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D4.1 Detailed protocols for
characterization of lines under growth-
chamber/green-house and field
conditions
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UNIVERSITY OF TARTU (UTARTU)

Deliverable description

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Glossary of terms

GM	Genetically Modified
GE	Genetically Engineered
PE	Plant establishment
HD	Heading Date
MD	Maturity Date
PH	Plant height
SpL	Spike length
GY	Grain Yield
BM	Biomass at maturity
TTN	Total Tiller Number
STN	Sterile Tiller Number
FTN	Fertile Tiller Number
SW	Seed weight
TGW	Thousand Grain Weight
Sm ²	Seed per unit area
SS	Seed per spike
StWt	Straw Weight
HI	Harvest Index
PAM	Pulse-amplitude modulated
PSII	Photosystem II
PSI	Photosystem I
F ₀	Minimum Fluorescence Yield
F _m	Maximum Fluorescence Yield
F _v	Variable Fluorescence
PPFD	Photosynthetic Photon Flux Density
Y(II)	Effective Photochemical Quantum Yield of PS II
Y(NPQ)	Quantum yield of light-induced non-photochemical fluorescence quenching
Y(NO)	Quantum yield of non-regulated heat dissipation and fluorescence emission
Y(NA)	Quantum yield of non-photochemical quenching due to acceptor side limitation

Y(ND)	Quantum yield of non-photochemical quenching due to donor side limitation
Y(I)	Effective Photochemical Quantum Yield of PS I
Cytb6f	Cytochrome b6f
ETR _{II}	Electron transport rate through PSII
ETR _I	Electron transport rate through PSI
FLV	Flavodiiron proteins
A	Absorbance
m	Mass
V	Volume
DL	Dilution factor
μmol	Micromole
ppb	Parts per Billion
E	Transpiration
gs	Stomatal conductance
A _{net}	Net Photosynthesis
PAR	Photosynthetically Active Radiation
ppt	Parts per Trillion
ppm	Parts per Million
RH	Relative Humidity
WRV	Water Retention Value
M_s	Saturated Mass
M_d	Dried Mass
CSA	Cross-Sectional Area
HRR	Heat Release Rate
THR	Total Heat Release

Field trials for characterization of barley plants

The field trials will be performed according to the project proposal (Table 1). Depending on the traits under study and seed availability, different sowing densities and nitrogen levels will be investigated.

Table 1. Locations of the field trials and planned experiments

Genetic materials	Starting experiments	Locations	Experiments
Non-GE/GM barley mutants with affected leaf angle (from WP1)	Year 1	Sweden (ULUND)	Scoring leaf erectness
Non-GE/GM barley mutants and lines with optimized light usage (from WP1), and different straw quality (from WP2)	Year 2	Italy (CREA) and Germany (KWS LOCHOW GMBH)	Standard and high densities, different N-inputs
GE/GM barley lines with reduced photorespiration penalty and enhanced O ₃ uptake (from WP1), and different straw quality (from WP2).	Year 3	Czech Republic (UP and Usovsko)	Standard and high densities, different N-inputs
Advanced barley breeding lines stacking natural and chemical-induced mutations (from WP3)	Year 5	Italy (CREA), Germany (KWS LOCHOW GMBH), Denmark (NORDIC SEED)	Standard and high densities, different N-inputs
Advanced barley breeding lines stacking GE/GM derived mutations (from WP3)	Year 5	Czech Republic (UP and Usovsko)	Different N-inputs

Availability and multiplication of seeds for field trials

The field trials will be carried out according to available seed amounts. Before the initial experiments and upon the development of new breeding lines, seeds will be multiplied to provide enough material for experiments. Non-GE/GM lines can be propagated by Nordic Seed, KWS, or CREA, eventually using breeding research stations in the Southern hemisphere. Transgenic/genome-edited lines will be multiplied by UP in cooperation with Usovsko, which operates the facility for field testing of transgenic barley.

Seed sowing

Depending on seed availability, alpha-design or augmented designs for plant growing will be applied. The barley lines can be sown:

- With a mechanical seeder in field plots (min 3 m²) with sowing density according to the local practices (e.g. 350 seeds/m² for autumn sowing in Italy), or higher (e.g. 500 seeds/m² in Italy). Depending on the genetic materials being tested, sowing density can be increased by reducing the space between the rows. In Usovsko, the seed density should be specified in the application for approval of new GM lines. For a limited number of lines, field plots can be sown manually.
- Manually in single/double rows (50 seeds/m). Boards for sowing plots at different densities can be constructed by Lund University and will be used for sowing of some mutant lines (e.g. erect mutants, such as *ert-k.32*, *ert-a.6*, *brh1.a*, *uzu1.a*, and their mother cultivars). The boards have rows of holes placed 5 cm apart, with various distances between the rows (15, 10, 7.5, and 5 cm).

Field management

In general, standard local management practices will be adopted (herbicide, pesticide, fertilization) to maximize crop health and final biomass/grain yield.

Depending on the genetic materials being tested, different levels of nitrogen fertilizers will be applied (e.g. standard and low N input). In this case, the content of nitrogen in the soil will be measured before the experimentation.

Monitoring meteorological conditions and ozone levels in fields

Air temperature, humidity, rainfalls, solar radiation, and wind speed will be recorded using meteo stations located in proximity to the fields. Data on ozone concentrations in the air will be collected from air quality monitoring services and will be used to estimate ozone uptake by barley lines. Detected ground ozone concentrations are available from the European Environment Agency (<https://www.eea.europa.eu/en>), which aggregates results of air quality monitoring from European countries. Locations of the stations that measure ozone are also available (<https://airindex.eea.europa.eu/Map/AQI/Viewer/>). Alternatively, archived data on ozone monitoring can be downloaded from the World Air Quality project (<https://aqicn.org/data-platform/register/>).

The air monitoring stations that are located in proximity to the sites of field trials:

- CREA: BESENZONE/ IT1924A (12.18 km distance)
- USO: Cervena hora/CZ0TCER (43.24 km)
- KWS: Allertal/DENI052 (25.21 km)
- Nordic Seed/Denmark: Århus/6153/DK0051A (23.68 km)
- Nordic Seed/Germany: Weserbergland/DENI041 (14.83 km)

Physiological and morphological traits to be measured in plants

The field trials will be focused on evaluating grain yield and biomass production in the barley lines under study. A set of plant traits (Table 2) will be measured to characterize plant development, morphology, biomass, and yield. In addition to these traits, depending on the availability of adequate instrumentation, plant performance in fields will be studied at some sites by measuring physiological parameters of interest (Table 3). Detailed protocols

for the characterization of plants in greenhouse and field conditions can be found in the corresponding chapters below. Erect leaf lines targeted by the project can be scored visually (Figure 1). Collected data will be stored and treated following the Data Management Plan (Deliverable 7.1).

