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Review

Linking stress with macroscopic and microscopic leaf response in trees: New diagnostic perspectives

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Received 16 August 2006; accepted 20 August 2006

Macroscopic leaf symptoms and their microscopic analysis as stress bioindications.

Abstract

Visible symptoms in tree foliage can be used for stress diagnosis once validated with microscopical analyses. This paper reviews and illustrates macroscopical and microscopical markers of stress with a biotic (bacteria, fungi, insects) or abiotic (frost, drought, mineral deficiency, heavy metal pollution in the soil, acidic deposition and ozone) origin helpful for the validation of symptoms in broadleaved and conifer trees. Differentiation of changes in the leaf or needle physiology, through ageing, senescence, accelerated cell senescence, programmed cell death and oxidative stress, provides additional clues raising diagnosis efficiency, especially in combination with information about the target of the stress agent at the tree, leaf/needle, tissue, cell and ultrastructural level. Given the increasing stress in a changing environment, this review discusses how integrated diagnostic approaches lead to better causal analysis to be applied for specific monitoring of stress factors affecting forest ecosystems.

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Keywords: Structural and physiological stress response; Histochemistry; Visual and microscopical stress diagnosis; Senescence and ageing

1. Introduction

Observing visible symptoms in the foliage of plants belongs to basic human experience about the natural environment and this still plays an important role in the diagnosis of plant disease. Aside from local previous reports, e.g. on silver smelters in ‘naturalis historia’ by Plinius secundus (23–79 after Christ), the toxic effects of anthropogeneous air pollution on trees started to attract attention in the United States of America with the observation of mottling on needles of declining *Pinus ponderosa* in the San Bernardino Mountains, which were attributed to air pollution and/or drought and

experimentally tested with elevated ozone concentrations (0.5 ppm, Miller et al., 1963). Since the 1970s in the USA macroscopic leaf and needle injury symptoms have been systematically monitored, with a variety of different survey approaches, and compared to measurements of ozone levels, as in the case of *Pinus ponderosa* and *P. jeffreyi* in California (Arbaugh et al., 1998). In Massachusetts, the occurrence of macroscopic symptoms observed in leaves of *Prunus serotina* increased in stands with higher site moisture and correlated with growth decline over a period of 31 years (Vollenweider et al., 2003b). Forest monitoring programs started all over Europe in the 1980s, using equal but not specific parameters, i.e. discoloration, foliage loss and tree growth. Field surveys of forest condition based on a specific ozone signature rather than on the stress-unspecific crown defoliation were implemented only at the beginning of this century by the Task Force of ICP Forests aiming to relate macroscopic ozone injury in the leaves and needles of natural forest vegetation in varying

Abbreviations: ACS, accelerated cell senescence; HR-like, hypersensitive-like response; OS, oxidative stress; OTC, open top chambers; PCD, programmed cell death; ROS, reactive oxygen species.

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Table 1
Macroscopic and microscopic stress symptoms in the foliage of broadleaved trees

Stress agent	Macroscopic injury	Microscopic stress symptom	Species	Reference
Sucking insects	Yellow, purple, brown patches in green leaves	Infestation by psyllids: toxic saliva secretion to mobilize nutrients; ultrastructural changes interpreted as 'resembling senescence'	<i>Eucalyptus camaldulensis</i>	Crawford and Wilkens, 1996
Frost in early spring (and ozone 65 ppb), climate chamber experiment	Wilting and inward folding; recovery possible, despite the structural injuries and local dehydration	Ultrastructural changes by frost: disintegration of the chloroplast envelope, vesiculation of the thylacoids, no effect on plastoglobuli; frost and ozone: reduction of chloroplast and starch grain size possible	<i>Betula pendula</i>	Prozherina et al., 2003
Drought (field samples)	Leaf curling (rolling) and dry necrotic leaf tip and rim	Strengthening of the xeromorphic structures; beech: increase of tannins in the epidermal cells (vacuoles and cell walls) during the vegetation season	<i>Fagus sylvatica</i> , <i>Fraxinus excelsior</i> , <i>Quercus ilex</i>	Dittmar et al., 2004; Grossoni et al., 1998
Salt , 100 or 150 mmol/l NaCl in shoot multiplication media	Lighter green color	Enlargement of the mesophyll cells; ultrastructural injury to chloroplasts including the dilatation of the thylacoid membranes (due to changes in the ionic composition of the stroma) and almost no grana	<i>Eucalyptus microcorys</i>	Keiper et al., 1998
0–50 μM Cd , hydroponic conditions in a growth chamber	Patchy chlorosis and necrosis particularly at the leaf rim increasing with leaf age; autoradiography: gradients of Cd accumulation in function of leaf age with less but evenly distributed Cd in younger foliage and more Cd preferentially accumulating in leaf veins of older leaves	Detoxification of Cd following active storage in the pectin-rich layers of the collenchyma cell walls (veins). Direct Cd effects: condensation and collapse of sieve tubes and companion cells in phloem (as a consequence of metal cycling); necrosis and acute OS (but no HR-like) in mesophyll and upper epidermis of the leaf blade, around discrete accumulations of Cd, as indicated by cell collapse, oxidation of cell walls, thickening of cell walls with polyphenols and sunscreen flavonoids. Indirect Cd effects: moderate OS (oxidized vacuolar proanthocyanidins) and ACS (size of vacuoles increased, condensed nuclei, chloroplast degeneration) in mesophyll cells, due to a low Cd content	<i>Salix viminalis</i> (clone 78198 tolerant to Cd stress)	Cosio et al., 2006; Vollenweider et al., 2006
3000 ppm Zn in the topsoil	Stippling on the upper leaf side, necrotic areas next to veins and/or the leaf rim; symptoms developing during the vegetation season in older foliage independent of light exposure	Increased Zn accumulation in the older foliage; little detoxification of Zn through storage in cell walls; preferential accumulation of Zn in the lower tissues of the leaf blade close to veins, often inside chloroplasts and rarely inside the vacuole. Direct Zn effects: phloem: folds in cell walls, degeneration of the cell content. Mesophyll: HR-like (discrete groups of necrotic cells, disruption of the cell content), OS (cell wall thickenings, inlays of polyphenolics, wart like protrusions); ACS (condensed nuclei, reduced chloroplast size) along the water pathway from the veins to the leaf blade and the lower epidermis which showed necrosis	<i>Populus tremula</i> , <i>Acer pseudo-platanus</i>	André et al., in press; Günthard-Goerg and Vollenweider, 2003

Ambient ozone ; OTCs ozone regimes: 50 or 92% ambient; 0, 50, 75, 90, 100 or 130 nI/l; ambient air +30, 40, 80, 90 or 130 nI/l ozone	Interveinal light-green, whitish, bronze or reddish stippling on the light-exposed leaf side, eventually developing to form necrotic dots, spots, flecks and colored areas; reduction of symptom development on leaf parts unexposed to direct insulation or closely shaded by another leaf or twig; increase of symptoms in leaves with a lower shoot position	Strengthening of the xeromorphic structures with ozone during leaf formation including condensation of the mucilaginous layer in epidermal cells, reduction of cell size and increase in the stomatal density; light-dependent gradient of chlorophyll degradation and cell injury in the mesophyll; light-dependent increase of oligo- and polymerized proanthocyanidins (bronzing) and/or anthocyanins (reddening); increased oxidation of the proanthocyanidins and cell content (OS) in light-exposed part of the palisade cells; depending on the species, irregular callose and cellulose thickening of cell walls and development of wart-like structures (with pectins, proteins and polyphenols) protruding into the inter-cellular space around and beneath the stipples; underlying the visible stipples: groups of, disrupted and collapsed palisade cells containing more or less disintegrated, resorbed and oxidized cell fragments often condensed in the form of apoptotic-like bodies, thus showing HR-like reactions; ACS homogeneously developing in mesophyll and epidermis around stipples (enlargement of the vacuome, increased amounts of vacuolar phenolic—often including tannins—and cytoplasmic lipids, enhanced electron density of cytoplasm/chloroplasts/nucleus/mitochondria, unclear delimitation of membranes (= membrane breakage) such as plasmalemma/tonoplast/nuclear envelope, injury to chloroplasts including a size reduction, abnormal and spherical shape, swelling and curling of thylacoids, increased stromal density, increased number/size/electron lucidity of plastoglobuli, disintegration of the mitochondrial matrix); daily course of starch accumulation disappearing with increasing cell injury, but enhancement of starch accumulation along veins; injury to the vein structure subsequently to strong injury in mesophyll including the accumulation of condensed tannins and callose and increased frequency of collapsed phloem cells	<i>Acer pseudo platanus</i> , <i>Ailantus altissima</i> , <i>Alnus glutinosa</i> , <i>Betula pendula</i> , <i>Carpinus betulus</i> , <i>Corylus avellana</i> , <i>Fagus sylvatica</i> , <i>Fraxinus excelsior</i> , <i>Pistacia lentiscus</i> , <i>Populus × euramericana</i> var. <i>Dorskamp</i> , <i>Prunus avium</i> , <i>Prunus serotina</i> , <i>Robinia pseudo acatia</i> , <i>Sorbus aria</i> , <i>Viburnum lantana</i>	Bussotti et al., 2005; Dittmar et al., 2004; Gravano et al., 2004; Günthardt-Goerg, 1996; Günthardt-Goerg et al., 1993, 1996, 1997, 1998, 2000; Matyssek et al., 2002; Mikkelsen et al., 1996; Pääkkönen et al., 1995a,b; Reig-Arminana et al., 2004; Vollenweider et al., 2003a
1.5× ambient ozone ± mild drought	Small bronze dots, no typical drought symptoms	Increase by both stress of the upper epidermal cell wall thickness, condensation of the mucilaginous layer in upper epidermis, number of pectinaceous cell wall protrusions on mesophyll cell walls (OS), and concentration of the vacuolar condensed tannins in the bundle sheath cells (ACS)	<i>Betula pendula</i>	Pääkkönen et al., 1998

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climates to different ambient ozone doses. Other stress symptoms such as drought have not been monitored on a large area scale in natural vegetation until now.

