INTERNATIONAL STANDARD

ISO 4979

First edition
2023-06

Water quality — Aquatic toxicity test based on root re-growth in *Lemna minor*

Qualité de l'eau — Essai de toxicité aquatique basé sur la repousse des racines chez Lemna minor

Reference number
ISO 4979:2023(E)

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Published in Switzerland

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 5, *Biological methods*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Lemna gibba and *L. minor* have been most extensively used in phytotoxicity testing and there are several standard methods which have been adopted by major international standardization agencies, for example, ISO 20079:2005, U.S. Environmental Protection Agency[1], Organization for Economic Cooperation and Development[2]. Tests with duckweed have typically favoured measurements of frond (e.g. their number, biomass, area, carbon uptake, chlorophyll content[3],[4]) and require standard exposure durations of at least 7 d to detect toxicity.

On the other hand, tests based on root elongation are some of the most widely used phytotoxicity methodologies for terrestrial angiosperms because of their simplicity and rapidity. Despite reports that roots of *L. minor* are highly sensitive to environmental stressors and that they play important ecological roles by providing stability[5],[6],[7],[8], little attention has been paid to the roots in *Lemna* since it was generally considered that root fragility made their handling for measurements difficult and that it was impractical to obtain sufficient numbers of individual plants with identical root lengths to initiate tests. However, the ecotoxicological significance of the root endpoint has been re-evaluated and root length was shown to be a sensitive, precise and ecologically significant endpoint in comparison with more traditional frond growth or biomass endpoints[7].

The proposed root re-growth bioassay differs in several key aspects from three internationally standardized methods (ISO, OECD and US EPA):

a) the test can be completed within 72 h;

b) the test vessel is a 24-well cell plate;

c) the required volume of test water samples is 3 ml;

d) roots were excised prior to exposure with subsequent measurements on newly developed roots. The technique of excising roots prior to exposure means that there is no requirement to pre-select roots of uniform length, which reduces the handling of these fragile roots.

Artificial severance of roots will possibly never happen in natural settings since root abscission in *Lemna* has not been reported previously. However, according to recent studies, the tiny globally distributed water ferns of the genus *Azolla* lost their roots under stress conditions[9], a phenomenon known as rapid root abscission. Such shedding sets its fronds free from root-entangled mats and facilitates their dispersion to a potentially better environment. Therefore, rapid root abscission is considered an important survival strategy of *Azolla*[9]. This can indicate that the endpoint root re-growth has its ecological relevance.

It is also well known that *Lemna* will thrive without any roots. Thus, *Lemna* roots appear nonessential organs, but are nonetheless important for plant anchorage, nutrient absorption and cytokinin biosynthesis. Therefore, the manipulation of roots by simple severance can be an unimportant issue and does not justify the conclusion that the removal of roots prior to ecotoxicological testing is inappropriate.

The three-day root re-growth test is useful for the rapid screening of either wastewater effluents or hazardous contaminants in natural waters[8] as it is easy to perform, quick to run and cost-effective to operate for wastewater toxicity screening and can have an operational benefit of testing time since management decision should be made in a timely manner in the case of unexpected pollution events.

The root re-growth endpoint from this 72-hour protocol is not a direct substitute for the seven-day growth rate/biomass endpoints.

The present protocol provides detailed information on how to set up and conduct the root re-growth test with *Lemna minor* as well as how to analyse toxicity data. This protocol is intended for use with *Lemna minor*, but it can also be applied to other *Lemna* species and *Spirodela* species with some modifications.

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WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted according to this document be carried out by suitably trained staff.

1 Scope

This document specifies a method for the determination of the inhibition of root re-growth in duckweeds (*Lemna minor*) by substances and mixtures contained in water or waste water. This method applies to environmental water samples including treated municipal wastewater and industrial effluents.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
axenic culture
monocultures of organisms from a single species, free from fungi, algae and other macrophyte species

[SOURCE: ISO 20079:2005, 3.1]

3.2
coefficient of variation
CV
relative standard deviation, expressed as a percentage

3.3
colony
aggregate of mother and daughter fronds, attached to each other, sometimes referred to as a plant

[SOURCE: ISO 20079:2005, 3.4]

3.4
effective concentration
EC_x
concentration of test sample at which an effect of x % is measured, if compared to the control

[SOURCE: ISO 20079:2005, 3.9, modified — Note 1 to entry deleted]

3.5
frond
individual leaf-like structure on a duckweed colony; the smallest unit (i.e. individual), capable to reproduce

[SOURCE: ISO 20079:2005, 3.10]

3.6
growth
increase in biomass over time as the result of proliferation of new tissues

Note 1 to entry: In this test, it refers to any parameter of observation.

