

Microscopical validation of ozone symptoms in beech leaves sampled during the 4th International Training Course on the Assessment of Ozone Visible Injury.

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Introduction

The ozone-like injuries on several branches of beech from a MTS site (LOM 3) in Moggio, Italy, were discussed during the field exercise of the 4th International Training Course on the Assessment of Ozone Visible Injury, on the 27th of August 2003. One of the branch samples presented an unusual distribution of an otherwise - for ozone symptoms - typical stippling (Fig. 1A, C, D: arrows) which raised some doubts among the participants

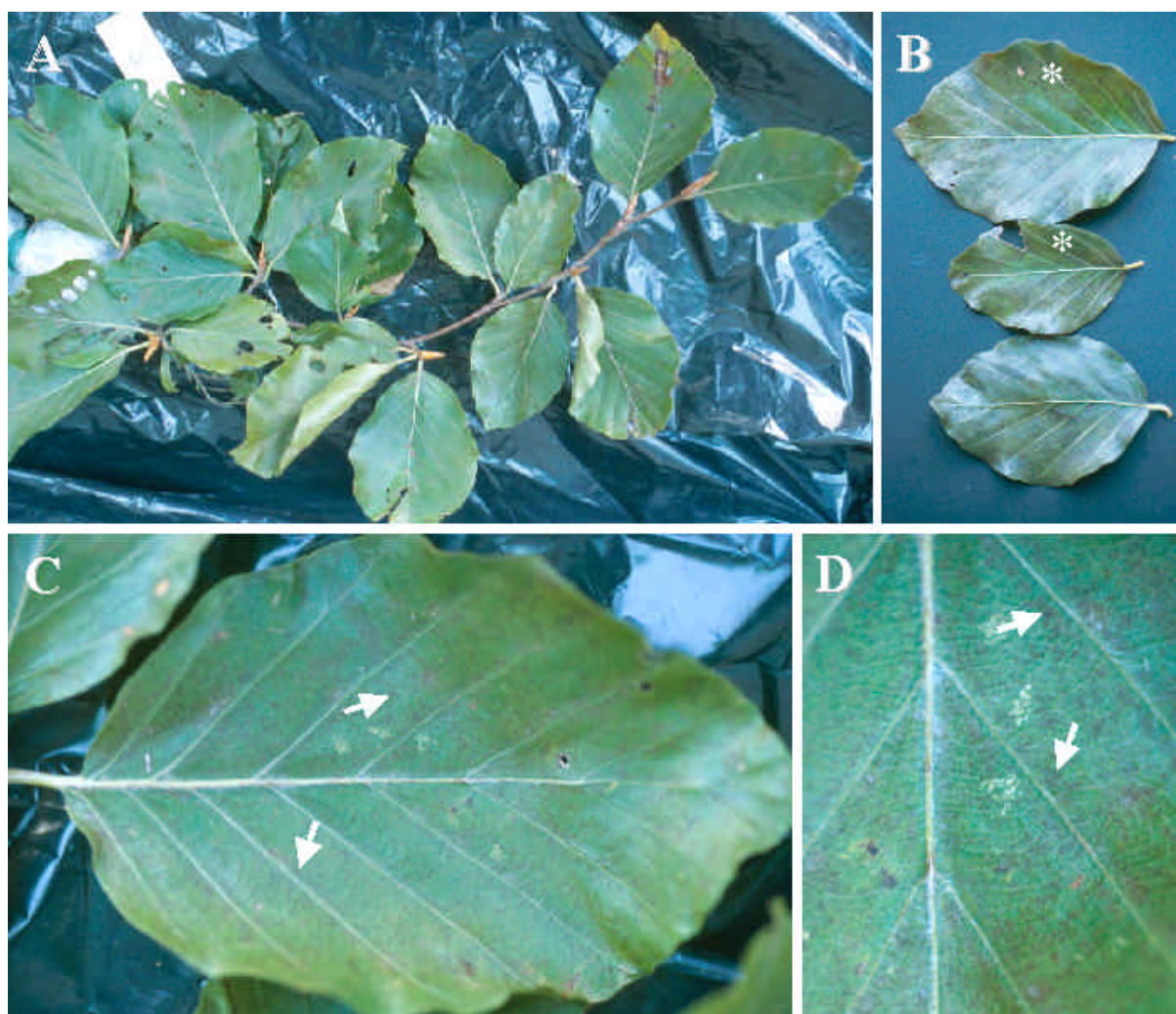


Fig. 1. Visible symptoms.