Unspecific symptoms, such as discoloration or increased crown transparency, reflect the general health status of trees resulting from the cumulative effects of stress events over the long term and, consecutively, do not indicate the nature of the particular stress agents involved (Godbold et al., 1993; Bussotti and Ferretti, 1998). As a consequence, the effects of anthropogenic influences, e.g. air pollutants, cannot be differentiated from those resulting from other environmental constraints (Badea et al., 2004). Therefore, the diagnosis of stress effects often remains basic and limited to paradigms like “All environmental stress leads to chlorophyll degradation” (Munné-Bosch and Alegre, 2004), or “If no pathogen can be found, an abiotic cause for the disease must be considered. However, abiotic stress factors interfere with normal physiological processes, causing unspecific symptoms and making it difficult to make a diagnosis, unless the history of the environmental conditions is known” (Agrios, 2005). Compilations of symptoms in textbooks (Skelly et al., 1990; Hartmann et al., 1995; Nienhaus et al., 1996; Sinclair and Lyon, 2005) allow practitioners, nevertheless, to identify some among the most common and easily identified diseases or deficiencies. The diagnostic efficiency is, however, reduced by limited understanding regarding plant responses underlying the symptoms. The development of diagnostic tools to discriminate between different stress factors is thus still required.

All different stresses eventually manifest themselves in macroscopic leaf symptoms. We have recently reviewed the diagnostic criteria, including the symptom expression at leaf, branch and tree level, for visible symptoms with an abiotic or biotic cause (Vollenweider and Günthardt-Goerg, 2006). The integrative evaluation of microscopic injury symptoms (causing macroscopic symptoms) for their potential to diagnose the causing agent still awaits exploration, because the macroscopic and microscopic levels have most often been studied independently from each other. This paper therefore intends to exploit the potential of connecting knowledge of macro to that of microscopic leaf, tissue and cell injury for a better understanding of the cause/effect relationship of symptoms in trees from the temperate (central European) zone. Indeed these long-living organisms present specificities important for stress diagnosis (as compared to herbaceous plants): (1) a vegetative development independent of the flowering activity letting symptoms better reflect the cumulative effects of an increasing stress dosage (Novak et al., 2003; Bassin et al., 2004), (2) sizable development of support tissues conveying important detoxification functions to cell walls for, e.g. oxidative stress or storage of heavy metal contaminants (Polle, 1997; Vollenweider et al., 2006) and (3) defense relying on a partly different foliage chemistry, noteworthy, with a more frequent involvement of condensed tannins (Waterman and Mole, 1994, p. 43). In the following sections the reader shall be introduced to background physiological changes occurring

in foliage and how the leaves and needles express them structurally. Referring to these basic considerations, new perspectives using consistent structural markers of several natural and anthropogeneous stress factors will show the potential of microscopic symptoms for a differential diagnosis of the causing agents in trees. To facilitate and initiate new insights, results from relevant recent references (since 1990) are condensed in Tables 1 and 2. They have been selected for their illustrations, subject relevance and connection with the visible symptoms; they are completed with figures derived from original microscopic work by the authors. This paper aims to promote new integrated approaches for better diagnosis of abiotic and biotic stress factors causing structural changes in leaves and needles by improving the causal analysis of stress in trees.

2. Background knowledge

2.1. Ageing

Research on leaf ageing is limited and poorly distinguished from senescence (Sections 2.2 and 2.3). As a tentative definition, ageing is a maturation process observed in long-living organs, during which fully functional tissues undergo progressive physiological and structural changes indicating extended use and declining vitality. Ageing tissues do not show stress symptoms or pronounced defense reactions and ageing foliage remains green. In foliage of trees, ageing is observed only in evergreens. An example of ageing symptoms was reported by Ruetze and Schmitt (1988) in more than 2-year old but still green conifer needles of *Picea abies*: in mesophyll cells, these authors observed increased aggregations of tannins and intercellular Ca-oxalate crystals but in up to 6-year-old needles only minor changes occurred in the chloroplast structure (increased number of plastoglobuli, but less thylacoids in grana). In the phloem, ageing was shown by the increased number of collapsed yearly cell rows. Indeed, cambial derivatives in needles differentiate abaxially only and form annual phloem but no xylem growth rings from the second year of growth until needle shedding, as shown for two American species of pines by Ewers (1982): during differentiation, derivatives first showed thickened cell walls with a lamellate structure and a dense cytoplasm (juvenile), then condensed nuclei and a clear cytoplasm (mature), later thin cell walls, definitive callose and no nuclei (senescent), before finally crushing (dead). The ontology of vascular tissues in conifers apparently shows only little variation between species as similar changes are visible in Norway spruce (Fig. 1(1–3), details see legends) or *Pinus cembra* from the Alps (authors' observations). Sutinen (1987) reported for *P. abies* that microscopic symptoms of ageing in more than 3-year old needles were evenly distributed in the mesophyll and looked similar at different tree height, in trees of different age or at different sites: with needle age the cytoplasmic and vacuolar lipid accumulations (Wulff et al., 1996a), proportion of cells with flat evenly electron dense chloroplasts (consecutively to a loss

of stroma) and less electron dense plastoglobuli (size and number not changed) increased whereas the cytoplasm and nucleus structure remained unchanged. These large electron translucent plastoglobuli contain the chlorophyll metabolite phytoen (Dahlin and Ryberg, 1986) and gather the waste products from chloroplast metabolism.

2.2. Ontological senescence

In contrast to ageing in green needles and leaves, ontological senescence is defined as the degenerative process organized in space and time at the final stage of development of the cell/organ (Orzaez and Granell, 2004), leading in the end to the abscission of oldest foliage. Macroscopically, symptom expression during senescence includes foliage yellowing, reddening, browning or blackening in deciduous tree species of the temperate zone, whereas only needle yellowing is observed in conifers. Yellowing results from the breakdown of chlorophyll and unmasking of carotenoids (Lee et al., 2003), reddening from the *de novo* synthesis of anthocyanins prior to chlorophyll breakdown (Matile, 2000), browning probably from the oxidation of cell components and blackening probably from the induction of phenoloxydases following the decompartmentation of cells. Full understanding of visible symptom development during autumn is, however, lacking. Foliage senescence proceeds along gradients at branch and crown level (Koike, 1990; Vollenweider and Günthardt-Goerg, 2006). The onset of autumnal senescence (decreasing leaf chlorophyll content) is mainly triggered by colder temperatures and reduced day length in deciduous trees (including *Larix* species, Rosenthal and Camm, 1997). At cell level, it is a controlled process, as shown by the maintained respiration for energy supply, with the mitochondrial integrity retained in senescent cells until only lipid droplets are left in an otherwise fully degenerated cell cytoplasm (Ojanperä et al., 1992). In senescent cells, oxidative stress (OS) is not increased and antioxidants (except of anthocyanins) are strongly decreased (García-Plazaola et al., 2003). This decrease is not the reason, but a consequence of senescence processes in the leaves (Dertinger et al., 2003). In control treatments, contrasting with symptoms due to various environmental stress factors, we found that cells in yellow leaves during autumn remained turgid and showed no cell wall thickening or oxidation of the cell content (Günthardt-Goerg et al., 1993). Information on microscopical changes during autumnal leaf senescence is, however, scarce and often needs to be extracted from studies where it was not the main focus (Mikkelsen et al., 1996). Microscopical senescence symptoms in photosynthetic tissue include chloroplast degeneration (dilatation and breakage of thylacoids, increase in number and size of plastoglobuli), vacuolization of the cytoplasm, nuclear chromatin condensation, portions of cytoplasm invaginating into the vacuole (autophagy processes) whereas the cell compartmentalization is maintained until the latest stage, during which lytic enzymes are found all over the cell (Gahan, 1981; Orzaez and Granell, 2004). According to the above definitions, leaves shed green have not undergone ontological senescence which

occurs later during autumn in deciduous trees. Remobilization processes, which culminate during senescence, however, start before the first macroscopic autumnal symptoms appear and show element-specific dynamics (Teissier du Cros et al., 1981, pp. 186–187; Marschner, 1995, pp. 466–477).

Macroscopic and microscopic symptoms of senescence also develop in evergreen conifers shortly before shedding of the oldest needles. They show some differences with the autumnal coloration found in broadleaved trees: only yellowing discoloration of needles is observed; yellowing does not occur all over the crown (as in deciduous trees including *Larix* sp.) but in a discrete way beginning in summer in the oldest needle age classes (e.g. in *Pinus cembra* as reported by Nebel and Matile, 1992); the accumulation of phenolic compounds (as occurring in different broadleaved trees) does not contribute to the symptom expression (own observations). The decrease in needle content of chlorophyll and proteins, the retrieval of nitrogen, phosphorous, magnesium and sulfur or the accumulation of calcium, manganese and zinc (Nebel and Matile, 1992) apparently proceed in a similar way in both broadleaved and conifer trees. During needle senescence, proteins are degraded homogeneously inside the degenerating mesophyll whereas vascular and epidermal tissues retain their structural integrity during a longer time (old chlorotic Fig. 1(5) vs. old green needle Fig. 1(4)).