Before threshing, 2 x 50 cm from central rows in each plot will be hand harvested, by cutting from the surface of the soil. The area must be representative of the whole plot with adequate density. In labs, the whole sample weight (= total biomass), total number of tillers with and without spikes, weight, and number of seeds will be measured. Based on these measurements, the harvest index, number of grains per m², yield components (spikes per m² and grains per spike), and average grain weight can be calculated (see Table 2 for more details).



Figure 1. An example of a mutant with erect leaves (the right panel) compared with the parental line (the left panel).

Collecting straw samples for further analysis

Straw from the lines of interest will be collected (a few hundred grams) and dried in a controlled environment (50% HR, 23°C), and then used for characterization of its biochemical composition (in particular lignin and proteins but also cellulose, hemicelluloses, see the detailed protocols below) and physical properties (i.e., density, dimensional, thermal and mechanical

properties, see detailed protocols below in corresponding chapters). Collected material (several kg) will be also used for innovative technologies to transform barley straw into high-value products in WP5 (Deliverables D5.1 to D5.3).

Table 2. Phenotypic traits that will be measured in the field trials

Trait	Abbreviation	Unit	Description
Plant establishment	PE	%	Ratio of number of plants established to number of plants sown
Heading Date	HD	Date	Measured when half of head emerged from the flag leaf in 50% of the plot (Z55).
Maturity Date	MD	Date	Measured when the peduncle is yellow in 50% of the plot (Z55).
Plant height	PH	cm	Measured on main culm from the crown level to the base of the spike
Spike length	SpL	cm	Height up to the tip of the spike - PH
Grain Yield	GY	t/ha	From the combine, normalized at 13% humidity
Biomass at maturity	BM	g	Harvest 2 x 50 cm from central rows, dry if necessary and weight
Total Tiller Number	TTN	count	Total number of tillers in the 2 x 50 cm sample
Sterile Tiller Number	STN	count	Number of tillers without the spike in the 2 x 50 cm sample
Fertile Tiller Number	FTN	count	TTN-STN
Seed weight	SW	g	Weight of the seeds from the 2 x 50 cm sample
Thousand Grain Weight	TGW	g	Weight of 1000 seeds from the 2 x 50 cm sample
Seed per unit area	Sm2	count	Estimated from TGW in the 2 x 50 cm sample, then referred to the whole plot
Seed per spike	SS	count	Estimated for the 2 x 50 cm sample

Straw Weight	StWt	g	BM-SW
Harvest Index	HI	%	SW/BM

Table 3. Physiological characterization of barley lines in field trials and greenhouses

Del.	Description of lines	Measurements	Plant part	Devices for measurements
D1.1	Reduced content of leaf chlorophylls	Photosynthetic performance	Leaves	SPAD, Spectrophotometer; Handy-PEA, Dual-PAM; Joliot Type spectrometer; ceptometer
D1.1	Erect leaf phenotype	Scoring leaf erectness		Ceptometer
D1.2	Faster adaptation to changes in light intensities	Photosynthetic performance	Leaves	Dual-PAM; Joliot Type spectrometer
D1.3	Reduced photorespiration penalty	Photosynthetic performance	Leaves	
D1.4	Increased leaf O ₃ uptake	Stomatal conductance, CO ₂ and O ₃ uptake	Leaves	Portable gas exchange device
D2.1- D2.3	Altered straw quality	Straw composition. Physical properties of straw fractions.	Straw	
D3.1- 3.2	Advanced barley breeding lines with a potential	Combinations of traits listed	Grain, Straw	

	increase in total biomass, O ₃ assimilation and improved straw quality	above for the parental lines		
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Measuring photosynthetic efficiency (University of Milan and University of Padua)

Equipment

The photosynthetic performance will be measured with Handy-PEA+ (Hansatech-instruments) for preliminary screening, and with Joliot Type spectrometer, JTS-10 (LAMBDA SYSTEM, Poland) and DUAL-PAM-100 (Walz, Effeltrich, Germany) for more detailed analyses (Figure 2).



Figure 2. Devices for characterization of photosynthetic performance (A) Handy-PEA+ (Hansatech-instruments). 1 – Control unit and sensor; 2 – Leafclips. (B) DUAL-PAM-100 (Walz, Effeltrich, Germany).

1 – Power-and-Control-Unit DUAL-C; 2 – Near infrared emitter unit; 3 – Detector units; 4 – two measuring heads. (Not in the picture: PC equipped with software DualPAM V3.20 - Windows 10). (C) Joliot Type spectrometer, JTS-10 (LAMBDA SYSTEM, Poland). 1 – Control unit; 2 – JTS optical bench.

Working principle

Handy PEA+ consists of a compact, light-weight control unit encapsulating sophisticated electronics. This instrument measures chlorophyll fluorescence parameters and allows to determine Photosystem II photosynthetic performance.

The DUAL-PAM-100 system consists of a high-performance Pulse-amplitude modulated (PAM) chlorophyll fluorometer, suitable for the analysis of photosystem II (PSII), and a dual wavelength absorbance spectrometer, capable of analyzing photosystem I (PSI) performance.

Plant growth conditions

Barley plants will be cultivated on acid soil (Vigor plant growth medium, based on Irish and Baltic peats, pH 6.0), supplemented with Osmocote fertilizer, under greenhouse-controlled conditions on a long-day photoperiod (250 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for 16 h, and 8 h dark). Temperatures will be set to 20 °C during the day and 16 °C at night, with controlled relative humidity.

Experimental procedures

- Handy PEA+:

Handy PEA+ is used in field and greenhouses analysis for preliminary screening. Before turning on the Handy PEA+ device leaf material is dark adapted for 30 minutes into the leafclips.

In-vivo PSII photosynthetic parameters are recorded on second/third barley leaves.

Starting-up the Handy PEA+ system:

1. Turn on the Control Unit.

Running the analysis:

1. Leaves are subjected to saturating light pulse to determine maximum photosynthetic efficiency of PSII.
2. Leaf-clips are opened to allow environmental illumination.
3. Leaves are then subjected to saturating light pulse to determine light-adapted photosynthetic efficiency of PSII.

Shutting down the Handy PEA+ system:

1. Copy your files from the system.
2. Turn off the Control Unit.

Data analysis:

1. Copy the dataset into the PEA+ software, Windows® program supplied with Handy PEA+ for system configuration, data acquisition and post-measurement analysis.
2. Use the PEA+ software to obtain the in-vivo PSII photosynthetic parameters such as F_0 , F_m , F_v , F_v/F_m .
3. Export the results.