concerning the origin of the stress factor. The stippling was apparently not distributed randomly in the intercostal region as usual, but was more frequently closer to the secondary veins. The other diagnosis criteria (leaf discolouration, shade and leaf position effects) were all indicating ozone as the cause. It was decided to analyse microscopically leaf strips from those leaves with the following aims: 1) to clarify if the stippling was connected to eventual changes in the veins or not, 2) to analyse the microscopical symptoms and to identify the stress agents and 3) to compare the results between labs. Three labs conducted microscopical analyses, namely Drs S. Manninen (University of Helsinki, Finland), F. Bussotti (University of Florence, Italy) and P. Vollenweider (WSL, Switzerland).

Materials, Methods and Results

Material

A symptomatic branch was taken back from the field exercise and kept in a black plastic bag till proper storing and fixing could be provided. Leaf disks of 1 cm diameter were stored in methanol in the evening or fixed 24 hours after sampling in 2.5% glutaraldehyde buffered at pH 7.0 with 0.067 M Soerensen phosphate buffer. Elevated temperatures and insufficient conserving conditions caused a browning of most of the leaf area (Fig. 1B asterisks). A pre-study was conducted on brown and still green leaf patches (P. Vollenweider) before smaller leaf strips were prepared from the still green areas and further dispatched to the three participating labs. A few days after the training course, new samples were collected (F. Bussotti) but this time preserved immediately after sampling.

1. Methods (WSL)

30-60 µm thick transversal sections were obtained with a hand microtome from samples kept in methanol or from samples fixed in 2.5% glutaraldehyde buffered at pH 7.0 with 0.067 M Soerensen phosphate buffer. Leaf disc strips from fixed material were dehydrated with 2-methoxyethanol (3 changes), ethanol, *n*-propanol, *n*-butanol, embedded in Technovit 7100 (Kulzer HistoTechnik) and sections 2 µm thick were cut with a Reichert ultramicrotome. Hand and plastic-embedded sections were specifically stained for proanthocyanidins (Table 1) and observed immediately or mounted in DePex for embedded sections respectively. All sections were observed in a Leica microscope Leitz DM/RB and micrographs were taken either with the micrograph system Wild MPS 48/52 using Kodak Ektachrome 400 Asa films or with a Leica DC 500 digital camera.

Stain	Color in transmitted light	Excitation (nm)	Application target	Fig.
Vanilin acid ^a	red	-	oligomeric proanthocyanidins	2A, 2B
H ₂ SO ₄ 3M ^b	purple-brown	-	polymerised proanthocyanidins	-
Toluidine blue ^b	blue	-	metachromatic stain	2C, 3A, 3B
<i>p</i> -Phenylenediamine ^b	dark blue	-	lipids	2C, 3A, 3B
Calcofluor White Mr2 ^b	-	340-380	cellulose	3E
PARS ^b	pink	-	polysacharides	3C, 3D

^a fresh hand-microtome cuttings

^b semi-thin Technovit embedded cuttings

Table 1. WSL staining methods.

Results (WSL)

As shown in Fig. 2, the delayed storage and fixation induced artefactual changes in the leaf structure. The leaf browning was mainly caused by deposits of oxidised tannins (intensive red in Fig. 2B versus Fig. 2A). The cell and tissue structure in the still green parts was only partly intact. Stipples were well recognisable (arrow in Fig. 2A) and were not connected to any reactions in veinlets. However, the lower mesophyll was damaged (Fig. 2C) as shown by the plasmolysed (asterisks) or partly collapsed (arrow) cell structure, the darkened chloroplasts or

the condensed nuclei. No biotic infection was visible.