2.3. Accelerated cell senescence

In stressed foliage organs, different physiological reactions (Pell et al., 1997) and structural changes (Gahan, 1981; Vollenweider et al., 2003a) at the macroscopical and microscopical level resemble natural senescence and have been interpreted as accelerated cell senescence (ACS) processes because they occur before autumn and/or in young needles. Structural criteria for ACS in mesophyll cells include (1) an increase in vacuole and vacuome size, (2) the progressive degeneration of cell constituents as shown by the increasing condensation of cytoplasm, nucleus or chloroplasts and (3) the accumulation of secondary compounds (Vollenweider et al., 2003a, Table 1). In vascular cambium and phloem of broadleaved trees, ACS is characterized by (1) reduced cambial activity, (2) folding of cell walls and partial cell collapse in phloem and (3) progressive condensation of cytoplasm and nucleus in sieve tubes and companion cells (Vollenweider et al., 2006). Regarding conifers, structural changes have been principally observed in the case of disturbances in the mineral nutrition. Thus, they are described in the corresponding Section 3.3. They resemble ontological senescence and can therefore be labeled as ACS but they largely differ from ACS in broadleaved trees as a consequence of the particular phloem ontogeny in conifers (see Section 2.1). Markers of ontological senescence, as well as those of ACS, thus vary according to the type of tissue. Similarly, it is questionable that the criteria of ACS and ontological senescence are matching in all cases, considering that ACS is often, and ontological senescence is not, associated with oxidative stress (see Sections 2.6 and 2.2, respectively). Future research should establish whether

Table 2
Macroscopic and microscopic stress symptoms in conifer needles

Stress agent	Macroscopic injury	Microscopic stress symptoms	Species	References
Freezing-thawing cycles by the end of the growing season and frost (–30 °C) during winter; experiments realized in growth chambers	Needle browning, grayish needles turning reddish-brown	Same injury on the adaxial and abaxial needle side; particularly in mesophyll crystallization of extra cellular ice resulting in the withdrawal of water from cells and cellular dehydration; damage progressively developing after thawing consecutively to the increased influx of ions and organic solutes into the apoplast: (i) cytoplasm darkening and fine tannin ribbons along the border of the central vacuole, (ii) irreparable disintegration of cell constituents, plasmolysis, disappearance of tonoplast, visual browning, (iii) death by rupture and collapse of cell wall	<i>Picea abies</i> , <i>Pinus sylvestris</i>	Ryypö et al., 1997; Wulff, 1996
Drought applied in growth chambers or during 2 months in OTCs under filtered air, sampling in July	No visible symptoms	Plasmolysis of mesophyll and transfusion parenchyma cells; chloroplasts: rounded form, local aggregation of stroma on the side facing the cell wall away from thylacoids; swelling of thylacoids and vesicle formation; lipid bodies concentrated around chloroplasts (in pine only)	<i>Picea abies</i> , <i>Pinus sylvestris</i>	Holopainen et al., 1992; Sutinen and Koivisto, 1995; Palomäki et al., 1994
Nitrogen deficiency	Uniform yellowing beginning in older needles	Ultrastructural changes in mesophyll and transfusion parenchyma: less endoplasmic reticulum and ribosomes in cytoplasm (first ultrastructural changes during shoot elongation in young needles); chloroplasts showing size reduction, irregular shape, less thylacoids, but more plastoglobuli and more electron translucent stroma (changes resembling ACS in yellowing needles); slightly swollen mitochondria; upon fertilization, recovering from chloroplastic but not from other cytoplasmic injury possible	<i>Pinus sylvestris</i>	Palomäki and Holopainen, 1995
Phosphorus deficiency	Necrotic spots in an advanced stage	Ultrastructural changes in mesophyll and transfusion parenchyma: swelling of cristae and subsequent dilatation of whole mitochondria in several needle generations; aborted development of chloroplasts; no regeneration despite fertilization during a full growing season	<i>Picea abies</i> , <i>Pinus sylvestris</i>	Palomäki and Holopainen, 1994; Utrainen and Holopainen, 2002
Potassium deficiency	Brown needle tips in an advanced stage	Potassium is the most important inorganic osmoticum to maintain the high turgor pressure inside sieve cells; symptoms looking similar but generally less acute than in the case of magnesium deficiency (see below)	<i>Pinus sylvestris</i>	Fink, 1991
Magnesium deficiency	Yellowing and, in second year needles, eventually tip necroses	First injury in current-year (c) needles: swelling and collapse of phloem parenchyma cells (Mg is involved in phloem loading), then in mesophyll: enlargement of vacuole, vesiculation of tonoplast, accumulation of condensed tannin bodies inside vacuoles and of starch inside chloroplasts, rounding of the chloroplastic shape, accumulation of plastoglobuli and of abnormal thylacoids with characteristic electron-translucent gaps, accumulation of lipid bodies in cytoplasm; in c + 1 needles: collapse of almost all sieve cells, enlargement of the adjacent parenchyma and cambium cells; reactivation of cambium and regeneration of chloroplasts after fertilization possible	<i>Picea abies</i>	Boxler-Baldoma et al., 2006; Fink, 1997; Hannick et al., 1993; Puech and Mehne-Jakobs, 1997; Palomäki, 1995
Simulated acid rain (pH 2.9, H ₂ SO ₄ and NO ₃ , 60 mm each, every 3 summer months during 7 years) under field conditions	Chlorosis probably caused by a slight nutrient deficiency induced by the acid rain treatment	Effects of acid rain on mesophyll ultrastructure: earlier onset of winter hardening as shown by the increased accumulation of large lipid bodies in the cytoplasm	<i>Pinus sylvestris</i>	Bäck et al., 1994
Spraying of flushing needles with a sulfuric acid solution down to pH 2.5, 10× during 1st day, then 1× per day during 1 month, greenhouse	Light-yellow spots on the needle surface starting from the needle tip and developing to light-brown spots	Sharp border between green and yellow tissues; injury beginning directly under the stomata; disorganization of membranes leads to death of mesophyll cells	<i>Pinus nigra</i> , <i>Pinus resinosa</i>	Zobel and Nighswander, 1991

1 mm acid mist pH 2.5 (containing H ₂ SO ₄ and NH ₄ NO ₃) during 4 consecutive days per week and 6 months), in OTC and in the field	Symptom present (no details)	Acid mist: decreased amount of calcium in outer epidermal cell walls (leaching), particularly in hypodermal subsidiary cells at the abaxial needle side; ultrastructural effects: increase in chloroplast width enhancing the chloroplastic sphericity (similarly than with drought); in OTC with acid mist earlier onset of winter hardening (reticulate appearance of cytoplasm, increase of cytoplasmic vacuoles)	<i>Picea sitchensis</i>	Wulff et al., 1996b
SO₂ field fumigation (8 h/day, 100–400 µg/m ³) and daily spraying with aq. NaF (50 mg/l) and nitrogen (NH ₄ NO ₃ , 200 mg/l) during 3 consecutive growth seasons	Chlorotic or necrotic needle tips in 3-year old needles with highest frequency in NaF-treated <i>P. sylvestris</i> (showing a dark band at the interface between the necrotic tip and healthy tissue)	Ultrastructural effect: increased frequency of cytoplasmic vesicles with SO ₂ (similar to winter hardening effect)	<i>Picea abies</i> , <i>Pinus sylvestris</i>	Wulff and Kärenlampi, 1996
Industrial environments with annual emissions of 12,000 t SO₂ or 5000 t SO₂ + 800 t NO_x + 22 t HF vs. clean air site	Chlorotic spots in ≥4-year old needles	Near the emission source, ultrastructural ACS effects in mesophyll cells, even those of younger needles, including large cytoplasmic lipid bodies, lipid droplets in vacuoles, accumulation of electron-opaque grains in the cell wall, spherically shaped chloroplasts (similar to that induced by drought), thylacoid swelling, electron-translucent plastoglobuli	<i>Picea abies</i> , <i>Pinus sylvestris</i>	Wulff et al., 1996a; Holopainen et al., 1992
Ambient ozone (0–71 ppb); ozone fumigation 1.2–2× ambient (100–600 nl/l)	Photobleaching and mottling at the light exposed tree, branch and needle side (large necrotic flecks at high ozone concentration) progressing from distal to basal needle part and increasing with needle age	Light-dependent gradient of chlorophyll degradation and cell injury in the outer mesophyll cells (photobleaching) beneath the hypodermis and the sub-stomatal cavities including changes indicative of ACS in the chloroplastic shape (smaller and/or irregular and rounder) and structure (increase in the electron density of the chloroplastic stroma and the abundance of ribosome-like granules), the hypertrophy of microbodies (probably peroxisomes), the condensation of nucleus and cytoplasm, the increase in the vacuole size and content of proanthocyanidins, the reduced resolution of the plasma membrane suggesting a decreased desaturation of fatty acids and finally the accumulation of lipid and starch in mesophyll and bundle sheath cells. Directly underlying the mottles: group of cells with a disrupted cell content and strong condensation of cell remnants to apoptotic-like bodies, indicating HR-like reactions. Epidermis, hypodermis and vascular tissue remaining longest intact; eventually intracellular accumulation of calcium oxalate	<i>Abies religiosa</i> , <i>Picea abies</i> , <i>Pinus cembra</i> , <i>Pinus elliotti</i> var. <i>densa</i> , <i>Pinus halepensis</i> , <i>Pinus sylvestris</i> , <i>Pinus taeda</i> , <i>Sequoia-dendron gigantea</i>	Alvarez et al., 1998; Anttonen and Kärenlampi, 1996; Anttonen et al., 1996; Dalstein et al., 2002; Evans and Leonard, 1991; Fitzgerald, 1993; Holopainen et al., 1996; Kivimäenpää et al., 2004; Soda et al., 2000; Sutinen et al., 1990; Vollenweider et al., 2003a
Elevated ozone and CO₂	Ozone: chlorotic mottling in previous-year needles	Ozone effects irrespective of CO ₂	<i>Pinus sylvestris</i>	Utriainen and Holopainen, 1998
Ambient ozone + ozone fumigation during 4 years, and/or drought ; experiments realized in OTC	No visible symptoms reported	Ozone effects: increase of peroxisome frequency at upper needle side, and mitochondria in younger needle generation (active defense against OS and increased maintenance respiration); no changes in chloroplasts. Drought effects: enhancement of nutrient unbalances as shown by the reduction of sclerenchyma inside the vascular bundle and increased proliferation of tonoplast	<i>Picea abies</i>	Kivimäenpää et al., 2003

This selection of research papers groups recent references (since 1990) about biotic and abiotic stress, which illustrate macro- and microscopic symptoms in trees and make the link between visible and (ultra-)structural injury.

