Protocol for monitoring visible ozone-induced foliar injury in tobacco plants (*Nicotiana tabacum* L. cv. Bel-W3) treated with wood distillate

Andrea Vannini, Michele Carbognani, Alessandro Petraglia
University of Parma (Italy)

Ignacio González Fernández
Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (Spain)

Yasutomo Hoshika, Barbara Baesso Moura
National Research Council, Institute of Research on Terrestrial Ecosystems (Italy)

Felicity Hayes
The UK Centre for Ecology & Hydrology (UK)

This protocol has been developed
as part of the ICP Vegetation Working Group
"*Biomonitoring ozone impacts by using wood distillate as an ozone-protectant*".

Contacts
Andrea Vannini, PhD, University of Parma, andrea.vannini@unipr.it
Felicity Hayes, PhD, UK Centre for Ecology & Hydrology, fhay@ceh.ac.uk

1. Aim of the current Protocol

Aim: To provide a practical protocol for monitoring ozone-induced foliar damage, senescence, and plant height in *Nicotiana tabacum* L. Bel-W3 cultivar treated with wood distillate. The aim is to:

i) Evaluate the efficacy of foliar treatments with wood distillates in protecting ozone-sensitive plants from its phytotoxic effects, starting from the concentrations suggested by the producer for use as a plant strengthener (corroborant);
ii) Identify, through appropriate screening, the most effective distillate concentrations in allowing the protective effect;
iii) Ensure that the wood distillates tested are produced in the same country, or preferably region, where the study is conducted. In the absence of such a distillate, products from neighbouring countries should be used. This approach allows assessment of whether wood distillates produced in the local context are effective in protecting crops from ozone damage;
iv) Use *Nicotiana tabacum* L. (cv. Bel-W3 and Bel-B) as a model species to evaluate the protective effects, given its high sensitivity to ozone, before applying the findings to agronomically important crops.

Expected duration of the biomonitoring study: It is anticipated that a single biomonitoring evaluation at a single site would take approximately three months, comprising one month of seed germination and establishment, followed by eight weeks of exposure to ambient air and wood distillate treatments. Treatments and plant actions should be carried out weekly and additional watering will be required daily. As flowering can alter the number of leaves, it is important to stop the trial before the first flower buds appear. In this case, the last valid assessment is that of no observed buds.

2. Definitions

Wood Distillate: Commonly referred to as pyroligneous acid, wood vinegar or liquid smoke, wood distillate is produced by the distillation of vapours produced during the **pyrolysis** of wood. This distillate typically contains acetic acid, methanol, acetone and various phenolic compounds. The composition of pyrolytic acid varies depending on the type of wood used and the specific conditions of the distillation process.

Pyrolysis: Pyrolysis is a thermochemical process in which organic material is decomposed at high temperatures in the absence of oxygen. This process breaks down the material into gaseous, liquid, and solid products, depending on the temperature and pressure conditions during decomposition.

Bel-W3 plant: cultivar of tobacco (*N. tabacum*) genetically selected for its high sensitivity to ozone (O_3). It is used as a bioindicator to monitor ozone levels in the environment, showing visible damage such as necrosis or leaf lesions when exposed to high concentrations of the gas.

Bel-B plant: tobacco cultivar with greater ozone tolerance. It is used alongside Bel-W3 to compare the effects of air pollution and to distinguish ozone-specific damage from that caused by other environmental factors.

3. Methodological Section

3.1 Materials needed

a) Seeds of Bel-W3 and Bel-B:

To ensure consistency and avoid variability in tobacco seed pools, it is recommended that seed of Bel-W3 and Bel-B cultivars be sourced from a common certified supplier: NiCoTa (www.NiCoTa.de).

b) At least 15 pots with a minimum size of 1.2 litres, ensuring all pots have the same volume);
c) Standard commercial potting soil;
d) 2 x Hand-held plant sprayers (one for wood distillate and one for water);
e) Wood distillate:

Common Italian wood distillates are sold by the litre. This is enough to treat approximately 5,000 plants at a typical application of 1:500, i.e. 0.2%. However, the wood distillate should first be tested/diluted according to the supplier's instructions.

3.1 *Germination of Bel-W3 and Bel-B seeds*

Germination environment: Both Bel-W3 and Bel-B tobacco seeds must be germinated in a dark, moist environment to promote successful sprouting. Germination can occur under controlled conditions or in a seedbed.

Note. Germination should be started considering the transplant period.

Optimum temperature range: Maintain the temperature between 24-28°C for optimal germination. Under these conditions, seeds will typically germinate within 7-14 days.