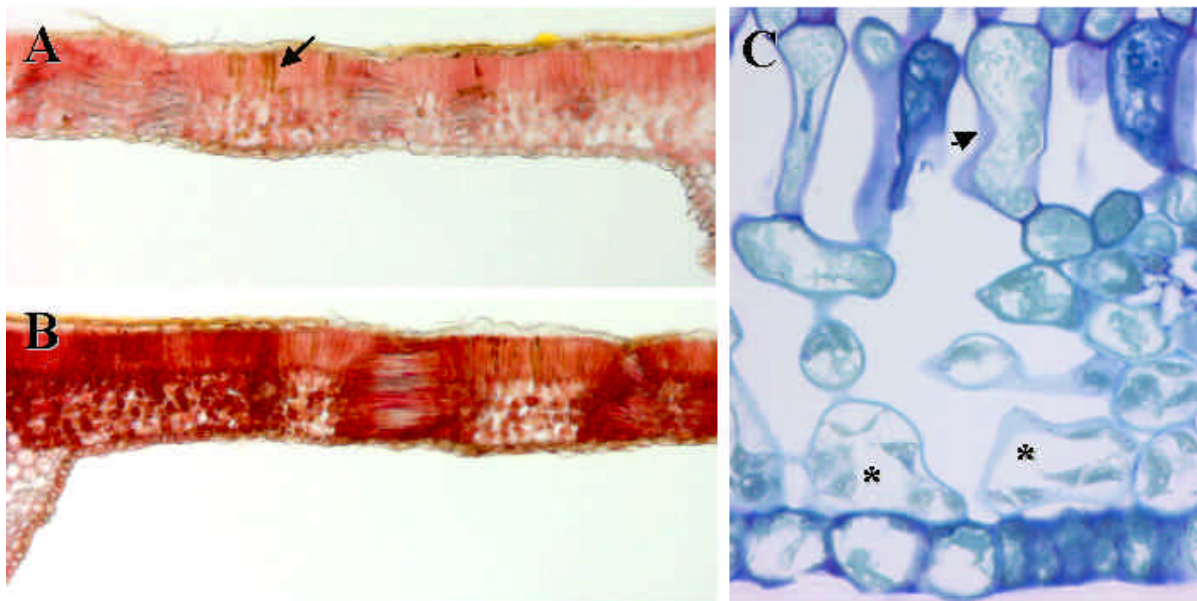


Fig. 2. Artefactual changes caused by the delayed storage and fixation.

Only the stippled areas showed markers attributable to ozone effects. Visible stipples consisted of groups of discrete necrotic cells in the palisade parenchyma (Fig. 2A arrow; Fig. 3B vs. 3A). Cells were partly or completely collapsed (Fig. 3C arrow) and the cell walls were irregularly thickened (Fig. 3D arrow) with cellulose (Fig. 3E arrow). Other cytological changes such as disruption of cell content, condensation of nuclei and chloroplasts were unreliable following the inadequate fixation conditions. However, the ascertained markers were sufficient to diagnose a hypersensitive response as frequently caused by ozone.