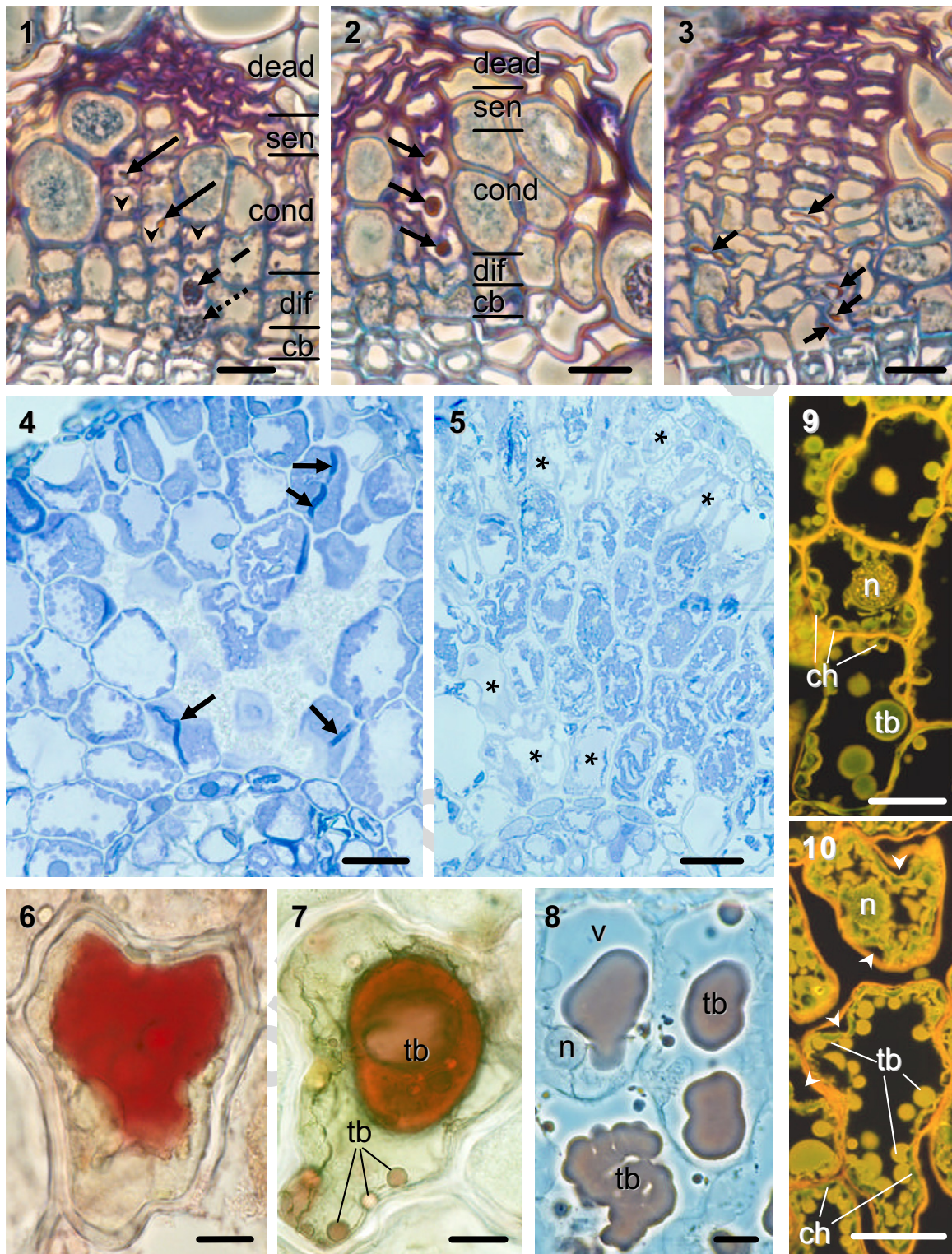


Fig. 1. Microscopical indications in spruce (*Picea abies*) needles sampled in the field. (1)–(3) Contrast between maturation, aging and senescence at cell and tissue level in the phloem of a green 2-year-old (1), vs. a green 7-year-old (2) and a senescent and yellowing 7-year-old needle (3). (1) Changes during the lifespan of sieve cells: cambial cells (cb) are identified by their uncondensed nucleus (dotted arrow), dense cytoplasm and thin periclinal cell walls; during differentiation (dif), nucleus condenses (dashed arrow) and cell wall thickens; mature conductive cells (cond) show highly condensed nuclei (plain arrow), clear cytoplasm and thick cell walls (arrowhead); senescent cells (sen) appear empty; dead cells (dead) are collapsed. (2) Aging in phloem is shown here by the missing secondary wall thickening inside mature phloem cells. (3) In yellow needles, the cambial, juvenile and mature phloem differentiation stage is no more recognizable. Senescence markers at the cell level include highly condensed nuclei (arrows), cell deformation, and thin cell walls. (4), (5) The same samples as (2) and (3), respectively, but stained for proteins (blue color). A fainter blue staining, especially in chloroplasts of numerous cells (*), is responsible for this senescence response in yellowing (5) vs. green (4) 7-year-old needles. Notice in (4) the blue colored lipoproteinous stylet sheaths of sucking insects (arrows; Günthart and Günthart, 1983). (6)–(8) Variability in the vacuolar structure of condensed tannins (= proanthocyanidins). (6) Tannin oligomers and tannin-building monomers, as observed in fresh

physiological and structural differences exist between ACS caused by different stress factors and to what extent ACS(s) differ from ontological senescence in the mesophyll or vascular tissues.

2.4. Accidental and programmed cell death

Dead is dead, but the process to death differs. In contrast to the controlled process of senescence causing death of cells and organs in a synchronic and controlled way, accidental cell death is a passive and uncontrolled form of rapid cell death not requiring energy. It results from mechanical injury (triggered for example by hail, frost or a micro-herbivorous activity) or exposure to excessive concentrations of chemicals including toxic pollutants. Accidental cell death consists of a sudden loss of membrane integrity and ionic homeostasis, disintegration of organelles and random degradation and oxidation of cellular contents (mostly passive following the penetration of air). Macroscopically, necrotic lesions form localized visible symptoms. In contrast, programmed cell death (PCD; Evans, 2004; Jones and Smirnov, 2005) is an ontological and endogenously regulated process by which cell material is degraded in a controlled way allowing resource recovery. As stated by Fukuda (2000), “PCD may be categorized by cytological features into three types: (1) apoptosis-like cell death (see Section 2.5), (2) cell death occurring during leaf senescence (ontological senescence; see Section 2.2), (3) (ontological) PCD ... in differentiating tracheary elements”. Ontological PCD also occurs in phloem of conifers prior to the onset of assimilate transport, as shown by the structural changes in sieve cells (see Section 2.1 and Fig. 1(1–3)). The term apotheosis (Evans, 2004) is used for cells undergoing PCD and showing a morphotype including chromatin condensation, cell shrinkage, membrane blebbing, oligonucleosomal DNA fragmentation (highlighted by the TUNEL reaction), and the formation of ‘apoptotic-like bodies’ (condensed and compacted cell remnants, Levin et al., 1996).

2.5. Hypersensitive response

Plants use PCD as a defense mechanism against biotic and abiotic stress. Rapid cell death can be thus observed during different plant–pathogen interactions (Lam et al., 2001; Levin et al., 1996) and constitutes a hypersensitive response (HR) of plants to microbial infections in order to restrict the growth of pathogens into healthy tissues (Heath, 2000). Sometimes, a HR is too slow or not efficient enough to prevent the pathogenic growth. Pathogenesis-related proteins are associated

with the HR (Orzaez and Granell, 2004). Defense PCD is named HR-like when the elicitor is an abiotic stress factor. Ozone (Schraudner et al., 1997; Sandermann et al., 1998; Rao et al., 2000; Vollenweider et al., 2003a) or some heavy metals (Piqueras et al., 1999) have been reported to induce HR-like reactions. Structural criteria to identify a HR-like reaction include: (1) the absence of pathogens, (2) the restriction of PCD to a small group of cells, (3) the disruption of the cell content, (4) nucleus degeneration and maximum chromatin condensation, (5) the collapse of cell walls and (6) the incomplete degradation of cell remnants and their condensation in apoptotic-like bodies as a consequence of rapid cell death (Fett and Jones, 1995; Levin et al., 1996; Alvarez et al., 1998; Fukuda, 2000; Vollenweider et al., 2003a).