Five plants of each genotype for three replicates are analyzed and average values plus/minus standard deviations are calculated.

● DUAL-PAM-100:

Before starting the DUAL-PAM-100 device plants are adapted for 30 minutes (Barbato et al., 2020). In-vivo PSII and PSI photosynthetic parameters are recorded on second/third barley leaves.

Starting-up the DUAL-PAM-100 system:

1. Turn on the computer and the Power-and-Control-Unit.
2. Open DUAL-PAM-100 program (light curve kinetic).

In this program leaves are subjected to increasing actinic light intensities (from 50 to 1500 PPFD $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 90s each light intensity.

Running the analysis:

1. Place the second/third barley leaves in between the optical unit.
2. Run the program.

Shutting down the DUAL-PAM-100 system:

1. Stop the software and turn off the Power-and-Control-Unit.
2. Copy your files from the computer and shut it down.

Data analysis:

1. Copy the dataset into the Excel template containing the formulas for calculating in-vivo PSII and PSI photosynthetic parameters such as F_0 , F_m , F_v , F_v/F_m , $Y(II)$, $Y(NPQ)$, $Y(NO)$, $Y(NA)$, $Y(ND)$ and $Y(I)$.
2. Export the results.

Three plants of each genotype for three replicates are analyzed and average values plus/minus standard deviations were calculated.

- Joliot Type spectrometer, JTS-10:

Before starting the JTS-10 device plants are dark adapted for 30 minutes (Gerotto et al 2016) before measuring *in vivo* Cytb₆f, Flavodiiron protein, PSII and PSI activity.

Starting-up the JTS-10 system:

1. Turn on the computer and the Control Unit.
2. Unlock the Control Unit.
3. Open JTS-10 program.
4. Upload user's parameters.
5. Select the specific command string to measure Cytb₆f, PSII or PSI activity.

Running the analysis:

1. Place the sample in the holder.

2. Run the program.

Shutting down the JTS-10 system:

1. Export data
2. Stop the software and turn off the Power-and-Control-Unit.
3. Copy your files from the computer and shut it down.

Data analysis:

1. Copy the dataset into the Excel template containing the formulas for calculating in-vivo PSII and PSI charge separation and photosynthetic parameters such as F₀, F_m, F_v, F_v/F_m, ETRII+ETRI, cyclic electron transport, FLV activity.
2. Export the results.

Three plants of each genotype for three replicates are analyzed and average values plus/minus standard deviations are calculated.

Literature

Barbato, R., Tadini, L., Cannata, R., Peracchio, C., Jeran, N., Alboresi, A., Morosinotto, T., Bajwa, A.A., Paakkarinen, V., Suorsa, M., Aro, E-M., Pesaresi, P. (2020) Higher order photoprotection mutants reveal the importance of Δ pH-dependent photosynthesis-control in preventing light induced damage to both photosystem II and photosystem I. *Scientific Reports*, 10, 6770. <https://doi.org/10.1038/s41598-020-62717-1>

Gerotto, C., Alboresi, A., Meneghesso, A., Jokel, M., Suorsa, M., Aro, E.-M. and Morosinotto, T. (2016) Flavodiiron proteins act as safety valve for electrons in *Physcomitrella patens*. *Proceedings of the National Academy of Sciences*, 113, 12322–12327. <https://doi.org/10.1073/pnas.1606685113>

Leaf chlorophyll concentrations (University of Milan and University of Padua)

Equipment

The leaf chlorophyll concentration is measured through SPAD-502Plus chlorophyll meter (Konica-Minolta, Tokyo, Japan) for preliminary screening, and with chlorophyll extraction and spectrophotometry analysis (Ultrospec™ 2100 pro, GE Life Sciences, Buckinghamshire, UK; Porra et al. 1989). The devices for measuring chlorophyll are shown in Figure 3.



Figure 3. Devices for measuring leaf chlorophyll concentrations (A) SPAD-502Plus chlorophyll meter (Konica-Minolta, Tokyo, Japan). 1 – Power-and-Control-Unit 2 – Clip on sensor. (B) Spectrophotometer (Ultrospec™ 2100 pro, GE Life Sciences, Buckinghamshire, UK)

Working principle

SPAD-502Plus chlorophyll meter is a portable, non-invasive measuring device for quantifying the chlorophyll content of leaves by measuring the absorbance of the leaf in two wavelength regions.

The chlorophyll extraction with acetone is followed by chlorophyll quantification with a spectrophotometer. The spectrophotometer light

source irradiates samples with proper wavelengths and allows the quantification of leaf chlorophyll content, directly proportional to the absorbance values recorded.

Experimental procedures

- SPAD-502Plus:

SPAD-502Plus is used in field and greenhouses analysis for preliminary screening.

Chlorophyll has absorbance peaks in the blue (400-500 nm) and red (600-700 nm) regions, with no absorbance in the near-infrared region. The SPAD-502Plus measures the absorbances of the leaf in the red and near-infrared regions. Using these two absorbances, the meter calculates a numerical SPAD value which is proportional to the amount of chlorophyll present in the leaf.

Chlorophyll concentration was recorded on second barley leaves.

Starting-up the SPAD-502Plus system:

1. Turn on the SPAD-502Plus system.

Running the analysis:

1. Inserting a leaf and closing the measuring head
2. Run the analysis

Shutting down the Handy PEA+ system:

1. Turn off the SPAD-502Plus system.

Data analysis:

1. SPAD-502Plus system can be used to measure chlorophyll content by measuring the absorbance of the leaf in two wavelength regions. Five plants of each genotype for three replicates are analyzed and average values plus/minus standard deviations were calculated.

Chlorophyll content through acetone 90% (v/v) extraction and spectrophotometer analysis:

Chlorophyll concentration is recorded on second barley leaves freshly collected.

Starting-up:

1. Prepare cold 90% acetone.
2. Prepare and clean quartz cuvettes
3. Place the plants in a dark room.

Running the analysis:

1. Take the second barley leaf
2. Quickly weigh the leaf on a precision scale, transfer into a clean Eppendorf tube and immediately freeze it in liquid nitrogen.
3. Grind leaf material in liquid nitrogen either in a mortar or by TissueLyser.
4. Resuspend the ground material in 1 ml of cold 90% acetone v/v.
5. Transfer in 1.5 ml Eppendorf tube
6. Centrifuge at 4°C at 13000 rcf for 10 minutes
7. Transfer the supernatant in a clean Eppendorf tube
8. To determine chlorophyll concentration, dilute 10 µl of the solution into 990 µl of 100% Acetone (dilution factor can change accordingly to the concentration of the extracted chlorophylls) and pour it into a 1 ml quartz cuvette.
9. Measure the absorbance with a spectrophotometer at 663 nm (Chlorophyll *a* absorption) and 646 nm (Chlorophyll *b* absorption). Moreover, 750 nm is used for determining the zero baseline.