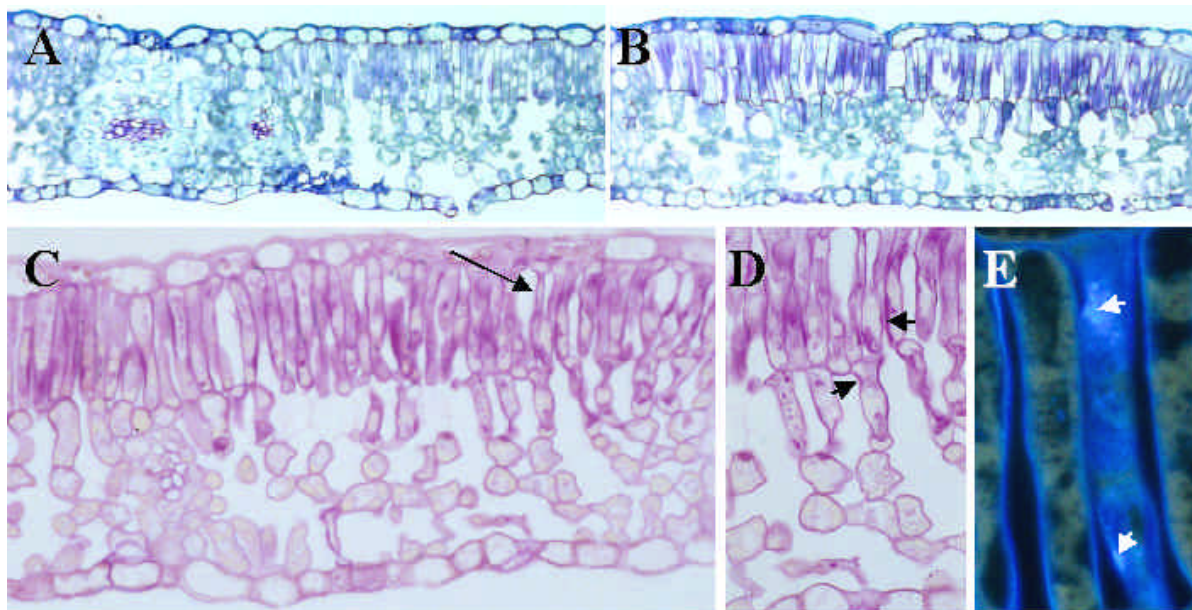


Fig. 3. Structural markers of ozone effects.

2. Methods and results (University of Florence)

We gathered foliar samples from beech at Moggio the 1 Sept. 2003. Leaves displayed “ozone-like” stipples. Samples were preserved in a refrigerated box and analysed in Florence the following day. The most relevant results are shown in Fig. 4.