[SOURCE: ISO 20079:2005, 3.13]

3.7
growth medium
pure water to which reagent-grade chemicals (micronutrients) have been added

3.8
non-axenic culture
monoculture of organisms from a single species (i.e. free from other macrophyte species), which has not been treated with antimycotic or antibiotic solutions to remove naturally associated bacteria and fungi

3.9
pre-culture
culture of duckweed used for acclimation of test plants to the test conditions and for the growing of the plants to be used in the inoculum

[SOURCE: ISO 20079:2005, 3.19]

3.10
pure water
deionized or distilled water

[SOURCE: ISO 19827:2016, 3.4]

3.11
root
plant part that normally grows under water and holds the plant in place

[SOURCE: ISO 20079:2005, 3.20]

3.12
stock culture
culture of a single species of duckweed to conserve the original *Lemna* species in the laboratory and to provide inoculum for the pre-culture

Note 1 to entry: It is necessary to use defined and verified strains, because of possible insecurities in species taxonomy.

[SOURCE: ISO 20079:2005, 3.21, modified — "An address list of suppliers is given in Annex C" has been deleted from Note 1 to entry.]

3.13
stock solution
solution with accurately known analyte concentration (s), prepared from chemicals with an appropriate purity

[SOURCE: ISO 5667-16:2017, 3.21]

3.14
test sample
discrete portion of sample (taken from, for example, receiving water, waste water, dissolved chemical substances or mixtures, products and compounds) pre-treated according to the needs of the test (e.g. dissolution, filtering, neutralization)

[SOURCE: ISO 20079:2005, 3.24]

3.15
test medium
aqueous solution that consists of a particular concentration of prepared test sample mixture of test water and the sample under test

[SOURCE: ISO 21427-1:2006, 3.4]

4 Principle

All roots shall be removed from *Lemna minor* fronds, grown in axenic or non-axenic cultures, prior to exposure to test samples and the growth of newly developed roots during the exposure period of 72 h will be measured.

To quantify substance-related effects, the root length in the test medium is compared with that of the controls and the concentration resulting in a relative inhibition of root length to be determined and expressed as the $EC_{(r)x}$.

5 Test organisms

The standard test organism of this test is duckweed, *Lemna minor*, which is a freshwater-floating plant.

6 Growth medium

6.1 Preparation of stock solution

Prepare stock solution by adding the weighed chemicals according to Table 1 to the desired volume of pure water for the growth medium and test compound solutions. Pure water should be used for the dilution of liquid media and test substances.

pH of liquid media shall be adjusted to 6,9 ± 0,1 after adding pure water to each stock solution. Nutrients should be added in the order of I-II-III-IV-V while preparing the solutions to prevent precipitation.

NOTE A pH of 7 is ideal for the re-growth of *Lemna* roots and pH 6,9 ± 0,1 is proposed to be the appropriate pH for the toxicity tests, as this is also the same pH range that is measured in the Steinberg medium.

Liquid media can be stored for up to one month at room temperature in the dark.

Table 1 — Main ingredients of Steinberg medium

Stock solution	Chemicals	Stock concentration g l^{-1}	Final concentration ml l^{-1}
Macro-elements			
I	KNO_3	17,5	20
	K_2HPO_4	4,5	
	KH_2PO_4	0,63	
II	$MgSO_4 \cdot 7H_2O$	5	20
III	$Ca(NO_3)_2 \cdot 4H_2O$	14,75	20

Table 1 *(continued)*

Stock solution	Chemicals	Stock concentration g l^{-1}	Final concentration ml l^{-1}
Micro-elements			
IV	H_3BO_3	0,12	1
	$ZnSO_4 \cdot 7H_2O$	0,18	
	$Na_2MoO_4 \cdot 2H_2O$	0,044	
	$MnCl_2 \cdot 4H_2O$	0,18	
V	$FeCl_3 \cdot 6H_2O$	0,76	1
	Na_2-EDTA$\cdot 2H_2O$	1,5	