Germination methods:

- In soil: Seeds can be directly sown into soil. It is recommended to use standard seedling pots filled with potting soil.

- On filter paper: Alternatively, seeds may be germinated on moist filter paper. Ensure the paper is kept moist with deionised or distilled water. Germination on filter paper may speed up the process slightly. If using filter paper, place seeds in a resealable plastic container to maintain moisture.

Watering: If germinating on filter paper, regularly check moisture levels and add water as needed to keep the paper moist. For soil germination, ensure the soil remains consistently moist but not waterlogged.

Note. For germination on filter paper: When the seedlings have grown to a shoot length of approximately 1 cm, carefully transplant them from the plastic container into seedling pots filled with soil. Use tweezers to handle the seedlings gently. It is advisable to moisten the soil thoroughly before transplanting. After placing the seedlings in the pots, cover the roots with additional moist soil to ensure good support and moisture retention. At this time, keep the pots with the seedlings indoors or in a glasshouse.

3.2 *Transplanting seedlings into growing pots*

When the seedlings reach a height of 3 to 5 cm, they can be transplanted from germination pots to larger growing pots. Larger seedlings are preferable as they tend to have better root development, making transplanting easier without damaging the root system. The growing pots must have a minimum volume of 1.2 liters and drainage holes in the bottom. The number of transplanted seedlings should be at least 10 Bel-W3 (at least five replicates for plants treated with wood distillate and five for untreated plants) and 5 Bel-B. At this time, keep the pots with the seedlings indoors or in a glasshouse.

Note. The larger the pot, the more the plant will grow, requiring a greater volume of foliar treatment solution.

3.3 Plants exposure and cultivation

Expose plants when average daily temperature is around 20°C. Plants should be watered daily after sunset. Irrigation should be avoided on rainy days. Plants should be fully shaded with green shade cloth (50% shade) - shaded plants are more sensitive to ground-level ozone concentrations (EN 16789 2016).

Note: To help determine when to start exposure, it is advisable to check the average temperature over the last five years.

3.4 Start of wood distillate treatments

Exposure to ambient air and wood distillate treatments should begin when plants have developed four fully mature leaves. Ideally, the start of treatments should coincide with the rise in ground-level ozone concentrations to significant levels (at least > 40 ppb). This timing will ensure that the plants are sufficiently developed to respond to the ozone exposure and that the wood distillate application can effectively mitigate the potential damage caused by elevated ozone exposure.

3.5 Treatment groups

These three groups of plants must be included in the biomonitoring study:

- Five replicate (minimum) plants of Bel-W3 must be treated with wood distillate;
- Five replicate (minimum) plants of Bel-W3 must be treated with distilled water;
- Five replicate (minimum) plants of Bel-B must be treated with distilled water.

Wood distillate must be applied by foliar application using a sprayer. Ensure that the spray covers the leaves evenly, concentrating on both the upper and lower surfaces. This technique will ensure thorough coverage of the leaves, targeting both the upper and lower surfaces. This is a well-established agricultural practice; foliar spraying allows efficient local uptake of the wood distillate solution, maximizing its protective effect against ozone-induced damage.

3.6 Treatment percentage

The first trial with wood distillate must be tested according to the supplier's instructions. If two dilutions or application volumes are suggested, one (mandatory) or both percentages should be tested. The application percentages suggested by the supplier's instructions are essential for investigating the protective effect of the products at their routinely used application percentages. However, other application percentages may be tested in addition to the selected application percentage(s). The additional application percentages should be two times higher or lower (or a multiple in the case of multiple application percentages) than those suggested by the manufacturer.

Note. If multiple percentages of application are tested, increase the number of plants to be transplanted: Five replicates (minimum) of Bel-W3 per treatment.

Example: Italian wood distillates usually have a suggested application percentage of 0.2% and 0.5%. The operator can choose to test one (mandatory) or both application percentages. If the operator wishes, he can also choose to test a third application percentage, which can be higher or lower than the suggested ones. In the case proposed, a third percentage to be tested can be or 0.1% or 1%, the first two times lower than 0.2% and the second two times higher to 0.5%; testing the 0.4% can be considered un-useful because close to the application with 0.5%.

3.7 Treatment frequency

There should be a minimum of one week between each application of wood distillate. This ensures that plants have sufficient time to respond to the previous treatment while maintaining the effectiveness of the protection against ozone injury. As summer temperatures tend to rise quickly in the morning, it is recommended that treatments are carried out either early in the morning (before dawn) or in the evening (around sunset) to avoid the heat and maximize the effectiveness of the application.

