeds

Seeds are surface-sterilized in a clean environment under a laminar flow hood using an Eppendorf tube (1.5 mL) and with a solution (SDS 0.05%, ethanol 70%) and then washed using ethanol 96%.

- Place a small quantity of seeds (an equivalent of around 50 μL or less) in an Eppendorf tube. Remember to mention the name of the seeds on it with date and cover the label with a transparent tape.
- Add 1 mL of the washing solution (SDS 0.05%, ethanol 70%) to the Eppendorf tube. Close the cap and shake for 1–5 min.
- With a pipette, push two times in the medium so that seeds are settled down. Then, pump out the solution carefully avoiding to take out seeds and discard the liquid.
- Rinse with 1 mL of pure ethanol. Shake the Eppendorf for 1 min minimum. Take out the solution with the help of a pipette.
- To dry the seeds, keep the Eppendorf open for at least 1 h under the laminar flow hood.
- Before storing seeds, check their dryness after sterilization. Seeds can be kept at room temperature, but ideally at 4 °C with low hygrometry.

Sterile seeds can be stored for up to 3 months. When kept for a longer time, the germination rate decreases with time, and seeds do not germinate synchronously.

5.4.4 Sowing Seeds

Seeds are sown onto the agar medium within petri dishes and grown under artificial light in a Peltier-cooled incubator.

5.4.4.1 Agar Preparation

The composition of the agar medium components is shown in Table 5.4.2.

Table 5.4.2: Agar medium.

Components	Amount per liter
Agar Sigma	8 g
Sucrose	5 g
Solution 1	20 mL

- The autoclaved solution 1 is added under sterile conditions.
- Complete to 1 L with deionized water. If a conventional microwave oven is planned for melting the agar, split the preparation into 500 mL bottles.
- Seal the bottles, add a piece of an autoclave tape, and put it in an autoclave for sterilization (120 °C, 30 min).

5.4.4.2 Preparing Agar Plates

Before melting a solidified growth medium in a microwave oven, do not forget to slightly unscrew the lid.² Heat the growth medium for around 3 min intermittently or observe until it becomes completely melted.³ ! Do not forget to wear thermoprotective gloves to handle the bottle. Wait until the agar medium is completely dissolved and melted.⁴

- Take a 50-mL Falcon™ tube and add 15 mL of agar solution.
- Mix in the tube with 300 µL MES buffer (50×) followed by 4 µL FeCl₂ (15 mM); 4 µL is the required volume for the desired concentration of iron at 4 µM.
- After a gentle mixing, the preparation is poured in the Petri dish.
- Label the Petri dish with “Fe₀” for zero iron stress, “Fe₄” in case of 4 µM stress, and so on.
- Wait for the agar medium to cool down until the gel is solidified.
- Sow a few seeds carefully into the medium and align them with a sterile wooden stick (see Figure 5.4.1).
- Seal the plate with microtape to prevent the evaporation of the water, yet allowing airflow.

² Without unscrewing the lid, there is a significant risk of explosion during the heating.

³ Do not heat the medium excessively as water will evaporate and thus change the concentration of the nutrients.

⁴ If needed, the bottle can be stored for a while in an incubator at 60 °C. At this temperature the growth medium will not boil nor evaporate, but still remains in a melting state.

5.4.4.3 Seedling Growth

The agar plate containing the seeds is kept under an artificial LED light box (Indoor Led) in a Peltier-cooled incubator (Memmert IPP + 110) with a 16 h photoperiod at 24 °C/21 °C day/night, respectively. To allow the root to grow on the surface of the medium, incline slightly the plate as shown in Figure 5.4.2. Figure 5.4.1 shows seedlings 4 days after sowing.

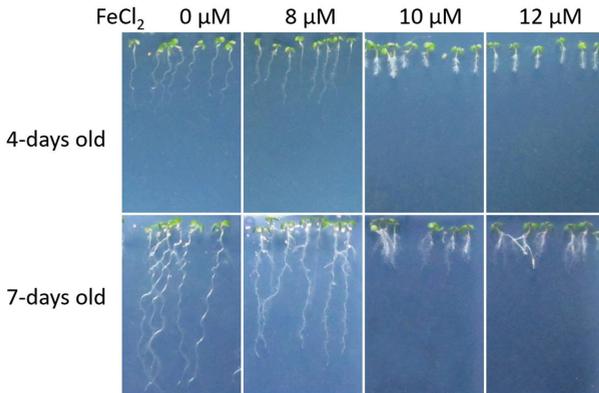


Figure 5.4.1: Four- and Seven-day-old seedlings grown on the solidified agar medium containing 8, 10, or 12 μM FeCl_2 . After 4 days under 10 and 12 μM FeCl_2 , a clear root growth arrest is observed; while at day 7, a reduced root growth is also observed under 8 μM FeCl_2 .