6.2 Storage and cultivation

Culture fronds of *Lemna minor* in growth medium (3.7) is shown in Table 1. The fronds (3.5) shall be cultured at (25 ± 1) °C, given 24 h continuous white light at an intensity of 30 µmol m^{-2} s^{-1} to 40 µmol m^{-2} s^{-1} (1,500 lx to 2,000 lx). The medium shall be replaced at an interval of 7 d and the storage culture can be kept continually unless uncontrolled contamination occurs. Cloudy medium in a *Lemna minor* stock culture indicates bacterial contamination, whereas contamination with mould is possibly not clearly evident until large colonies appear in the medium or a slime layer develops on the vessel. Contaminated *Lemna minor* cultures shall be discarded.

7 Apparatus

The test requires usual laboratory equipment and the following.

7.1 Temperature-controlled cabinet or room, with a white fluorescent light, providing, continuous, uniform illumination in accordance with the requirements specified in Table 2.

Table 2 — Summary test conditions for the *Lemna* root re-growth test

Type of test:	Static; 72 h test
Lighting:	90 µmol m^{-2} s^{-1} to 100 µmol m^{-2} s^{-1} (4,500 lx to 5,000 lx; 400 nm to 700 nm)
Photoperiod:	Continuous cool white fluorescent light
Temperature:	(25 ± 1) °C
Salinity:	0 psu
pH:	6,9 ± 0,1
Test vessel:	24-well plate (85,4 mm × 127,6 mm; well dimension 15,6 mm diameter)
Growth medium:	Steinberg medium
Volume of test solution:	3,0 ml/well
Number of colonies per well:	One colony; an individual colony has two fronds to three fronds each with one root
Number of replicates:	At least three (6 control replicates are recommended)
Endpoint:	Root length

NOTE As 25 °C is optimal for the re-growth of *Lemna minor* roots, the temperature range was determined, taking into account temperature changes due to electricity in the culture chamber.

The test shall be performed after adjusting the pH if the pH is outside the acceptance range.

7.2 Light-meter, to be used to measure in photon irradiance expressed in micromoles per square meter per second (µmol m^{-2} s^{-1}) or in lx.

Measure the light intensity once per test in at least five characteristic points of the test area in a realistic test environment. It shall not vary by more than ±15 % of the selected light intensity. The measurement of light with a spherical head quantifies all light that would reach the plants if the test solution is clear.

The use of a random design with changes at the observation times is recommended but does not compensate high deviations of light intensity and temperature between different places of the test area. Before a toxicity test is conducted with new test facilities, it is desirable to conduct a non-toxicant test, in which all test vessels contain control medium.

7.3 pH meter, for the adjustment of pH during the preparation of cultures and test solutions and to measure pH at the beginning and end of a test.

7.4 Tweezers, for handling fronds.

7.5 Stainless scissors, for excising roots.

7.6 Beakers.

7.7 Graduated cylinders.

7.8 Pipettes.

7.9 Conical tubes.

7.10 Plastic tank.

7.11 Exposure dishes, for example, 24-well cell plates with 3,0 ml per well (a diameter of 15,6 mm can be suitable).

Cell plates shall be sealed with sealing tape for prevention of evaporation of medium and test solution. In the case of volatile organic compounds, separate cell plates should be used to avoid transfer of volatile compounds between the wells.

NOTE Cross contamination is possible if the test substance is highly volatile.

7.12 Sealing tape, to seal around the exposure dishes.

7.13 Microscope slide glasses, for putting fronds on for taking measurements of root length.

7.14 Image analysis system, with a magnification of 4 times to 6 times, for measurements of the root length.

8 Experimental methods

8.1 Preparation of medium

To prepare 1 l of Steinberg medium, refer to Table 1. Chemicals shall be reagent-grade.

The medium shall be stirred until all the contents are dissolved. Adjust the pH to 6,9 ± 0,1 with 1 mol l^{-1} of hydrochloric acid (HCl) or that of sodium hydroxide (NaOH). Stock solution should be kept in the dark.