Data analysis:

Chl *a* and Chl *b* concentrations were measured by spectrophotometry according to Porra et al. (1989) and normalized relative to fresh leaf weight.

The chlorophyll content (expressed in $\mu\text{g/ml}$) was determined using the following equations:

$$\text{Chl } a = (12.25 \cdot A^{663} - 2.55 \cdot A^{646}) / (m \cdot V) \cdot \text{DF}$$

$$\text{Chl } b = (20.31 \cdot A^{646} - 4.91 \cdot A^{663}) / (m \cdot V) \cdot \text{DF}$$

$$\text{Chl } a + \text{Chl } b = (17.76 \cdot A^{646} - 7.34 \cdot A^{663}) / (m \cdot V) \cdot \text{DF}$$

Where:

- A is the absorbance at 663 or at 646 nm
- m is the mass of the fresh material (mg)
- V is the volume of acetone (mL)
- DL is the Dilution Factor (volume of extract/tot vol)

Five plants of each genotype for three replicates are analyzed and average values plus/minus standard deviations were calculated.

Literature

Porra, R.J., Thompson, W.A., Kriedemann, P.E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta*, 975, 384-394. [https://doi.org/10.1016/S0005-2728\(89\)80347-0](https://doi.org/10.1016/S0005-2728(89)80347-0)

Measuring stomatal conductance, net CO₂ uptake, ozone uptake, and assessment of ozone tolerance in barley plants at the laboratory conditions (University of Tartu)

Equipment

The custom-made gas exchange device for large plants (shown in Figure 4; Hõrak *et al.*, 2017), tanks with pressurized nitrogen and oxygen. A device for measuring water conductivity.

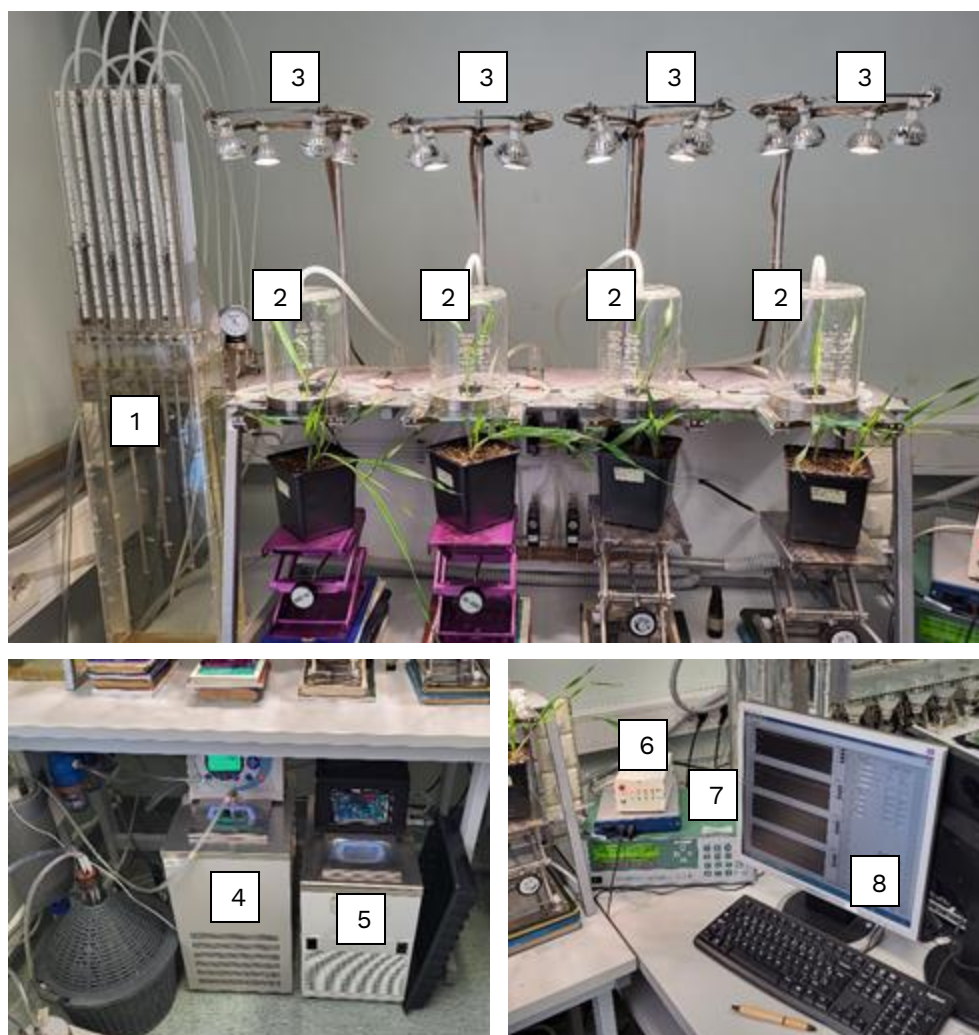


Figure 4. The gas exchange system for barley plants. 1 – The manostat water column controlling CO₂ and ozone levels in the chambers; 2 – the thermostated chambers; 3 – the adjustable light source, 4 – the thermostat with a spiral for controlling air humidity; 5 – the thermostat circulating water in the water-jackets of the chambers and regulating temperature in the chambers; 6 – the device

connecting the system with the computer; 7 - Li-COR LI 7000 CO₂/H₂O Analyzer; 8 – the computer with software controlling the system and collecting data.

Working principle

The device consequently measures the airflows passing and bypassing the chambers containing plants. Differences in the concentrations of CO₂, water, and ozone in these airflows are detected and used to calculate stomatal conductance, CO₂ and ozone uptake, stomatal ozone conductance, and ozone accumulated doze, based on the plant area.

Plant growth conditions

Seedlings are germinated on wet paper towels and are transferred to large pots filled with the peat: vermiculite mixture (2:1). Plants are grown in growth cabinets at 12 h/12 h photoperiod, 23°C / 18°C day/night temperature, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, and 70% relative humidity. At the age of approximately 2 weeks, plants can be used for experiments.

Experimental procedures

Before starting the gas exchange device:

*Check how much water is in the thermostat spiral controlling air humidity in the device – by using a large syringe, remove all water from the spiral and add about 5-7 mL of distilled water.

*Adjust the water levels in the manostat water column, water pipe on the wall and both thermostats.