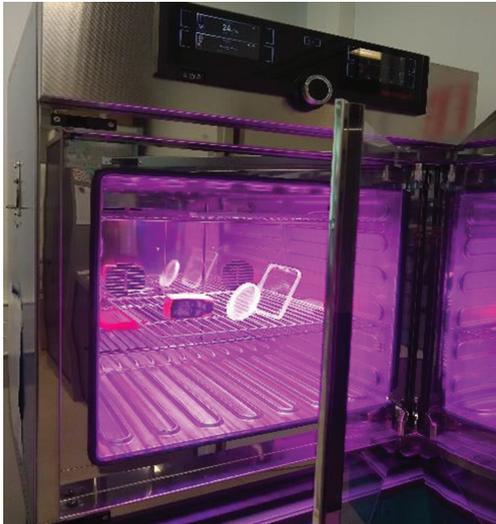


Figure 5.4.2: Slightly inclined squared agar plates in the Peltier-cooled incubator with an artificial LED light box.

5.4.4.4 Seedling Stress

We perform experiments after a 2 h stress by transferring seedling from Fe_0 agar plate to an agar plate with a given stress condition (Fe_0 , Fe_5 , Fe_{10} , etc.). After 2 h, plants are mounted on a glass slide.

! Because it takes about half an hour to do the experiment on each plant, careful planning of the daily experiment is required.

5.4.5 Mounting Root Samples on Glass Slide

Mounting the root samples on glass slides is the most delicate step in this experiment. The goal is to maintain plant roots immobile on a glass slide but keeping a physiological liquid buffer environment.

- Take two standard glass slides plus two rectangular cover slips.
- Add a superglue drop in the middle of both glass slides.
- Position the cover slips on the glass slides as shown in Figure 5.4.3; make sure that both the coverslips are at the same height in the middle of the slide and that two-thirds of width is touching the slide and one-third of it is outside the slide.

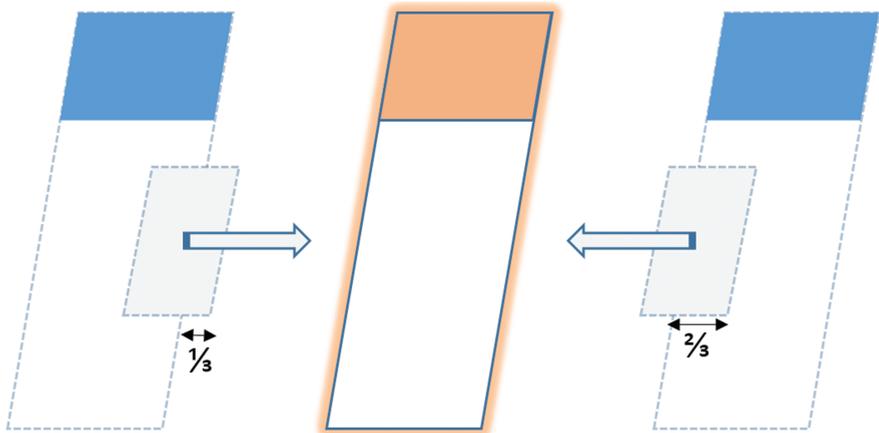


Figure 5.4.3: Principle of assembly of glass slides to mount plant seedlings for further mechanical analysis with AFM.

- Take a third glass slide and place it in the middle of the other two glass slides such that a little rectangular space is left in between (Figure 5.4.3).
- On the middle glass slide, deposit a uniform layer of silicone adhesive (NuSil MED1-1356) approx. 90 μL is spread carefully with the help of a coverslip (square #1, Figure 5.4.4).

- !! The silicon adhesive tends to solidify with time, so it should not be stored in big bottles. Aliquot NuSil in small glass tubes (preferably 2 mL) so that a tube will serve for a single day of work.
- Wait for 20–25 s, so that the adhesive starts drying, yet it is still wet for the sealing purpose. The goal is to place the root on the adhesive when it is semisolid so that it stays on the surface and does not dip into the adhesive.
- Take one seedling from the Petri dish and, using a dedicated tweezer,⁵ gently lift it from the shoot area preferably under the cotyledon without pinching the seedling.
- With the help of a binocular, place the plant on the slide such that the root tip is placed first, followed by the shoot part.