Note. If rain is forecast, avoid treatments at least 48 hours before the event. If rain cannot be avoided, protect plants from direct rainfall for 48 hours.

3.8 Treatment volume

For each plant, the volume of treatment solution should be sufficient to ensure complete treatment of all leaves on each plant. As a rough guide, this is likely to be 100 mL per plant when the plants are fully established.

3.9 Mandatory measurements

Morphometric measurements: Plant height must be measured as the distance between the growing medium and the petiole of the topmost leaf, regardless of its length. The number of leaves (i.e., leaves under evaluation, LUE) should be counted from top to bottom: only leaves longer than 6 cm and with a degree of ozone-induced leaf injury (i.e. necrotic areas) <50% are counted (see Vannini and Petraglia 2024 for details).

Leaves Injury Index (LII): LII must be recorded according to Paoletti et al. (2009) using the following formula:

$$LII = (LA \times AA)/100$$

Where LA indicates the percentage of injured leaves per shoot, and AA represents the percentage of injured leaf surface per symptomatic leaf.

Note. LII is only applied to LUE leaves.

Injury to Specific Leaves (ISL): Measurements must be done for all plants on the same counted leaf. Leaves should be counted from top to bottom. The last leaf of the Bel-W3 plant, characterized by the plant with the lowest number of leaves, is selected (see **Figure 1** for a specific study case). The leaf to be measured must be selected each time monitoring is carried out. The degree of damage can be reported as a percentage (**Figure 2**). This measure is used to detect the occurrence of changes in leaf injury between treatments if the LII has not changed.

Note. It is recommended to take pictures of all selected leaves for ISL measurements. ISL is only applied to LUE leaves.

General note: For each plant, plant height, the number of viable leaves, the percentage injured leaves per shoot, the average percentage of injured leaf per symptomatic plant, and the Leaves Injury Index should be recorded weekly.

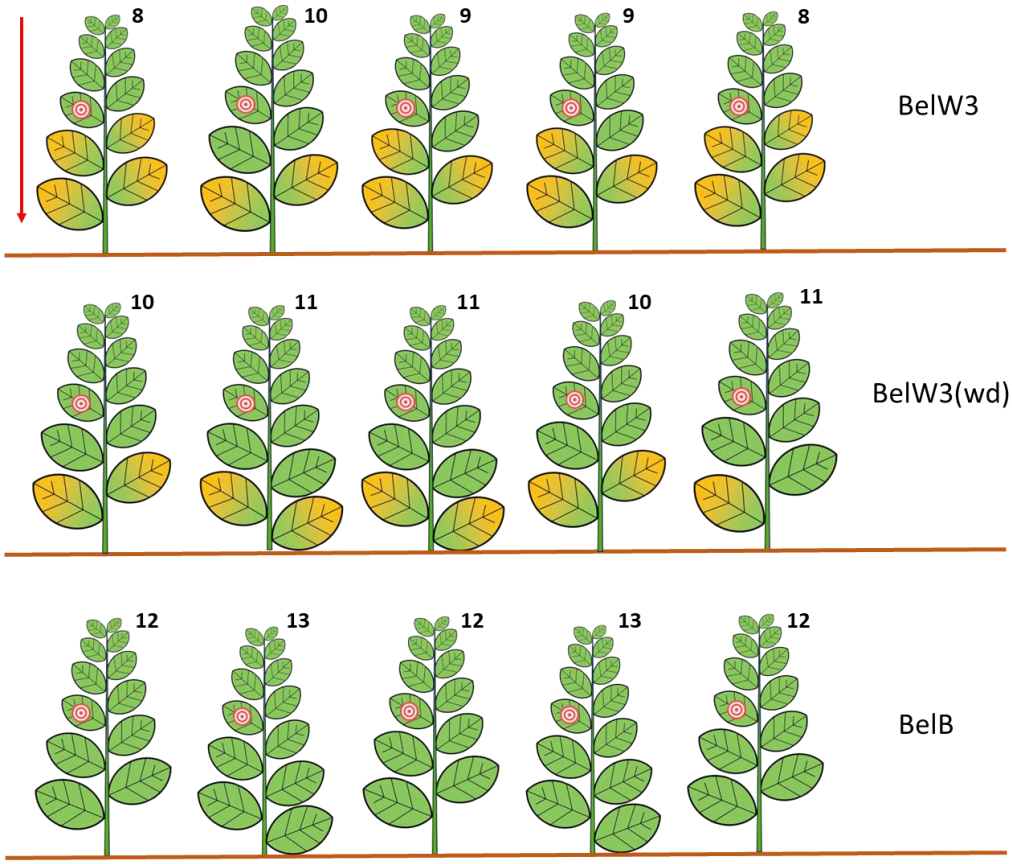

Figure 1. Experiment with five Bel-B and 10 Bel-W3 tobacco plants (five untreated (Bel-W3) and five treated plants (Bel-W3(wood distillate))). In this case, the Bel-W3 plant with the lowest number of leaves had only eight leaves damaged by less than 50%. Therefore, injury assessments and photosynthesis measurements for all plants were made on the eighth leaf of the plant, counted from top to bottom (see Vannini and Petraglia 2024 for details). The target indicates the leaf selected for this example.