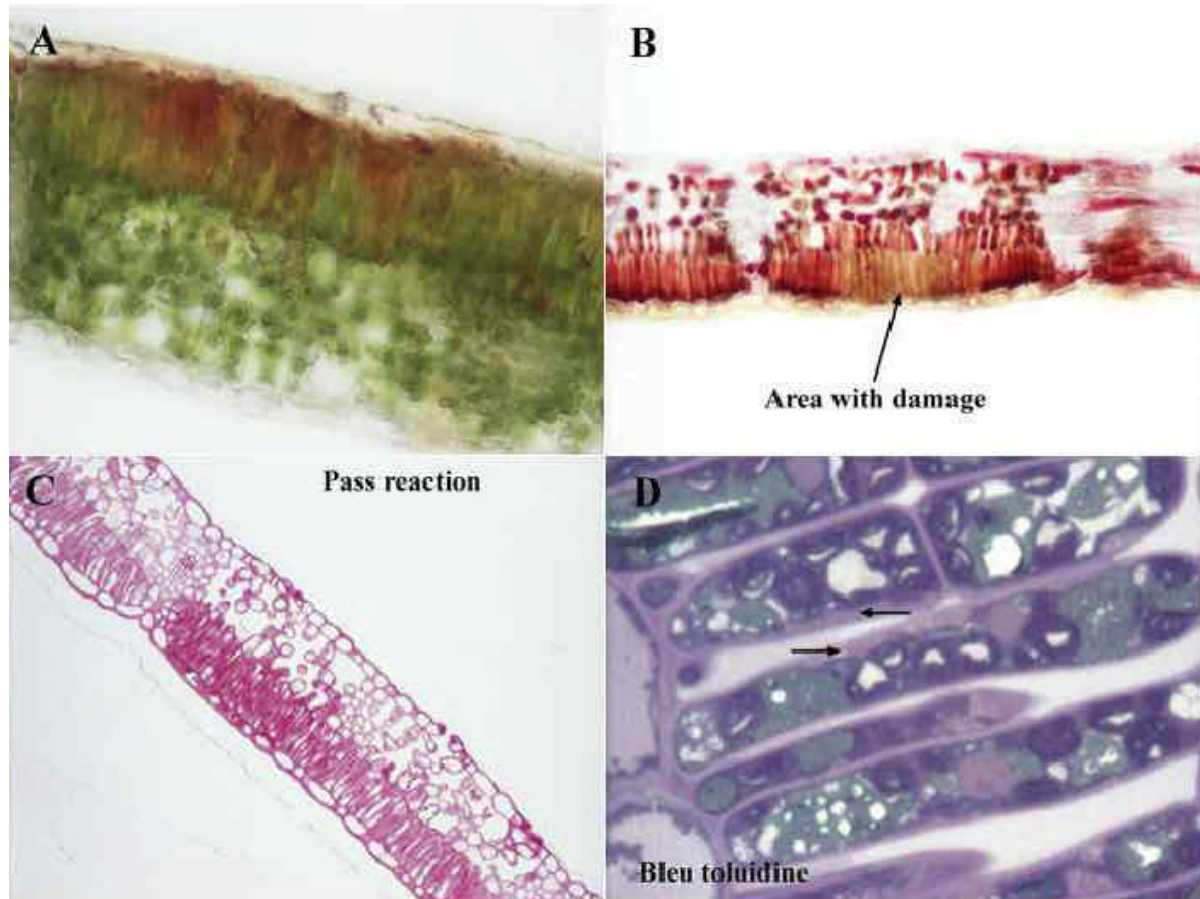


Fig. 4

A. Fresh-cut section, without any staining. The orange/brown area is located inside of a stipple. It may indicate the accumulation of some metabolites in the palisade cells.

B. Fresh-cut section stained with vanillin, for tannins. Tannins are present, especially in the palisade tissue. In the stipple, the response is different. Probably a different compound interacts with the tannins in the affected cells.

C. Pass (= PARS) reaction, to stain polysaccharides. The response is more intense in the stipples.

D. Toluidine blue. This image may be considered as a detail of the previous. You can note that the cell wall is thicker in the stipples.

The samples sent from Pierre are shown in Fig. 5. We were only able to observe some structural features. The most relevant aspect was the thickening of the cell wall in the palisade mesophyll, as shown by arrows in C and D.