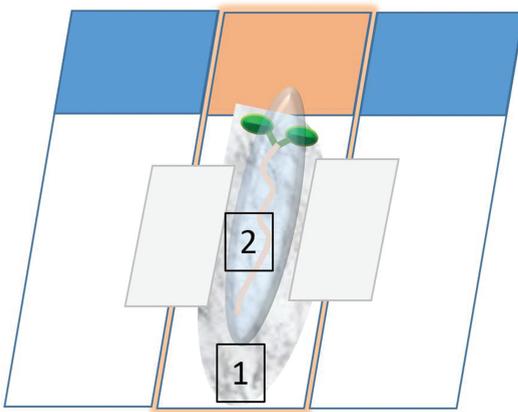


Figure 5.4.4: Principle of mounting plant seedling on glass slides. The adhesive layer is shown in light gray and labeled with the number 1. A long primary root in orange and two green cotyledons schematize the seedling. The seedling is finally covered with a growth medium shown in light transparent blue color and labeled with the number 2.

This is the challenging technical step:

- After placing the root on the glass slide, it is necessary to seal it with the semi-solid adhesive, starting from the top, then the middle and the very end (root tip) to prevent any movement of the root (Figure 5.4.5). !! Sealing by adhesive is done such that the height shall remain constant throughout the slide. With an excess of adhesive, the tip may touch the adhesive during the positioning under the AFM scanner or the edge of cantilever support may also touch the adhesive that will bias nanomechanical measurements.

⁵ NuSil is a glue and it is difficult to fully remove from the tweezer. Thus, the tweezer would not be able to be used for picking cantilevers for instance.

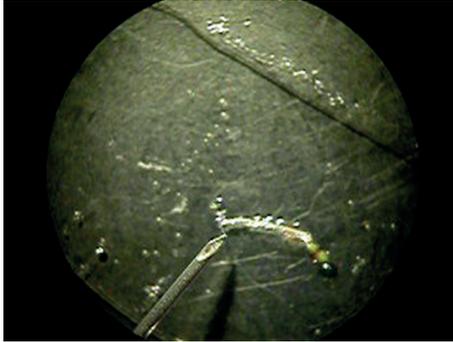


Figure 5.4.5: Optical magnification of deposited seedling roots on adhesive over a glass slide. A thin needle (bright color on the left) is used to fasten the position of the root on the adhesive located all over underneath the root by picking hardening adhesive over the root. Several fastening strips are required along the root but keep a significant room for the investigation zone, which is about 500 μm from the root tip. Note that the diameter of the seedling root is about 120 μm .

- Cover entirely the plant root (at least 200 μL) with growth solution (square #2, Figure 5.4.4) to prevent drying during measurement. !! Do not use water to cover the root as it will generate a stress of high cellular turgor pressure.

The mounted seedling is then positioned under the AFM so that the plant is still alive, and experiment on living root tissues can be performed.

5.4.6 AFM Protocol

Due to the nature of the sample, it is necessary to use an AFM scanner over the top of the sample. In our case, we use a Dimension 3100 AFM (Bruker, Santa Barbara, USA) with a Nanoscope V controller and a Nanoscope 7.3 software. The advantage of this system is the large motorized sample stage, which is perfect for plant root analysis. Before starting measurements, there are several parameters and calibration steps that need to be performed each time.

5.4.6.1 Set Up

When experimenting with AFM cantilever in fluid, the probe holder DTFML-DD (Bruker, Santa Barbara) prevents the scanner tube from coming into contact with the fluid environment. Triangular pyrex nitride cantilevers with pyramidal tips of 10 nm nominal

radius and a half-opening angle of 35° were used. The reasons for using this tip are its symmetry, its low sharpness, and its proper spring constant for plant tissues.

5.4.6.2 Laser Alignment

Aligning the laser onto the cantilever is one of the most critical parts of the AFM setup. A good laser alignment helps control the signal-to-noise ratio, the sensitivity of the cantilever deflection or amplitude, and ultimately the quality of any data acquired using the AFM. A poor alignment may cause excessive applied forces or the presence of optical interferences (fringes) from the liquid surface of the sample. Check the instructions of your system for optimal laser alignment. In our case, a SUM value of 3.5–4 V is usually achieved with PNP-TR cantilevers. At the end, the laser signal is centered on the photodetector using proper adjustment knobs.