2.6. Oxidative stress

Defense and senescence PCD are mediated by reactive oxygen species (ROS). ROS formation constitutes an unavoidable by-product of aerobic and chloroplastic metabolism (Elstner, 1996) which causes oxidative stress when antioxidative reactions are outbalanced. Accumulation of ROS leads to rapid and uncontrolled reactions with biomolecules forming an oxidative burst in some cases (Sandermann, 1996; Pellinen et al., 1999) and resulting in lipid peroxidation, inactivation of proteins (e.g. enzymes of the CO₂-fixation or Ca²⁺-ATPase, which increases cytoplasmic Ca²⁺) and strand breaks of DNA (Bartosz, 1997). ROS are formed in different cell compartments (such as the apoplast, mitochondria, peroxisomes, chloroplasts and endoplasmic reticulum; Bartosz, 1997; Dietz et al., 1999; Pellinen et al., 1999) along the penetration pathway of the stress factor into the foliage organ, as shown for ozone (Schraudner et al., 1997; Pellinen et al., 1999). Markers for OS and OS effects include: (1) wart-like and strand protrusions on cell walls (Günthardt-Goerg et al., 1996, 1997); (2) the accumulation of antioxidants (Sandermann, 1996), (3) the increased oxidation of cell content and H₂O₂ accumulation in cell walls, on the outer surface of plasma membranes, in adjacent cytoplasm and even chloroplasts (Pellinen et al., 1999; Oksanen et al., 2003) and (4) the sequential degeneration of chloroplasts, peroxysomes and mitochondria (Sutinen et al., 1990). Metabolites produced during oxidative burst also have signaling functions and thus modulate the gene expression (Vollenweider et al., 2000; Weber et al., 2004). With plant hormones, they contribute to determining which acclimation, defense or PCD (Evans, 2004) responses will be implemented by the plant. Antioxidative systems are localized in apoplast, cytosol, peroxisomes,

unfixed samples, are less oxidized (red tones) than polymers and occur as solutes in the vacuolar medium. (7) Tannin polymers form different kinds of segregated and more or less oxidized bodies (tb) in the vacuole, as indicated by red to brown tones. (8) Only insoluble tannin bodies (in brown) are observable in the vacuole (v) of fixed and plastic-embedded samples, n, nucleus. (9)–(10) Beginning plasmolysis (arrow heads) in green needles from young trees grown on acidic silty forest soil (10) (pH 3.9 in 0.01 M CaCl₂) vs. those grown on calcareous sandy forest soil (9) (pH 7.59 in 0.01 M CaCl₂) in a controlled experiment. The tonoplast is outlined by tannin bodies (tb) ribbons or globules, often capping chloroplasts (ch) or nucleus (n). Samples were collected during summer. Revelation methods: (1)–(3) Toluidine Blue (Feder and O'Brien, 1968); (4),(5) Coomassie Brilliant Blue R-250 (Fisher, 1968); (6),(7) Acid vanillin (Sarkar and Howarth, 1976); (8) 3 M sulfuric acid (Gutmann, 1993); (9),(10) Coriphosphine, viewed under excitation 450–490 nm (Günthardt-Goerg, 1996). Bars: 10 µm (1)–(3) and (6)–(8); 25 µm (9) and (10); 50 µm (4) and (5). (For interpretation of the references to colours in figure legends, the reader is referred to the web version of this article).

chloroplasts and vacuoles (Sandermann et al., 1998; Palma et al., 2002; Vollenweider et al., 2006).

3. Cause–effect relationship between stress factors and responses in tree foliage

3.1. Responses to biotic stress factors

The detection of plant diseases caused by microorganisms became possible after Antonius van Leeuwenhoek invented the first microscope in 1674. Two-hundred years later, De Bary (1861) demonstrated that the potato blight was due to a fungus. A plant pathologist nowadays is educated to identify (mainly by eye or by microscope) pests and pathogenic organisms. Several databases also offer online diagnosis (e.g. <http://www.waldschutz.ch/pbmd/>; <http://www.plantdoctors.com/faq.htm>). Infections and insect feeding can also be diagnosed by the determination of macroscopic leaf injury, which most often consists of confined necrotic or colored patches in green leaves. Handbooks exist for determining typical macroscopic leaf symptoms (e.g. Skelly et al., 1990; Hartmann et al., 1995; Nienhaus et al., 1996; Sinclair and Lyon, 2005). The type of injury as a consequence of piercing by insects is mechanical (lipoproteinous stylet sheaths formed by sucking insects, Fig. 1(4) unless worsened by chemicals secreted during piercing some of which interfere with the plant physiology (e.g. hydrolytic enzymes, toxins, growth regulators). Structurally, plants restrict the penetration and growth of pathogens with constitutive or inducible physical and chemical barriers and defenses. Cell walls can be thickened and have suberin, callose or polyphenols inlaid (Fig. 2(15); Dai et al., 1996; Benhamou and Nicole, 1999; Silva et al., 1999), whereas secondary compounds (Fig. 2(12,16)) including condensed tannins (Fig. 2(13)) accumulate in the vacuole (Fig. 2(13,14)); hypersensitive cell death can confine some infections (Section 2.4). Typically, structural reactions to pathogenic infections are irregularly distributed in the leaf (Fig. 2(13–15)) as they culminate where the infection develops (Fig. 2(15)). Cells adjacent to those injured generally show ACS (symptoms of ACS in chloroplasts were reported for a viral infection; Hernández et al., 2004) decreasing with distance from the penetration spot. Sucking insects, as reported for psyllids (Crawford and Wilkens, 1996), suck nutrients through their piercing stylets after salivating hydrolytic enzymes into vacuoles; the induced breakdown of the tonoplast increases the solubility and mobility of nutrients; proteins from chloroplasts are also retrieved. Such a breakdown of the plant cell structure through parasitism differs in many points from degradation processes controlled by the plant (ACS). Mesophyll feeding insects can suck the cell content without inducing plant defense reactions: microscopically, groups of empty cells with a few oxidized remnants of the cell content (Fig. 2(11)) resulting from accidental cell death are found within an otherwise asymptomatic tissue. These cells, filled with air, cause visible symptoms in the form of white feeding traces inside otherwise green tissues (Vollenweider and Günthardt-Goerg, 2006). Besides fungal diseases

generally diagnosed using the morphology of fruit bodies, infection by vegetative mycelium causing stress reactions is often present in tree foliage (Fig. 2(12)), but remains unidentified because of missing sporulation and avirulence. Frequency and effects of such endophytes are still poorly understood but they rarely penetrate inside cells and consequently induce more or less advanced ACS. Similarly, bacterial infections, which can be recognized when isolated or when couples of hyphen-like structures together with ACS markers are observed (Fig. 2(15)), are not rare in the foliage of forest trees and need further investigation. In situations where more than one stress factor affects the cell physiology in foliage, the interaction between stress factors or the individual and combined plant responses, are far more difficult to predict as knowledge is generally available on the effects of isolated stress factors only. Research using stress combinations, noteworthy a biotic infection in trees stressed by an abiotic stress factor (e.g. ozone or drought) is particularly needed in the current era of environmental change. So far, tolerance or susceptibility of plants to biotic stress seems to be positively or negatively influenced by a previous abiotic stress (Sandermann, 1996).

3.2. Responses to adverse climate events

Extreme climatic events like frost (Fig. 3(17,18)), ice storms (Fig. 3(20)), hail (Fig. 3(19)), heat or drought (Fig. 3(22,23)) can cause a large range of symptoms in tree crowns from the breakdown of crown branches to the recoverable changes in still green foliage. The effects of each stress factor present diagnostic features which vary slightly according to the species. Isolated events like spring frost, hail or ice storms cause either more or less severe mechanical injuries but hardly interfere with the leaf or needle physiology. Climatic events which occur more chronically during a part of the year, like drought or winter frost, elicit different hardening, defense, and stress plant responses and cause structural changes at the micro- and visible symptoms at the macroscopic level.

Partly irreversible cell injury occurs when temperatures drop below the cold tolerance levels, as chilling stress (subtropical and tropical plants, $<10-15 > 0^{\circ}\text{C}$) or freezing stress (reviewed by Fink, 1999, pp. 456–468). For deciduous trees few observations have been published (Vollenweider and Günthardt-Goerg, 2006; Table 1). Investigations relating findings using crops to processes in deciduous trees are still missing. Macroscopic leaf symptoms appear as dark flecks, wilting or chlorosis, sometimes days or weeks after the temperature has dropped. Cellular injuries are thought to result from disturbances in osmotic homeostasis resulting from changed membrane permeability: with frost, segregation of water in the form of ice crystals strongly reduces the extracellular osmotic potential and cells plasmolyse. Therefore at the ultrastructural level, injuries are mainly found in membranes. They are more frequent in the mesophyll than in bundle sheath cells. In the cytoplasm, electron density increases, the plasma membrane and tonoplast form vesicles, ribosomes detach from