Starting-up the gas exchange system:

1. Turn on the computer, the controller device, Li-COR LI 7000 CO₂/H₂O Analyzer, the lamps (white round knobs on the right side), and the chamber ventilators (metallic switches on the right side).
2. Open the nitrogen tank to 1 bar to create the reference flow in the Li-COR gas analyzer (cell A).

3. Start the air humidity thermostat containing the spiral. Adjust its temperature to obtain the desired air humidity level in the system (usually 65-70%).
4. Turn on the main system pressure pump. Adjust pressure/air flow in the system if needed.
5. Turn on the water-jacket thermostat. Set the desired air temperature for the experiment.
6. Check light intensity in the chambers and adjust if needed (usually $300 \mu\text{mol m}^{-2} \text{s}^{-1}$).
7. Open the oxygen tank and start the ozone generator. Calibrate the manostat water column to obtain desired ozone levels before starting the experiment.
8. Start the software on the computer.
9. Insert plants into the chambers. Depending on plant size, a whole plant or two top leaves can be used. The chambers with plants should be sealed airtight with putty.
10. Start the small pump creating the airflow from the system to the Li-COR gas analyzer (cell B). Immediately start measuring in the software.

Treatments:

1. Plants are stabilized at the standard conditions ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 65-70% relative air humidity, 23-24° C, ambient CO₂ level) for 1 h or longer.
2. Long-term exposure to relatively low ozone doses (50, 100 and 200 ppb) will be applied to plants during the light period (up to 12 h). Additionally, stomatal closure in response to 3-min 500 ppb ozone pulses will be studied. Water, CO₂, and ozone levels in the reference and passing through chamber airflows as well as temperature in the chambers will be recorded before and during the treatments.
3. In addition, stomatal responses to other stimuli, such as spraying with abscisic acid, elevated CO₂, low air humidity, and various light levels, can be studied upon a need.

Shutting down the gas exchange system:

1. Stop the software and turn off the small pump.
2. Shut down the thermostats, the pressure pump, the lamps, the vents, the ozone generator, the controller box, the Li-COR gas analyzer. Close the nitrogen and oxygen tanks.
3. Copy your files from the computer and shut it down.
4. Measure the accurate plant leaf area by taking a photograph with a scale and using ImageJ.

Data analysis:

1. Copy the dataset into the Excel template containing the formulas for calculating time-resolved stomatal conductance, net CO₂ exchange, ozone stomatal uptake, ozone cumulative dose (Kollist *et al.*, 2007).
2. Export the results.

Characterization of ozone-treated plants

Plants after ozone treatments can be characterized by various methods. Ozone-induced lesions can be assessed visually in 2-3 days after treatments. Additionally, ion leakage can be measured in the ozone-treated and untreated plants:

1. Place a fragment of a barley leaf into a 50-ml falcon tube filled with mQ water. Incubate the leaf in water during 3 h.
2. Measure water conductivity with a conductivity meter.
3. Freeze and unfreeze the tube with the leaf sample to destroy the plant cell and obtain a maximal ion concentration.
4. Measure water conductivity again.
5. Ion leakage is expressed as a relative value for water conductivity measured in 3 h and after tissue destruction.

Literature

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Kollist, T., Moldau, H., Rasulov, B., Oja, V., Rämme, H., Hüve, K., Jaspers, P., Kangasjärvi, J. and Kollist, H. (2007) A novel device detects a rapid ozone-induced transient stomatal closure in intact *Arabidopsis* and its absence in *abi2* mutant. *Physiologia Plantarum*, 129, 796–803. <https://doi.org/10.1111/j.1399-3054.2006.00851.x>

Measurements of stomatal conductance and CO₂ and O₃ uptake in barley grown in field conditions (University of Tartu)

Equipment

The custom-made gas exchange device KaRaL (shown in Figure 5). The device consists of a measuring head, a backpack housing the device, and a mobile phone with an operating app (Karal 2.0). The head has a light intensity sensor, temperature sensors for both the leaf and ambient environment and a flow meter. The backpack contains a modified LI650 Infrared gas analyzer, a bag for holding reference air, a power bank (20V and 12V outputs), a pump, a pump control module, a pump switch, and a system control module.

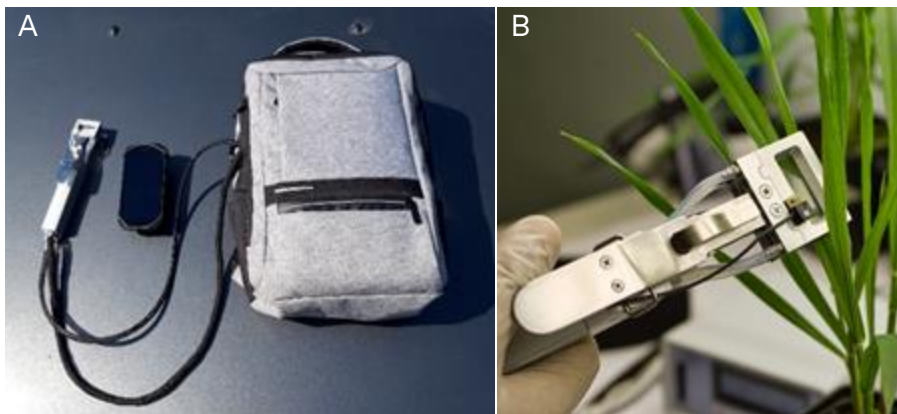


Figure 5. The gas exchange device KaRaL. (A) The overall look of the device, (B) The measuring head clamped to a plant.

Working principle

The air surrounding the soon-to-be-measured leaves is pumped into a special “reference” bag that is impermeable to both water vapor and carbon dioxide. The concentrations of H₂O and CO₂ in the bag are measured, and the values are used as a reference. Once a leaf is clamped to the measuring head, the air in the bag is pumped through the cuvette, and the concentrations of

H₂O and CO₂ are measured again using the LI650 Infrared gas analyzer. The flow rate and total volume of the gas tubes and the cuvette are adjusted to ensure that a reliable sample value can be obtained in just 8 seconds. Using the initial reference measurement and subsequent samples, the program then calculates the values for transpiration (E), stomatal conductance (gs), and net photosynthesis (Anet). The volume of one "reference" bag is enough for the cycle measurements of 20 leaves. The collected data include values for E, gs, and Anet, as well as measurements for light intensity (photosynthetically active radiation; PAR), leaf temperature, and ambient temperature for the entire cycle. Stomatal O₃ uptake can be calculated based on measured stomatal conductance and ground O₃ concentrations (Laisk et al., 1989).