Individual stock solutions can be stored in the refrigerator (4 °C) for up to one month. Prepared medium can be stored for up to one month at room temperature in the dark.

8.2 Preparation of toxicant solution and test dilutions

8.2.1 Test dilutions

Test dilutions can be prepared in volumetric flasks (or conical tubes) and then distributed to the replicate test vessels.

8.2.2 Selection of test concentrations

In cases of uncertainty about sample toxicity, it is beneficial to run a range-finding test for choosing concentrations of definitive test.

A wide range of concentrations (e.g. ≥ an order of magnitude) should assist in the selection of the concentrations for the definitive test.

In case the test solution is an effluent or liquid material, set the concentration to 0 %, 6,25 %, 12,5 %, 25,0 %, 50,0 % and 100,0 %.

The concentration should be selected so that different levels (4 to 5 sections) of growth can occur in the range of less than 10 % to over 90 %.

8.3 Control

All experiments require a negative control with the identical culture medium, test conditions and procedures, but exclude the test substance.

8.4 Transfer of test organisms

Select fronds of duckweed that are dark green in colour and consist of 2 to 3 identical attached leaves.

Bring stainless scissors (7.5) in parallel to the bottom of the floating fronds, lift up the fronds, cut the roots and gently lay down the rootless leaves on the surface of the medium (see Annex A, Figure A.1).

Place one colony, consisting of 2 to 3 fronds, in each cell of 24-well plates by using tweezers (7.4).

Care shall be taken to ensure that the plants do not adhere to the side of the well.

The transfer of *Lemna minor* to test solutions shall be done in random order across the replicates within a concentration.

Cover the plate and seal with sealing tape to avoid evaporation of medium or test solution.

8.5 Culture

As a standard condition, the test organism shall be cultured at (25 ± 1) °C with continuous white light of 90 µmol m^{-2} s^{-1} to 100 µmol m^{-2} s^{-1} (4,500 lx to 5,000 lx).

The duration of the *Lemna minor* root growth inhibition test shall be 72 h.

The test is a static type so that the test solutions should not be changed for the duration of the test.

8.6 Method of measurements

After 72 h, use tweezers to pick up *Lemna minor* fronds with regrown roots and place them on the slide glass, with the upper part of frond facing the slide glass (see Annex A, Figure A.2).

As the fronds are wet with water, the roots can be easily spread straight even with a light touch. Images of *Lemna minor* fronds with regrown roots are captured by an image analyser (e.g. Image J). The length of the longest roots from one colony shall be measured. As there can be reproduction effects at 72 h, frond counts as an observation endpoint is also recommended.

Adjust and fix the distance between a camera and a slide glass on which a frond with regrown roots is spread. Calibration can be made by a ruler placed next to the slide glass when shooting an image.

NOTE 1 The range of expected growth for the control root length is (28,9 ± 20,2) mm (see Table B.1).

NOTE 2 'Image J software' (NIH Bethesda, MD, USA) is the name of an image analysis software which is freely provided by the US EPA and is available on the Internet.

9 Tests on effects

9.1 Reference chemicals

The routine use of a reference toxicant or toxicants during 72 h exposure is practical and necessary for quality assurance and to check the sensitivity of the *Lemna minor* being used.

For internal quality control, reference tests using $CuSO_4$ (CAS Registry Number®[1)] 7758-99-8) as a reference chemical shall be run periodically to determine if the *Lemna minor* being tested are responding to a known chemical in the expected manner, for example, the acceptable range of EC_{50} for $CuSO_4$ is 0,199 mg l^{-1} to 0,475 mg l^{-1} based on the results of the international ring test (see Annex B, Table B.2). Test with the reference chemical should be run every three months.

It is advisable to use control charts for measuring within laboratory precision and monitoring culture health.

9.2 Statistics

Calculate EC_{50} and standard deviations preferably using non-linear regression with the experimental data.

If the data do not meet the assumptions (describe what assumptions are meant) required for regression analysis, a non-parametric model or other statistical technique (describe what other technique is meant) should be used.

Differences between the levels of a factor (or factors) shall be analysed by an appropriate statistical test (e.g. Williams or Dunnett's test or – if the normality and homoscedasticity assumption does not hold – Jonckheere-Terpstra test).