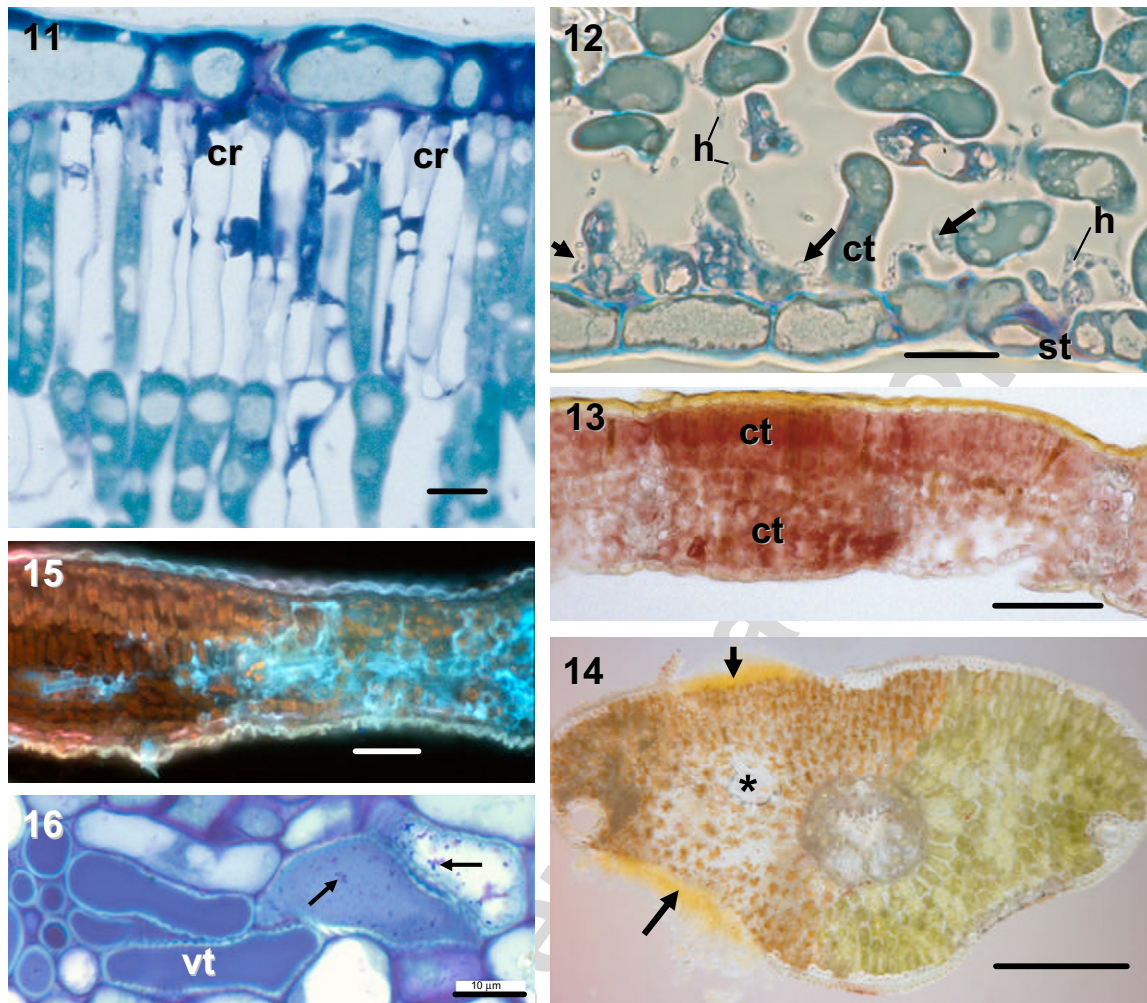


Fig. 2. Different biotic injuries in leaves and needles from field samples. (11) Injury caused by a mesophyll feeding leafhopper species (Homoptera, Auchenorrhyncha, Cicadellidae). Cells are sucked out by insect piercing. Cells walls are not collapsed and a few condensed (dark blue colored) cell remnants (cr) remain visible. (12),(13) Fungal infection in beech leaves. Probably a rust fungus penetrated through the stomata (st) and progressed with intercellular hyphae (h). Host cells have been probably parasitized with haustoria as indicated by swollen hyphae portions (arrows). Plant cells reacted to the stress with different types of defense mechanisms including the inlay of condensed tannins (ct), especially in the leaf blade areas where the fungal parasite had an enhanced development (12). (14) A conifer needle infected with a parasitic and host-specific ascomycete fungus. Vegetative fungal structures are restricted to the brown and necrotic needle tissues (*). By the time of sampling (spring), the fungus had developed fruiting bodies (arrows). (15) Injury caused by mite parasitism. Mite colonies developed on the leaf lower side from where they repeatedly punctured the host tissues. Leaf thickness was reduced, cells were badly injured (reduction of the red chlorophyll autofluorescence) and massive polyphenolic thickenings were inlayed in cell walls (in blue). (16) Bacterial infection in leaf veins. Bacteria were visible as single or twin hyphen-like structures (arrows). Plant cell defense is indicated by inlayed vacuolar tannins (vt). Parasitic species: unknown except in (14): *Rhabdocline pseudotsugae*. Host species: (11)–(13) *Fagus sylvatica*; (14) *Pseudotsuga menziesii*; (15) *Populus tremula*; (16) *Quercus petraea*. Revelation methods: (11), (12), and (16) Toluidine blue and *p*-phenylenediamine (Kivimäenpää et al., 2004); (13) Acid vanillin (Sarkar and Howarth, 1976); (14) Fresh unstained preparation; (15) autofluorescence (excitation at 340–380 nm). Bars: 10 µm (16); 15 µm (11); 50 µm (12) and (15); 100 µm (13); and 250 µm (14). (For interpretation of the references to colours in figure legends, the reader is referred to the web version of this article).

membranes and mitochondria show swollen cristae and vacuoles (as in the case of severe water stress). The size of the nuclei increases as a consequence of the de-condensation of chromatin. Chloroplasts show vesiculation and disruption of the inner envelope, disruption of grana, thylacoid swelling and distortion (no shrinking), and accumulation of lipid droplets. Later, cells drop their turgor, the cytoplasm becomes vacuolated and decompartmentalized after rupture of the tonoplast. How far the cell retains control over such degeneration effects during chilling (similarities to PCD) was discussed by Kratsch and Wise (2000) mainly for herbaceous plant species. Similar changes in thylacoid ultrastructure

have been reported as a consequence of salt uptake (NaCl stress, Table 1), whereas visible symptoms (leaf rim necroses) due to salt spray (Fig. 3(21)) more resemble those of drought (see below). However, information for trees is again scarce (reviewed in Fink, 1999).

Trees of the temperate and boreal climate zone are adapted to cope with winter-frost damage. In conifer needles, frost hardening is shown by different structural markers including an irregular amoeba shape of chloroplast (Wulff et al., 1996a), the absence of starch, chloroplasts clumping together with other organelles, a reticulate appearance of cytoplasm due to proliferation and vesiculation of the endoplasmic

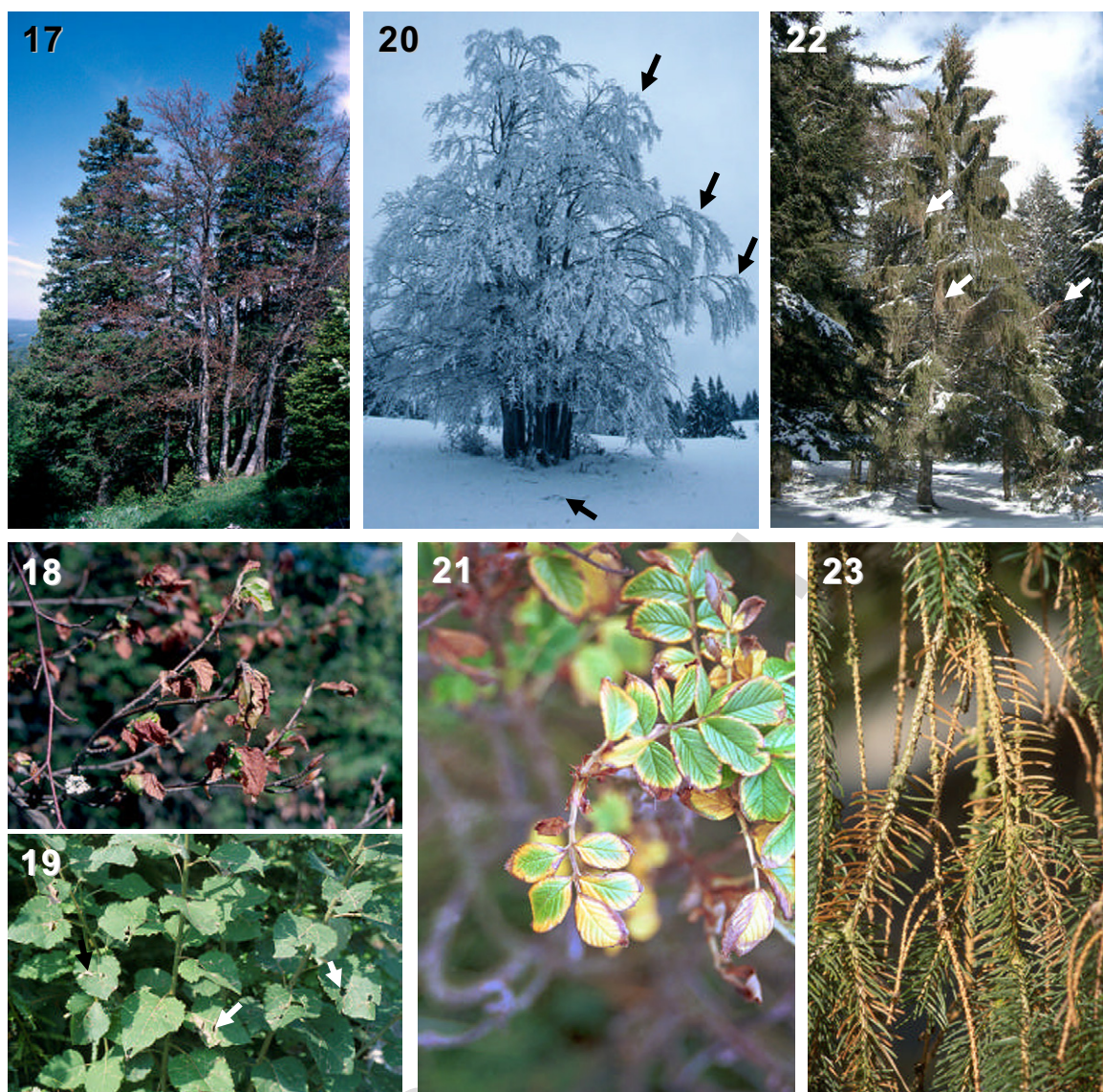


Fig. 3. Visible symptoms in trees caused by adverse environmental conditions. (17), (18) Destruction of new foliage after severe spring frost during budbreak. (19) Leaves torn and punctured by hail (arrows). (20) Crown bending (arrows) due to ice overloading and branch breaking (arrows) during an ice storm. (21) Chlorosis and rim necrosis by sea salt sprayed inland by storms. (22), (23) Drought symptoms in a thinning crown as shown by drying needles (23) and branches (arrows in (22)) observed in February 2004, several months after the exceptionally hot and dry 2003 summer. Plant species: *Fagus sylvatica* ((17), (18) and (20)), *Populus tremula* (19), *Rosa rugosa* (21), *Picea abies* ((22) and (23)). Field material from the mountain ((17), (18), (20), (22), and (23)) and sub-mountain (19) vegetation zones of Switzerland and the northern German sea coast (21).