Experimental procedures

Starting the system:

1. Power on the device by pressing the powerbank button once. The powerbank indicators should remain illuminated. Otherwise, ensure that both wire plugs are correctly connected to the powerbank output - 12V to the 12V socket and 20V to the 20V socket.
2. Switch on the phone.
3. Allow the system to warm up for 15 minutes before conducting measurements. The LI650 gas analyzer requires time to reach the appropriate measurement temperature.
4. Prior to the first measurement of the day at the plant growth location, pump the reference air bag full of air once and then completely exhale the air again (see as described below for controlling the "reference" bag). This is essential to ensure that there is no residual air (with potentially different parameters) from previous experiments.

Gas exchange measurements:

1. Launch the Karal 2.0 app from the home screen of the phone. Ensure proximity of the phone and the system at all times. The phone will

establish a Bluetooth connection with the system control module when the "Connected" button becomes greyed out, and the "Get reference" and "Next spot" buttons become available for pressing. To modify the settings, refer to the "Settings" section below.

2. Enter the file name by either typing it manually or by accessing a pre-built list. To access the pre-built list, press the parameter indicators buttons such as "crops," "P1," and "P2," and select the desired options. When selecting from the pre-built lists, all the chosen parameters will be combined into a sequence and used as the file name. This feature allows for modifying only one parameter in the sequence without needing to alter the others. It is particularly useful for streamlining workflow when the repetition and treatment remain the same, but the line or variety changes.
3. Extend the rod for reference air intake from beneath the measuring head and release all the air previously present in the airbag by pushing the switch towards "E". The pump will automatically stop once the airbag is empty. To inflate the airbag with reference air, direct the rod away from yourself, positioning it at the desired level in the plot that you want to measure. Inflate the airbag by pushing the black switch away from "E." The pump will automatically stop when the bag is full. Retract the rod.
4. Close the measurement chamber by unclamping the lock.
5. Click the "Get reference" button - there should be a click/vibration, and the phone display should show "Airinging 40s" with a progress bar (If not, please refer to the Errors section in this protocol). This will be followed by another cycle and progress bar for measuring reference values. When it is completed, you can see the reference parameters (H₂O ppt, CO₂ ppm, temperature, PAR, and air flow) on the app screen. After the last cycle, the system emits a beep sound and displays "Get expose". Now it is ready to start measuring leaves.
6. To measure a leaf, open the chamber and place a leaf inside. Ensure that the glass area is fully covered by the leaf, and then close the

chamber. Any folds or crumples in the leaves can cause leaks through the soft membrane seals, and the system will notify this with "double beeps". It is important keep the leaf in the same approximate orientation and location during the measurement as it was naturally. When the leaf has been measured and the data has been collected, the system emits a single beep.

7. Repeat the measurements until the system emits two beeps and displays "Spot completed."
8. To begin the next spot, press the "Next spot" button and then start from step 2 in the current section.

Shutting down the system:

1. Deflate the "reference" bag completely.
2. Hook the clamp to hold the chamber in open position (important!).
3. Hold the power bank button down for ~3 seconds until the lights go out.
4. Recharge the power bank. You can leave it overnight or longer for a full charge.
5. Recharge the phone

Settings

Settings can be accessed when both the system and app are running. Once you open the app, there is a "Settings" button in the bottom right corner. By pressing this button, a new screen will open. Here, you can change the number of exposures per plot (recommended is 20), leaf area, and cuticular conductance. The latter two should be left unchanged.

Once you have entered new desired values, press "OK" and then go back. The app will refresh the system shortly. However, please note that for the app to communicate with the system, both need to be running and in close proximity.

Retrieving data

Files with the measurement results are located on the phone. On the home screen, there is a File Catalog app located next to the Karal app. The file directory is Main Storage/Internal Storage -> Android -> Data -> appinventor...Karal2.0 -> Files.

For each date, the app creates a separate folder. Within each folder, there are two files: one with the .TXT extension and one with the .LOG extension. The LOG files contain raw data, while the TXT files contain the measured and calculated values for each measurement/leaf in rows.

The file names are generated based on the name you provide in the app, the date (only a day of the month), and the time of measurements.

Notes for correct gas exchange measurements

- Organization of gas exchange measurements

Recording gas exchange characteristics should not be started too early when stomatal conductance is very high and photosynthesis is not very active. Late hours when dew is formed should be avoided too. In Estonia, the most reliable measurements can be obtained starting from 11 am until 3 pm. Under other climatic conditions, a preliminary experiment with repeated gas exchange measurements throughout 2 days might be necessary.

The performance of Karal is sufficient to analyze 28-32 plots per day, within a 4-5 hour period each day. The same plots ideally should be analyzed twice during the same day.

- Leaf position

When measuring leaves, it is absolutely important to clamp a leaf in same orientation and position as it was originally. This occasionally requires some foresight to position the measuring head in accordance with the leaf angle.

- Sun/cloud

Photosynthesis (CO₂ uptake) primarily depends on the intensity of sunlight in healthy leaves. To obtain comparable values for different plots, measurements must be conducted under the same conditions or using the same principle. Specifically, when a cloud affects the sun's exposure, the CO₂

uptake of sun-exposed leaves may vary due to differences in momentary light intensity. In such cases, it is advisable to measure leaves in all possible orientations throughout the plot, including shaded areas and those exposed to sunlight. However, on clear days or when cloud cover is uniform, it is possible to measure only the leaves exposed to direct sunlight and still obtain comparable data.

- Rain, dew, humidity, and temperature

It is not possible to obtain accurate values for transpiration or stomatal conductance when the leaves are wet or the humidity is excessively high (above 85% relative air humidity). Under such conditions, the stomatal conductance values may appear higher than expected or even abnormally high. If the leaves appear less healthy, but exhibit stomatal conductance values of 1000 units and above, it is likely that they were wet or had condensation on them. Moreover, in situations where the ambient temperature is significantly hotter than the leaf temperature (5-6 degrees), the stomatal conductance values tend to reach thousands, possibly indicating the presence of microscopic condensation on the leaves. Although stomatal conductance may pose challenges, the measurement of CO₂ uptake can still be accurately performed.

Handling error messages

The app controlling Karal is still under development. Although it is functional and stable, some minor bugs and unclear error messages can be observed. To fix errors created by users (such as entering a wrong file name even after starting the reference) and small software bugs, there is a button located in the bottom left corner marked as "R". To initiate a system reset, press and hold this button for 2 seconds. If the error persists, you can consider the following troubleshooting steps to determine the cause and potentially resolve the issue. Alternatively, you can contact University of Tartu for further assistance in addressing and resolving the issue collaboratively.

- The app shows “Data mode” and does not start airing the device

Close the app completely (press the button that displays all the apps in the background and swipe the Karal 2.0 app window to stop it running in the background). Then, go to the phone settings → application settings → Karal 2.0 settings → Force stop. Find the gray controller box and press left and right arrows simultaneously to reset the controller. Then start the Karal app again from the home screen and try again.