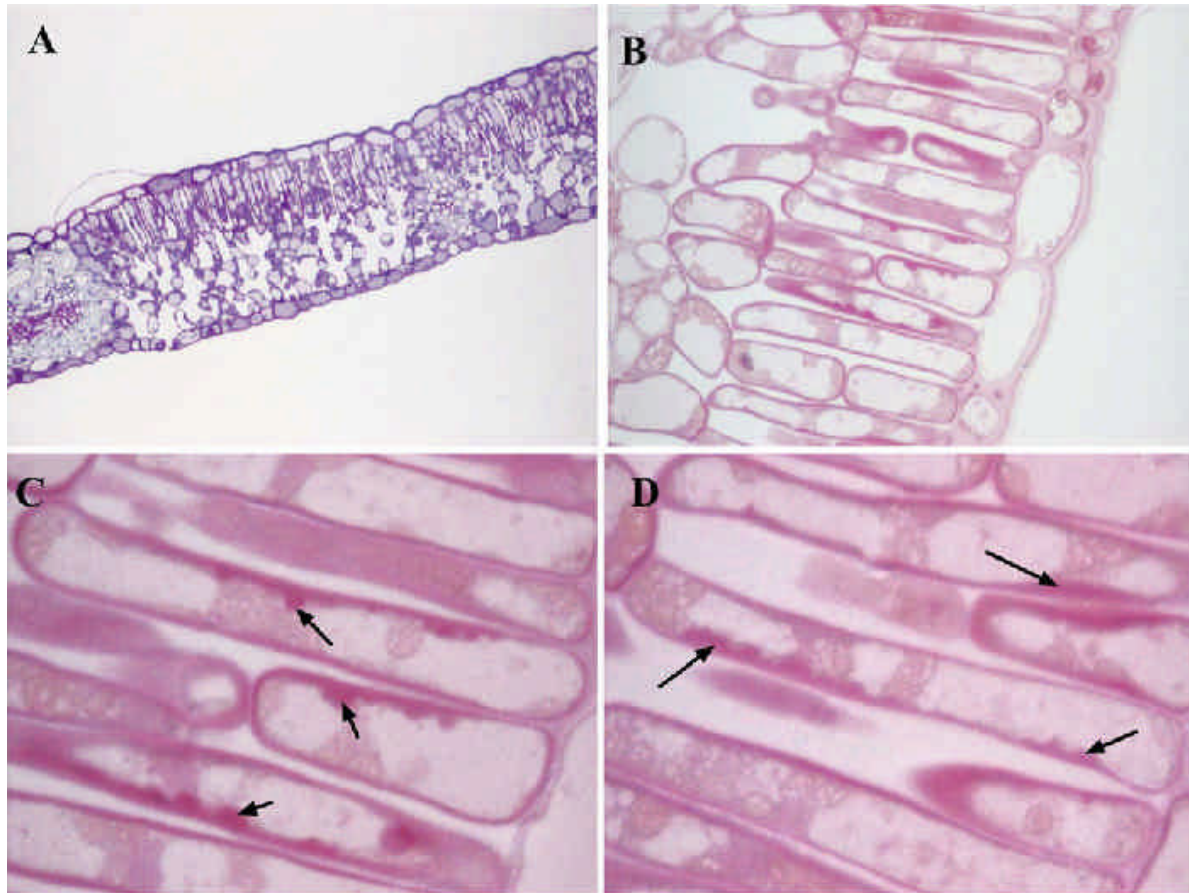


Fig. 5

3. Methods and results (University of Helsinki)

I received a letter from Professor Satu Huttunen with her comments on the Moggio samples. She found tannin accumulation, some dead cells and signs of possible proanthocyanidin accumulation. In other words, she thinks that the leaves exhibit ozone injuries.

Discussion

In the samples collected in Moggio on the 27th of August, a delayed sample preparation caused important artefacts to the leaf structure. In the mesophyll outside the stipples, reactions to excessive heat and dessication obliterated any marker of an accelerated tissue senescence. This latter plant response is generally associated with ozone stress. Its occurrence is suggested by the partial discolouration of the leaves. The artefacts observed also concealed any possible gradient between the more light exposed upper mesophyll and the lower shaded one; another important indicator of photo-oxidative stress. However, material conservation was sufficient to exclude a biotic infection which is the most likely cause of a stippling connected to veinlets.

Only the stippled areas in the palisade layer provided reliable markers of an ozone effect. The identification of hypersensitive responses caused by abiotic stress agents in the better light exposed portion of mesophyll is a reasonably sure indication of ozone stress. Such injuries thus result from the synergetic effects of ozone and photo-oxidative stress. The reactive oxygen species produced by these two sources can exceed the antioxidant capacities in the cell apoplasm and symplasm. The resulting oxidative cascade chain reaction can trigger the different defence responses observed in the analysed samples.

The three participating labs have used different approaches. However, they all lead to similar conclusions. The results are further supported by the analyses conducted on the leaves sampled a few days later: the visible and microscopic symptoms indicated typical ozone-induced changes too. Adequate sample preparation, if possible immediately after leaf removal from the plant, can be thus crucially important for a reliable symptom validation.

In conclusion, despite insufficient storing and fixation conditions, several characteristic markers for ozone were identifiable in the leaves sampled on the 27th of August. Stress agents other than ozone were not identified. The particular distribution of the stippling in the intercostal fields should be then attributed either to the ecotype characteristics or to the physiological conditions at the time of symptom formation. This study documents the plasticity in the symptom expression in beech at the macroscopical and microscopical level.