9.3 Validity of test

The experiment should be repeated if the root length of the control is outside the mean determined by interlaboratory tests or the test is considered valid, if the CV between replicates of the same test concentrations is ≤30 % to 40 %.

1) Chemical Abstracts Service (CAS) Registry Number® is a trademark of the American Chemical Society (ACS). This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Assuming that all the recommended procedures and conditions are followed, the mean root length in the controls shall be ≥8,6 mm and the acceptable EC_{50} range for a reference toxicant ($CuSO_4$) is 0,199 mg l^{-1} to 0,475 mg l^{-1}.

NOTE These values are the minimum length of the regrown roots in the controls and the EC_{50} value range obtained from the ten different laboratories.

9.4 Precision

The interlaboratory variability from *Lemna* toxicity test using a reference toxicant ($CuSO_4$; CAS 7758-99-8) was 21,3 % for repeatability and 27,2 % for reproducibility (see Annex B, Table B.1).

10 Expression of results

10.1 Test results

Record root length values of each replicate, mean values and standard deviations. Calculate the coefficient of variation, the standard deviation expressed as a percentage of the mean, to estimate the precision and reproducibility of the tests.

Plotting concentration response curves is highly recommended to provide the basis for determining the effective concentration, EC_x, values for the inhibition of root growth.

10.2 Expression of results

The values calculated for EC_x (e.g. EC_{50}) and the corresponding standard deviation and CVs shall be displayed with the required significant precision (digits).

The values should be given as mg l^{-1} for tests with individual chemicals or as percentage for effluent samples.

11 Test report

This test report shall contain at least the following information and the data and test endpoint estimates shall be based on nominal test concentrations:

a) the test method used, together with a reference to this document, i.e. ISO 4979:2023;

b) name of the laboratory performing the test;

c) recent reference substance testing;

d) date and period of test;

e) test organisms (e.g. scientific name, strain, source, holding conditions);

f) test details;

- culturing apparatus and incubation procedure;
- culture types (static, static-renewal etc.);
- composition of medium;
- preparation of test sample (e.g. pH, salinity of effluent sample) and treatments;
- concentrations tested;
- replicates per concentration;

- number of fronds per replicate;
- size of cell plates;
- solution volume;
- light intensity and quality;
- pH of test solutions including the controls at start and end of test;
- salinity;
- temperature range during incubation;
- method of measuring root length;
- results;
- table outlining root length in each cell plate at each measuring point;
- mean root length for each test concentration (and control) at each measuring point including the validity of the test criteria;
- relationship between root length and concentration in table and graphical representation;
- EC_x values with standard deviations and the corresponding CV values including details of the statistical method used to estimate EC_x values including Software tools;