- Error message: "Pipe broken".

It happens when the app has lost connection with the system control module: you move too far away from the system with the phone or when the system unexpectedly shuts down. To resolve this issue, please ensure that the system is turned on and try force closing and restarting the app.

- The air flow is too low, and the app keeps beeping.

This error can have multiple causes and you can consider the following:

- First, check whether the reference air bag has air. If there is no air in the bag, the air flow will be greatly reduced or stopped.
- Next, inspect the cuvette seals. If they are visibly worn out and the app stops beeping when you apply some force to tighten the cuvette, the seals should be replaced. An extra set of seals should be available.
- Additionally, a tube may have become loose either from inside the backpack or from the handheld part.

Literature

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Physical, mechanical and thermal/fire properties of barley straw (IMT Mines Alès)

Measuring the moisture content of raw and fractionated barley straw

The moisture content of raw barley straws (from WP4) and barley straw fractions (from WP5) will be measured on 1 gram of dried materials using an infrared moisture balance (MA160-1, Sartorius; Figure 6) after conditioning barley samples at 50% RH and 23°C. A centrifugation method adapted from standard ISO 23714 will be used to evaluate the Water Retention Value (WRV) of barley straw after immersion in water. A few grams of straw will be immersed in deionized water for 5 min at 23 °C, then placed in tubes to be centrifuged at 3000 g for a period of 30 min, and finally weighed to determine the saturated mass M_s (in g) of barley straw. *WRV* values will be determined after drying the saturated sample in the moisture analyzer and weighing of the dried mass of barley straw M_d (in g).



Figure 6. Moisture Analyzer MA160-1 (Sartorius)

Measuring the density of raw and fractionated barley straw

The density ρ of raw barley straws (from WP4) and barley straw fractions (from WP5) will be measured on a few grams of dried materials using a gas

pycnometer (AccuPyc 1330, Micromeritics; Figure 7) in a helium atmosphere. The helium pycnometer enables precise determination of the volume of a solid sample (solid, divided or porous). Knowing its mass, it is then possible to access its density. This method is particularly well suited to measure the volumes and densities of powders (as barley fractions) or porous solids, thanks to the gas's ability to penetrate cavities. Helium was chosen because its small atomic diameter enables it to reach the smallest cavities. Regardless of the gas used, it can only access open pores (i.e. those that open outwards). This means that, in the presence of closed pores and interstices, the volume measured does not correspond to the bulk material volume, but to a so-called skeletal volume.



Figure 7. Gas pycnometer AccuPyc 1330 (Micromeritics)

Measuring the hygro- and hydro-expansion of raw and fractionated barley straw

The granulometry and swelling-in-water of barley straw fractions (from WP5) will be analysed with a laser granulometer (LS 13 320, Beckman Coulter; Figure 8) in air and water flow. This will allow to determine the hydroexpansion coefficient of the different barley straw fractions based on their volumetric swelling and Water Retention Value (WRV).



Figure 8. Laser granulometer LS 13 320 (Beckman Coulter)

Optional: If fibre bundles can be extracted from raw barley straws (from WP4), a fibre dimensional analysis system (FDAS770, Diastron Ltd, Hampshire, UK; Figure 9) will be used to measure the cross-sectional dimensions of fibre bundles and thus estimate their cross-sectional area (CSA). This experimental method based on automated laser scanning of the fibre bundle, as well as the sample preparation protocol, is described in detail in (Garat, 2018). During the measurement, the fibre bundle sample is put under slight tension to maintain it straight, and then translated and rotated, so as to collect hundreds of values of apparent diameter per revolution for each cross-section. In the end, several thousands of apparent diameters are collected for each fibre bundle allowing to take into account the irregularity of the fibre bundles along their length and the non-circularity of their cross-section. In order to analyse the influence of relative humidity on the cross-sectional dimensions of the fibre bundles (Garat, 2020), the dimensional analysis apparatus (FDAS770) is placed in a climatic chamber supplied by ETS (Electro-Tech Systems Inc, Pennsylvania, USA) to control the temperature at 23 °C and relative humidity between 20 and 80%. An immersion cell (DSM, Dynamic Swelling Module, Diastron Ltd., Hampshire, United Kingdom) can also be implemented on the FDAS device to measure the cross-sectional

dimensions of the fibre bundles immersed in deionized water at 23 °C. It should be noted that under immersion conditions, the device allows the dimensional analysis of the sample for only one cross-section at a time. For each type of barley straw and each hygro/hydrothermal condition, at least 20 fibre bundles should be characterized after being acclimatized 13 h at 23 °C and at the studied RH or at 50% RH before immersion.

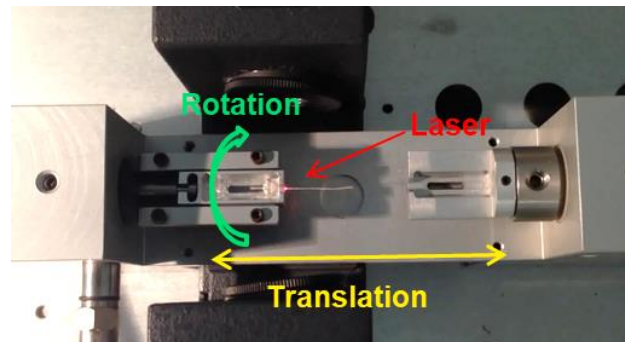


Figure 9. FDAS770 (Diastron Ltd, Hampshire, UK)

Measuring the mechanical properties of raw barley straw

Optional: If fibre bundles can be extracted from raw barley straws (from WP4), their mechanical properties, i.e tensile strength and Young's modulus, will be determined from micro-tensile tests, using a micro-tensile device (LEX820, Diastron Ltd, Hampshire, UK) equipped with a 20N capacity load cell and controlled by UvWin 3.60® software (Diastron Ltd, Hampshire, UK). Force to stress conversion will be based on the aforementioned CSA calculation. Tests will be carried out at a displacement speed of 1 mm/min and a gauge length ranging from 4 to 30 mm depending of the actual length of fibre bundles. The Young's modulus and the maximum strength and strain at break will be determined according to the NF T25-501-3 standard.