reticulum and the accumulation of spherical lipid droplets (Holopainen et al., 1992). The fragmentation of the central vacuole in numerous small vesicles dispersed inside cytoplasm may reflect the accumulation of cryoprotectants (frost hardening, Fink, 1999, p. 461). Whilst hardening reactions are reversible and limited to winter time, irreversible cellular injuries caused by frost are accumulating with increased needle age (Sutinen and Koivisto, 1995). Foliage organs are especially sensitive to frost occurring very early (Fig. 3(17,18)) or late during the vegetation period: without hardening, microscopical freezing injury usually begins with ice formation in xylem elements and intercellular space and affects the entire needle cross section (Wulff, 1996; Ryyppö et al., 1997; Fink, 1999; Table 2). It first includes tannin precipitation along the central

vacuole (not a stress specific change, Fig. 1(9,10), details see legends), later the protoplast contracts and the cell walls finally collapse. According to Perkins and Adams (1995), nutritional imbalances may promote winter injury by causing alterations to the cellular permeability and/or the osmotic gradient between the intra- and intercellular compartment; particularly potassium deficiency may play a critical role. It implies that trees growing on poor acidic soil or at sites with acid precipitation can be more threatened by frost injury, as reported by Kukkola et al. (1997).

When drought occurs during leaf formation, the leaf anatomy is changed, often resulting in smaller leaf area and lower stomatal density (Grossoni et al., 1998; Pääkkönen et al., 1998). When drought affects fully expanded leaves, visible

symptoms include leaf rolling, necrosis starting at the end of the water way (leaf tip and rim, twig tips, tree top) and leaf shedding (Hartmann et al., 1995; Vollenweider and Günthardt-Goerg, 2006). Possibly senescence interferes with the symptom expression when symptoms are increased towards the basis of the yearly shoot increment in species with indefinite shoot growth. In conifers, browning and soon afterwards shedding of the older needle generations can be observed during the months following the drought period, as observed in spruce following the record summer of 2003 (Fig. 3(22), detail Fig. 3(23)). The distinction between heat and drought effects still awaits further investigation, whereas those resulting from ozone and drought stress are distinguishable at the macroscopic symptom level (Dittmar et al., 2004; Vollenweider and Günthardt-Goerg, 2006). Consequently, at least partly different microscopic symptoms can also be expected. Mild drought promotes OS as shown in leaf mesophyll by cell walls irregularly thickened, whatever the ontogeny phase, with pectic warts (Pääkkönen et al., 1998) or by the formation of H_2O_2 occurring with increasing stress in the xylem, mesophyll cell walls and plasma membrane, but not in chloroplasts (Munné-Bosch et al., 2001). Finally, transfusion parenchyma and mesophyll cells show plasmolysis. When leaves affected by drought show yellowing, both symptoms of drought and senescence are found (e.g. nutrient remobilization, chromatin condensation, chlorophyll degradation, accumulation of plastoglobuli, thylacoid swelling and distortion, decreased grana stacking) which indicates ACS (reviewed by Munné-Bosch and Alegre, 2004). In conifer needles, specific drought symptoms have been reported for chloroplasts (see Table 2).

3.3. Disturbance in the mineral nutrition: deficiency in macro- and micro-nutrients

Visible symptoms in foliage frequently result from a disturbed mineral nutrition. Kabata-Pendias and Pendias (2001) indicate that, in such a case, they are more frequently caused by a deficiency than by an excess of mineral elements. Macroscopic symptoms include stunting and different types of foliage discoloration. The precise symptom distribution at the leaf or needle, twig and crown level plays an important role in diagnosis (Hartmann et al., 1995; Nienhaus et al., 1996; Vollenweider and Günthardt-Goerg, 2006). Macroscopic chlorosis in older (or younger, in the case of sulfur) foliage and the underlying microscopical symptoms due to nutrient deficiencies have been reviewed by Fink (1999, pp. 489–504), Holopainen et al. (1992), Briat et al. (1995) on iron and earlier by Hecht-Buchholz (1983) and Bussler (1981). In conifer needles, symptoms caused by a deficiency in phloem-mobile elements like N, P, Mg and S show similarities with those resulting from ontological senescence observed during nutrient retranslocation (Fig. 1(5)) and are therefore examples of ACS. As indicated in references cited in Table 2, differences with ontological senescence include ultrastructural changes in mesophyll and transfusion parenchyma, namely early degeneration of the endoplasmic reticulum and ribosomes in the cytoplasm in the case of nitrogen and the early swelling/

dilatation of mitochondria in the case of phosphorous. Symptoms from a deficiency in magnesium and potassium (Table 2, Holopainen and Nygren, 1989) first appear in the vascular cylinder with a collapse of almost all sieve cells, hypertrophy of the adjacent parenchyma and cambium cells and ACS in transfusion and albuminous cells. Reactivation of cambium and regeneration of chloroplasts after fertilization is possible. Macro- and microscopic symptoms caused by a deficiency in phloem-immobile elements differ from those occurring with recyclable macro- and micro-nutrients: calcium, boron and manganese deficiencies lead to deformation of the shoot apex and young leaves resulting from thin-walled or abnormally lignified cells and hyperplasia of the undifferentiating cambium cells (Fink, 1999). For boron, this effect may be explained by a decreased borate cross-linking of pectin (O'Neill et al., 2004). Deficiencies in other mineral elements are liable to cause macro- and microscopic symptoms in foliage of plants. However, since the available evidence has been mostly obtained using agricultural plants, comparable data about several element deficiencies in trees appear to be missing, these include sulfur, copper, iron, molybdenum and zinc.

3.4. Disturbance in the mineral nutrition: excess of heavy metals

Macro- and microscopical symptoms of heavy metal stress show more complexity than those resulting from mineral deficiencies (Section 3.3) as their cause includes, not only a disturbed mineral nutrition, but also element- and species-specific decontamination, defense and stress reactions. Furthermore, the toxicity depends on the species and ecotype, ontogeny, edaphic and climatic factors (Greger, 2004). Kabata-Pendias and Pendias (2001) reviewed the visible symptoms in foliage, influencing soil factors, critical concentrations in plant tissues, and interactions between major (particularly iron and manganese) and trace elements for crops and a few tree species. Most commonly reported visible symptoms of intoxication by heavy metals include stunting and leaf chlorosis (Sanità di Toppi and Gabrielli, 1999; Kabata-Pendias and Pendias, 2001) which often result from only indirect effects on foliage such as the inhibition of root growth (Cosio et al., 2006). Chlorosis induced by an excess of cadmium, cobalt, copper, lead, nickel or zinc and other elements at a deficiency level such as aluminum and manganese (Das et al., 1997; Arduini et al., 1998; Fink, 1999; Krupa et al., 2002; Myšliva-Kurdiel and Strzałka, 2002) can result from interferences in the mineral nutrition between the elements in excess and the other elements such as iron, nitrogen, phosphorus potassium, calcium, magnesium, copper or zinc. Whereas poorly mobile elements (aluminum, chromium, copper, lead) directly injure the roots and only indirectly the leaves, mobile elements (cadmium, nickel, zinc) can affect foliage directly and thus cause more specific visible symptoms (Fig. 4(24); Table 1). Because toxic metals enter the leaves via the transpiration stream, they often show accumulations in the limb in the form of gradients connected to the vein system. Metal (re-)cycling via the phloem, as shown for nickel and zinc (in contrast to cobalt, cadmium