5.4.6.3 Calibration and Deflection Sensitivity

The deflection sensitivity S is used to convert photodiode measurements (in V) into distances (nm), which in turn can be converted to forces (N) upon further multiplication by the spring constant k (eq. (5.4.1)). The calibration of photodetector sensitivity is first done in air by acquiring a deflection versus distance curve against a hard homogeneous surface (like glass slide), whose effective stiffness is orders of magnitude greater than the cantilever stiffness. Refer to the instructions of your system to perform this step properly. Detailed information can also be found in Chapter 3.1.3 on calibration issues:

$$F = k \cdot S \cdot \Delta x \quad (5.4.1)$$

where F is the force (N), k is the cantilever-specific spring constant (N/m), S is the sensitivity of the position-sensitive photodetector (m/V), and Δx is deflection (V).

A typical force versus distance curve from which the deflection sensitivity can be obtained is shown in Figure 5.4.6. The relationship between parameters is shown in eq. (5.4.1), an adaptation of Hooke's law in which the deflection is comprised of two terms: the deflection sensitivity as measured in meter per volt and the deflection as measured in volt (Schillers et al., 2017).

The cantilever is ramped over a very stiff surface. Accordingly, all downward piezoelectric motion will be equal to cantilever bending (right part of the graph). The inverse of the measured slope (red line) corresponds to the sought deflection sensitivity (in nm/V) (Gavara, 2017).

Measuring the stiffness of the cantilever is necessary to determine the applied force during the experiment; and this is done using the thermal tuning methods where spring constant is calculated. Finally, the deflection sensitivity is determined

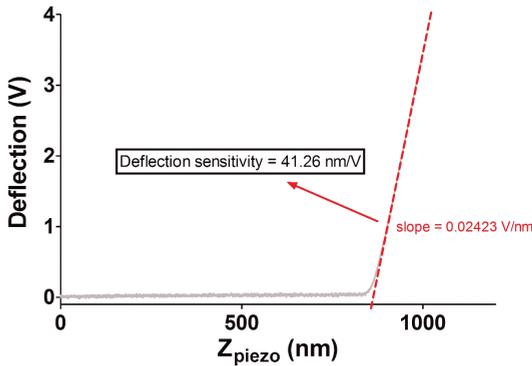


Figure 5.4.6: Calibration of the deflection sensitivity.

again in liquid medium because indentations are performed on samples while they are immersed in the liquid medium and its value is often lower than that in the air. For the case of PNP cantilevers and Nanoscope 7.3 software, it is not possible to determine the spring constant of the cantilevers in liquid medium (low resonance frequency of PNP cantilevers). The value determined in the air is used instead. See Chapter 3.1.3 on calibraton issues for complete description of calibration procedures.

5.4.6.4 Recording the Force–Distance Curves

Before positioning the plant root under the AFM cantilever, make sure that the cantilever is retracted enough to allow a safe distance between the cantilever and the plant root. Lift the scanner head carefully and insert the glass slide with the plant root. Buffer is already on the top of the root, and so carefully lower the scanner head so that the cantilever will not bend upon contact with water (adding a drop of buffer on the cantilever is usually necessary to have a smooth insertion in liquid). Orient the glass slide so that the root orientation is close to vertical, and its tip is on the top of the camera (Figure 5.4.7). The target working area (elongation zone of the root) is located approximately 500 μm away from the root tip. This distance can be estimated knowing the length of PNP cantilever (about 200 μm). Use the stage positioning controls to move the cantilever on the right zone. Because the root is cylindrical and the tip height rather small (3.5 μm), it is not advised to probe the root beyond the longitudinal median line of the root. Note that there is small setback of the tip on PNP cantilever (4 μm).

During the experiments, the deflection setpoint is kept at 0 V while the initial vertical deflection on the photodiode is set to -2.5 V. For all the analysis, the ramp size for the force–distance curves is kept at 3 μm (maximum z -piezo range of 5 μm). Trigger is set off and no trigger values are used. The later point implies that it is often

necessary to adjust the z -start value for the ramp each time a new engagement has been done.

Because the AFM tip has a setback of $4\ \mu\text{m}$, it is not possible to ascertain the exact target area for indentation (edge or middle of cells). A trick is to perform force–distance measurements in 16 different nodes on the elongation zone (Figure 5.4.7). Each time, curves are recorded with the auto ramp feature in 2×2 matrix, having four force–distance curves at each node separated by $50\ \text{nm}$ in distance between each point. Between each of these 16 nodes, the piezo move in X - and Y -directions using the offset facility of Nanoscope 7.3 according to the pattern is shown in Figure 5.4.8. Because the plant root is living, even when fixated by nusil on the glass slide, the root continue to grow. Thus, it is important to avoid going up and down along the root during the acquisition. We choose to go down in a single direction as shown in Figure 5.4.8. On our system, force–distance curves are recorded after withdrawing and re-engaging at each matrix node. In rare cases, it is necessary to withdraw and move the cantilever away ($5\ \mu\text{m}$) to probe another area. In practice, it usually takes 25 min to record a full set of force–distance curve for a single plant root. It is important to keep the time for acquisition as short as possible to avoid additional stress effect.