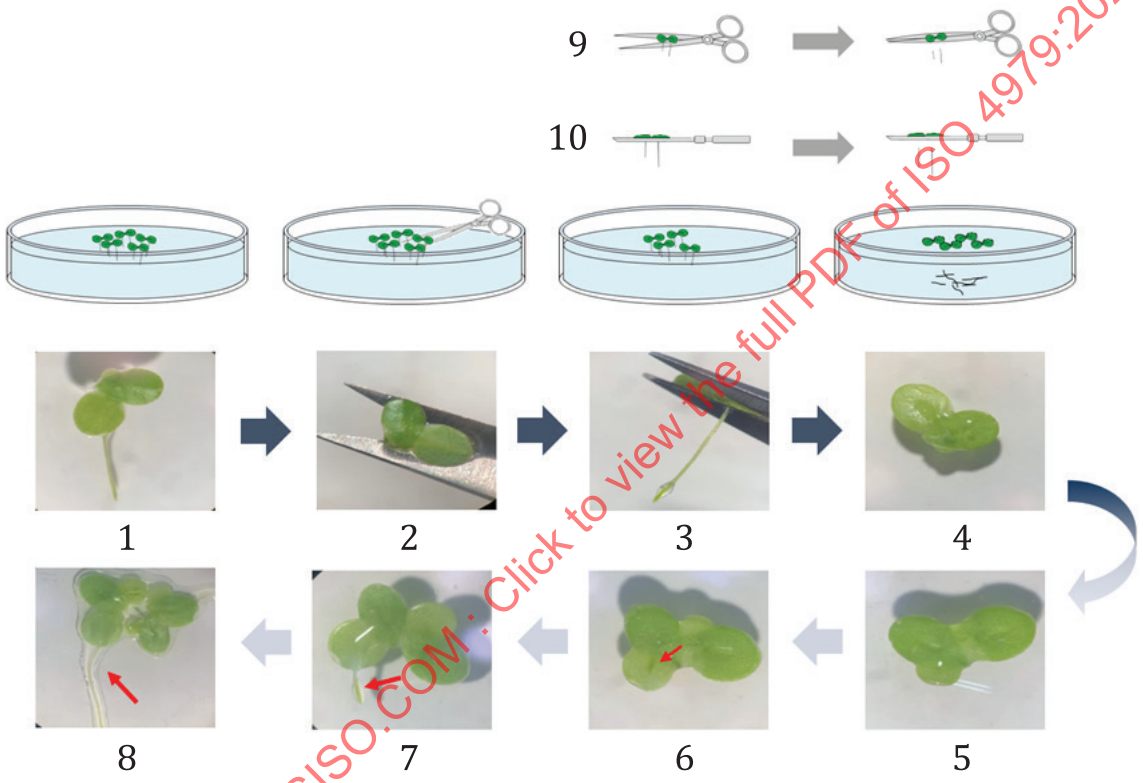
g) any deviations including other observed effects.

Annex A
(informative)

Root excision and re-growth length measurement

A.1 Excision procedure

Figure A.1 shows an excision procedure followed by re-growth of roots in *Lemna minor*.



Key

1 isolate fronds
2 lift up the fronds
3 cut the root
4 rootless leaves
5 rootless leaves after 12 h culture
6 regrown root after 24 h culture
7 regrown root after 48 h culture
8 regrown root after 72 h culture
9 top view
10 side view

Figure A.1 — Root excision and re-growth

A.2 Root length measurements

In Figure A.2 panel (a) shows the roots straightened on the glass slide, (b) shows an image analyser (right) and screen with captured images of *Lemna minor* fronds with roots (left). Panel (c) shows the fronds and roots placed onto a glass slide next to a ruler. The length of the longest roots is measured using an image analyser.

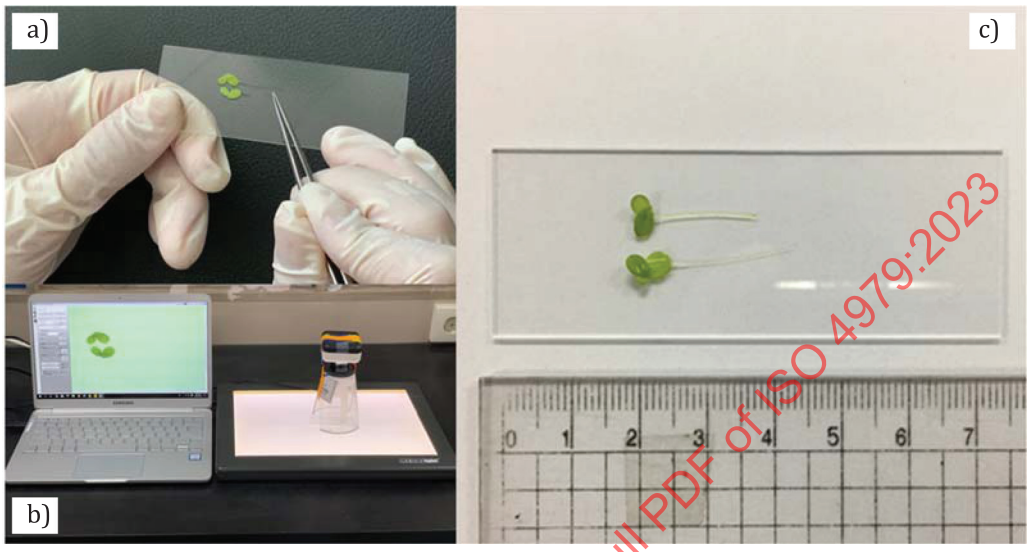


Figure A.2 — Root length measurements