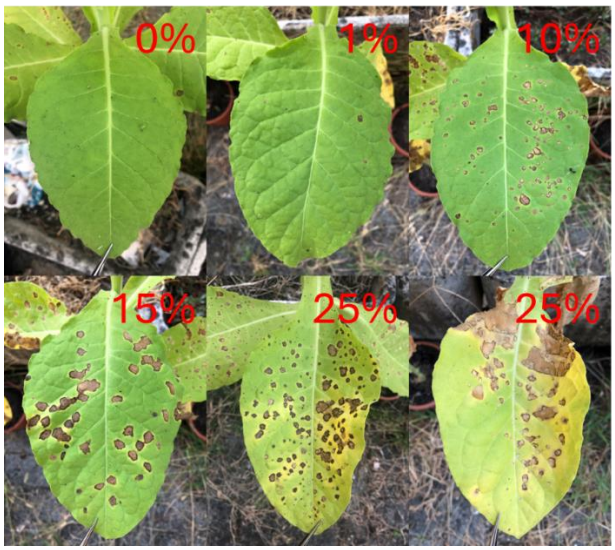

Figure 2. Ozone-induced damage to tobacco leaves, cv. Bel-W3. The percentage is estimated by considering the necrotic area in relation to the total leaf area. Chlorosis is not considered as it is not directly caused by ozone (EN 16789 2016).

Phytomass: Plant biomass (stem + leaves; grams) can be measured at the end of the biomonitoring study. Both fresh and dry biomass are required. Dry biomass must be measured by weighing the plants after drying at 70 °C for at least 24h.

Ozone concentrations: At least the maximum hourly ozone concentrations should be collected. Data may be collected from air quality biomonitoring stations located in the immediate vicinity (at the most 3 km) and at a similar altitude to the field/experiment.

Meteorological data: Data may be collected from air quality biomonitoring stations located in the vicinity (at the most 5 km) and at a similar altitude to the field/experiment.

3.10 Optional measurements

Photosynthetic measurements: Chlorophyll content (or index) and photosynthetic system functionality must be measured for all plants on the same identified leaf. Leaves should be counted from top to bottom, and the leaf used for measurements should be the last leaf of the Bel-W3 plant with the lowest number of leaves (see **Figure 1** for a specific study case). The specific leaf to be monitored must be selected each time measurements are taken.
Photosynthesis measurements can be taken weekly or just before treatment. At least five randomly selected measurements should be taken for each leaf, ensuring that they are taken from the outer part of the leaf, away from the node, and avoiding the leaf veins (**Figure 3**).

Figure 3. The red area indicates where photosynthesis measurements are required (see Vannini and Petraglia 2024 for details).

Any instrument capable of providing information on chlorophyll concentration or expression (e.g. atLeaf, CCM 300, etc.) and photosystem functionality (e.g. Handy PEA) can be used for these measurements.

Note. Instrument settings must be explained in the Excel file for data sharing.

Ozone concentrations: Average hourly ozone concentrations can be collected to calculate the Accumulated dose of ozone Over a Threshold of 40 ppb (AOT40). AOT40 is calculated as

the sum of the differences between hourly ozone concentrations exceeding 40 ppb and the threshold itself, during daylight hours and the exposure period. The result should be expressed in ppb·hours.

References

EN 16789 (2016). Ambient air - Biomonitoring with Higher Plants - Method of the standardized tobacco exposure.

Paoletti, E., Castagna, A., Ederli, L., Pasqualini, S., Ranieri, A., & Manning, W. J. (2014). Gene expression in snapbeans exposed to ozone and protected by ethylenediurea. *Environmental pollution*, *193*, 1-5.

Vannini, A., & Petraglia, A. (2024). Wood Distillate Mitigates Ozone-Induced Visible and Photosynthetic Plant Damage: Evidence from Ozone-Sensitive Tobacco (Nicotiana tabacum L.) Bel-W3. *Horticulturae*, *10*(5), 480.