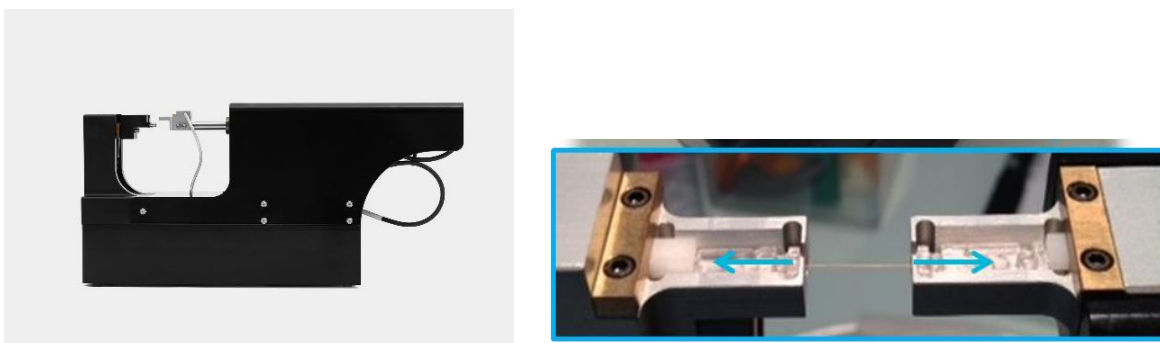


Figure 10. LEX820 (Diastron Ltd, Hampshire, UK)

Measuring the flammability of raw and fractionated barley straw at micro and bench-scale

The flammability of raw barley straws (from WP4) and barley straw fractions (from WP5) will be measured at microscale using a Pyrolysis-Combustion Flow Calorimeter PCFC (FTT, UK) and at bench-scale using cone calorimeter (FTT, UK). Both apparatus follow international standards, namely ASTM D7309 and ISO 5660, respectively, and are shown in Figures 11 and 12.



Figure 11. PCFC (FTT, UK)



Figure 12. Cone calorimeter (FTT, UK)

a. Working principle of Pyrolysis-Combustion Flow Calorimeter

A sample (around 3 mg) is heated at a constant heating rate (typically 1 K/s) under anaerobic (N_2) or aerobic (N_2/O_2) atmosphere up to 750 °C. Gases released from the pyrolysis of the sample are sent to a combustor and burnt at a constant temperature in the presence of oxygen in excess. Typically, the combustor temperature (900 °C) and the residence time of gases in the combustor (10s) are enough to ensure complete combustion. The heat release rate is measured using the well-known Huggett's relation, i.e. the oxygen depletion method: 1 kg of oxygen consumed during combustion corresponds to 13.1 MJ of energy released (Huggett, 1980). The heat release rate (HRR) is usually plotted versus the pyrolysis temperature. Total heat release (THR) corresponds to the area under the curve $HRR = f(\text{temperature})$. The initial and final mass of the sample are independently measured using a balance to calculate the residue content. The energy of complete combustion is calculated as the ratio between THR and the mass loss.

Combustion temperature can be reduced to obtain incomplete combustion according to a methodology developed in our lab (among others). Combustion gases can be analyzed using a special coupling with a Fourier transform infrared spectrometer.

Details about the apparatus can be found in (Lyon, 2004) and (Sonnier, 2023).

b. Working principle of cone calorimeter

A sample is usually horizontally exposed to a heat flux from an electric resistance (radiant cone). The heat flux can be monitored from 10 to 80 kW/m² typically. The special shape of the resistance ensures a homogeneous heat flux over the whole exposed surface. The ignition is ensured using a spark igniter. Gases are sent to a chimney (flow rate 24 L/s) and gases sampling allows calculating oxygen, CO and CO₂ contents. Oxygen consumption allows calculating the heat release rate according to the Huggett's relation as already stated. Heat release rate (HRR) is plotted versus time. Total heat release is measured as the area under the curve $HRR=f(t)$.

The sample is continuously weighted using a balance. The ratio between THR and the mass loss allows calculating the effective heat of combustion (to be compared to the heat of complete combustion measured in PCFC).

Smoke opacity can be assessed from the extinction coefficient measured using a laser diode. Burning gases can be analyzed using a special coupling with a Fourier transform infrared spectrometer.

Details about the apparatus can be found in (Schartel, 2007). Typically, a sample has a surface of 100 cm² and its thickness can reach several centimeters. Its mass can vary from a few grams to several hundreds of grams. For fibers in bulk, a special procedure has been developed in our lab to prepare the sample. The sample holder was filled by cautiously scattering fibers as to reach a given thickness (up to 70 mm) in order to reach a constant density through the whole thickness. The procedure as well as the modelling of the flammability of such thermally thin materials in cone calorimeter can be found in (Sonnier, 2023).

Literature

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Characterization of straw composition

Measuring the protein content of barley straw (CREA)

The protein content of barley straws from field trials (WP4) will be measured on a subsample of dried materials that is: 1- cut at an approximate length of 2 cm with a shredder (if available) or with pruning shears; 2- milled for 10 seconds with an IKA® M 20 universal mill (Figure 13).



Figure 13. Cut straw for the analysis of crude protein content

The analysis of crude protein content will be performed through Near-infrared spectroscopy (NIRS) using a FOSS NIRS™ DS2500 F (Figure 14). The instrument can quantify different compounds (Fat, Protein, Moisture, Ash, Starch, Crude Fiber, NDF, ADF, and more) on different types of sample (Grains/cereals, Forages, Vegetal by-products and more). Whenever necessary, chemical analysis of nitrogen content (e.g. Kjeldahl, Dumas) will be outsourced for confirmation of NIRS results.



Figure 14. FOSS NIR5™ DS2500 F

Measuring the lignin content of barley straw (UNIVDUN)

The lignin content of barley straw will be measured to confirm the selection of 10 high lignin and 10 low lignin cultivars. To be consistent with previous lignin content measurements, Lignin content will be measured in straw tillers with leaves removed. These will be shredded using a shredder scissors or a crosscut paper shredder and then milled in a Cyclone Mill Twister (Retsch Ltd. UK) or a TissueLyser (Quiagen) depending on straw volume.

We envisage using the Klason lignin determination method for reference lignin quantification since it is a well-established and accurate gravimetric method (Jung et al., 1999). The Klason method we have used previously is based broadly on that of Dence (1992). Extract-free samples are first prepared by exhaustive extraction with water, then ethanol. Samples are then hydrolysed in sulphuric acid for 2 hours with stirring to solubilize the cell wall carbohydrates and leave the insoluble lignin. After washing, filtering and drying, the residue can be weighed to estimate the lignin content.

We envisage using Klason lignin determinations of a variety of straw samples to calibrate NIRS (near-infrared spectroscopy) for a simpler, more rapid, high-throughput lignin determination method for barley straw that can be used by other partners. NIRS requires little sample preparation and is non-

destructive. Once a calibration has been performed to correlate the NIRS spectra of a variety of straw samples with their known Klason lignin data, quantitative predictions of the lignin content of unknown samples can be made from their NIRS spectrum alone (Jin and Chen H, 2007).

Literature

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