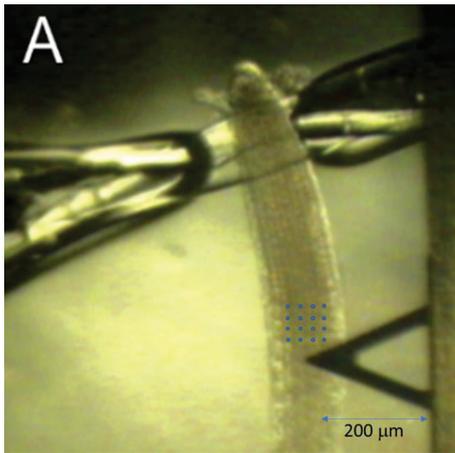


Figure 5.4.7: Camera-taken snapshot of a plant root under an AFM cantilever (triangle on the right). The fastening of the root tip can be seen on the top of the image. The elongation zone where indentation must be performed is located approximately $500\ \mu\text{m}$ from the end of the root tip. This distance can be estimated using the length of the cantilever ($200\ \mu\text{m}$ here for PNP-TR). The blue dots represent the redundant measurement performed on the plant root (data points not to scale).

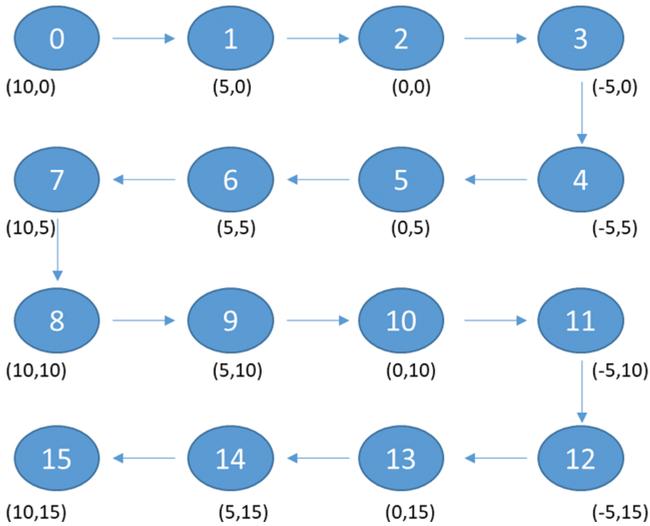


Figure 5.4.8: Schematic diagram used to perform the redundant indentation measurements. An experiment starts at position 0 and goes through the last point 15. The X,Y offset (in μm) needed to move from one point to the next one is indicated in parenthesis. Coordinate system is according to the piezo of Dimension 3100.

5.4.7 Plant Phenotype

Detailed results from experiments on plant roots will be presented in Chapter 6.11. In brief, it has been found that the presence of iron (under low phosphate condition) leads to a phenotype of plant root growth arrest (Balzergue et al., 2017). It is therefore an important step in this experimental protocol to check whether the phenotype “root growth arrest” is observed. Experiments were performed on 4-day-old seedlings, and it is necessary to check next day for the expected phenotype. With a pen, label the position of the root tip of the remaining seedlings on the +Fe agar plates and check the next day for the presence or absence of root growth. In Figure 5.4.9, roots transferred from Fe_0 to Fe_0 (left) show a normal growth, that is, the end of the root is beyond the marker line, whereas when roots were transferred from Fe_0 to Fe_{15} (right), no root growth is observed after 1-day post-AFM experiments.

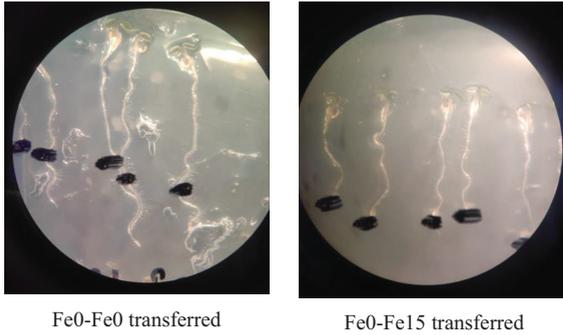


Figure 5.4.9: Agar plates after mechanical measurements. Several seedlings remained on the plate to confirm the presence of the expected phenotype (here, the root growth arrest under high concentration of Fe and low phosphate). To check the phenotype, a mark is made with a pen on the day of the mechanical experiment, and the growth of plants is checked 1 or 2 days later. Picture taken 24 h after transfer.

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