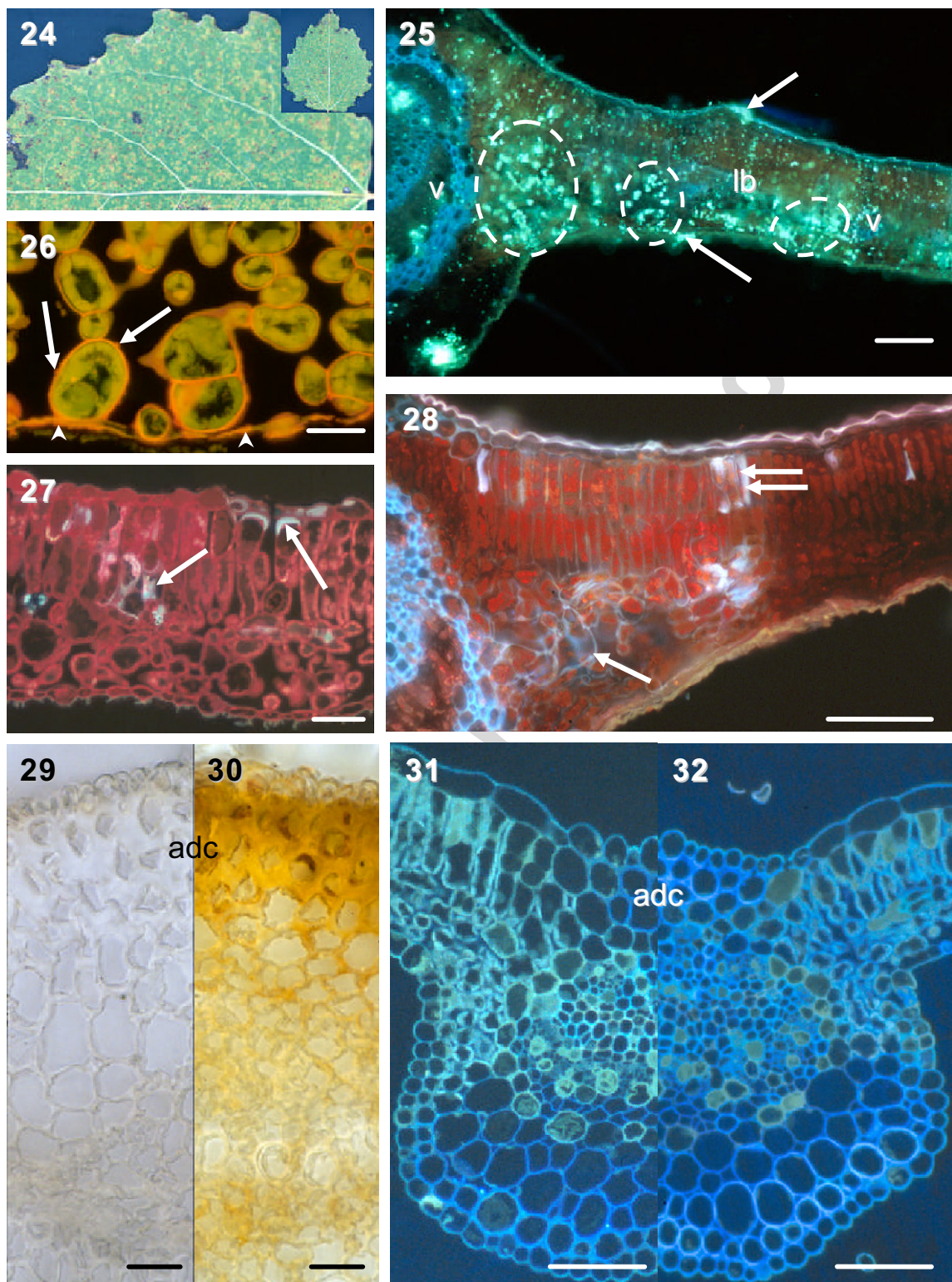


Fig. 4. Heavy metal allocation and cellular reactions in leaves of two *Salicaceae* species. (24)–(28) Zn allocation and stress reactions in sensitive *Populus tremula* plants exposed to several heavy metals in a controlled large lysimeter experiment. (24) Gradients of chlorotic and brown stippling increasing towards the shoot basis were observed on the adaxial leaf side. Brown dots were especially frequent near veins. (25) Zn (fluorescing in blue green; arrows) was the main metal contaminant imported to the leaves. It reached all leaf blade tissues but was not homogeneously distributed at the microscopical level in the leaf blade (lb), with characteristic large deposits (dashed circle) near veins (v). (26)–(28) Oxidative stress in apoplasm is shown by cell wall thickening with wart-like pectinaeous protrusions (arrows in (26)), callose (arrows in (27)) or polyphenolic (arrows in (28)) inlays. Notice the typically collapsed lower epidermis in (26) (arrow head). The decreasing gradient of polyphenols (28), starting from the vein, matches that of Zn frequency (25). The double arrow in (28) indicates a stipple in the palisade layer. (29)–(32) Cd allocation and adaptation of cell walls to Cd storage in a Cd-tolerant clone of *Salix viminalis* exposed to 0 ((29) and (31)) and 10 ((30) and (32)) μM Cd under hydroponic conditions. (30) vs. (29) Yellowish to brownish tones indicate where Cd preferentially accumulated in Cd-treated samples

and manganese) in wheat (Riesen and Feller, 2005), also occur in trees as shown for cadmium in *Salix viminalis* (Cosio et al., 2006).

To distinguish between direct and indirect effects of heavy metals at the leaf level, microlocalization as well as structural and ultrastructural studies have to be carried out in parallel. Unfortunately, published evidence combining metal detection and structural reactions in tissues is scarce, in particular for tree species, although clones of *Salix* can extract cadmium from contaminated sites (Pulford and Watson, 2003) in an efficient way. Barceló and Poschenrieder (2004) have reviewed ultrastructural injury by heavy metal exposure, particularly in chloroplasts. They report that the organelles in chlorotic leaf areas resemble either immature plastids (injury during the chloroplast formation) or senescent chloroplasts (decreased number and swelling of chloroplasts, reduced thylacoid membrane area and grana stacks, distortion and swelling of thylacoid membranes, increase of plastoglobuli). In the case of mobile heavy metals like cadmium or zinc, contaminants can be detected cytochemically, which allows the observer to also detect the cell and tissue reactions in the same portion of the investigated leaf sample and relate the micro- to the macroscopical symptoms (Fig. 4(24–32)). Logically, gradients of cell reactions frequently match those of metal accumulation (André et al., in press; Vollenweider et al., 2006; Fig. 4(28 vs. 25)). Plant responses to intoxication vary largely depending on heavy metal, plant species and ecotype. Reactions of (1) detoxification, (2) stress resulting from direct and indirect heavy metal effects and (3) defense, mix inside the same leaf portion. Detoxification can be shown by storage of contaminants in different structures, e.g. cell walls (Carrier et al., 2003; Fig. 4(30)), vacuoles (Clemens, 2001) or hairs (Choi et al., 2001), and can contribute to the plant's tolerance. In a cadmium-tolerant clone of basket willow, cadmium was preferentially stored in leaf cell walls of the vein collenchyma away from the more metabolically active and vital leaf tissues (Fig. 4(30 vs. 29)). An orderly increase in cell wall thickness including cellulosic inlays was observed (Fig. 4(32 vs. 31)). These willows could thus bear exposure to 10 μ M cadmium in hydroponics, corresponding to cadmium contamination levels in the soil solution to be found in environments with the highest pollution only (Sanità di Toppi and Gabrielli, 1999). Contrastingly, direct and acute stress can result from unrestricted metal allocation even to highly OS-sensitive sites, such as mesophyll and chloroplasts (examples for Zn in André et al., in press; Martin et al., 2006). In a sensitive provenance of European aspen, Zn contaminants thus reached, rather homogeneously, most leaf blade parts (Fig. 4(24)) leading to increased stress (Fig. 4(25–28)) including HR-like reactions developing

independent of light exposure (stipple, Fig. 4(28)). Indirect stress reactions seem more likely to increase ACS (Vollenweider et al., 2006). According to our knowledge defense reactions to zinc or cadmium, in the form of proanthocyanidin accumulation in vacuoles and cell walls, have been observed in different tree species (Günthardt-Goerg and Vollenweider, 2003; André et al., in press; Vollenweider et al., 2006) whereas they often are missing in herbaceous plants under similar treatments (Martin et al., 2006). The diagnostic and bioindication potential of the different symptoms still needs further research. Evidence about conifers is missing.

3.5. Response to air pollutants enhancing acidity stress

Direct effects from strong acid precipitation (acid mist, acid rain) on tree foliage have been shown for pH below 3.5: with simulated acid rain, chlorotic spots and later necroses were induced in upper epidermal cells of young leaves of poplar and oak; adjacent parenchyma cells showed abnormal cell proliferation (hyperblasia) and hypertrophy as a wound reaction (Evans et al., 1978; Evans and Curry, 1979). In yellow poplar treated with acid mist, cell collapse increased in all tissues; nearby cells, which retained their shape, showed tannin bodies in vacuoles, condensation of the cytoplasm but no injury symptoms in chloroplasts (Crang and McQuattie, 1985). In needles of *Pinus nigra* and *P. resinosa* sprayed with sulfuric acid, visible symptoms (brown spots) developed on the needle surface at the end of the needle flushing period; they followed the degeneration and necrosis of mesophyll cells near the stomata, thus along the penetration pathway of the stress factor inside the needle (Zobel and Nighswander, 1991, Table 2). After the sensitive period in spring during shoot growth, conifer needles with thick cuticle, epicuticular wax layer and stomata sunken below the surface (Günthardt-Goerg et al., 1994) should be better protected from acid precipitation than leaves in broadleaved trees. Other effects in needle mesophyll or vascular tissue (for details, see Table 2) are indirect effects of acid precipitation and related to a possibly earlier onset of winter hardening, nutrient disorders (Section 3.3), or fertilizing effects (nitrogen and sulfur).

Responses to sulfur dioxide (SO₂) and other gases promoting acidity stress have been particularly studied in conifer needles. These air pollutants are more toxic than acid rain as shown by Wulff and Kärenlampi (1993) who found more visible injuries in needles of *Picea abies* when excluding precipitation. Elevated concentrations of SO₂ and even worse fluorides (Wulff and Kärenlampi, 1996, Table 2) lead to chlorosis, rim necroses and tip burn symptoms (which can be similar to drought, potassium deficiency, salt or metal toxicity macroscopically (Sections 3.2, 3.3, 3.4, Table 2). Microscopically, the mesophyll

whereas no signal was observed in control (29). Especially, the cell walls in collenchyma (adc, adaxial collenchyma) provided a safe Cd storage compartment away from sensitive and metabolically more active leaf tissues. (32) Leaves of Cd-treated samples showed adaptation to the storage of Cd by a regular thickening of cell walls with cellulosic material as indicated by a more intense blue fluorescence of the cell walls in (32) vs. (31). Revelation methods: (25) new specific stain for Zn (in preparation); (26) Coriophosphine (Günthardt-Goerg, 1996); (27) PARS (Gahan, 1984) and Aniline blue (Gerlach, 1984); (28) autofluorescence (excitation at 340–380 nm); (29) and (30) physical development (15 min, Vollenweider et al., 2006); (31) and (32) Calcofluor White Mr2 (Munck, 1989). Bars: 15 μ m (26), 25 μ m ((29) and (30)), 30 μ m (27), 50 μ m ((31) and (32)), 100 μ m ((25) and (28)). (For interpretation of the references to colours in figure legends, the reader is referred to the web version of this article).

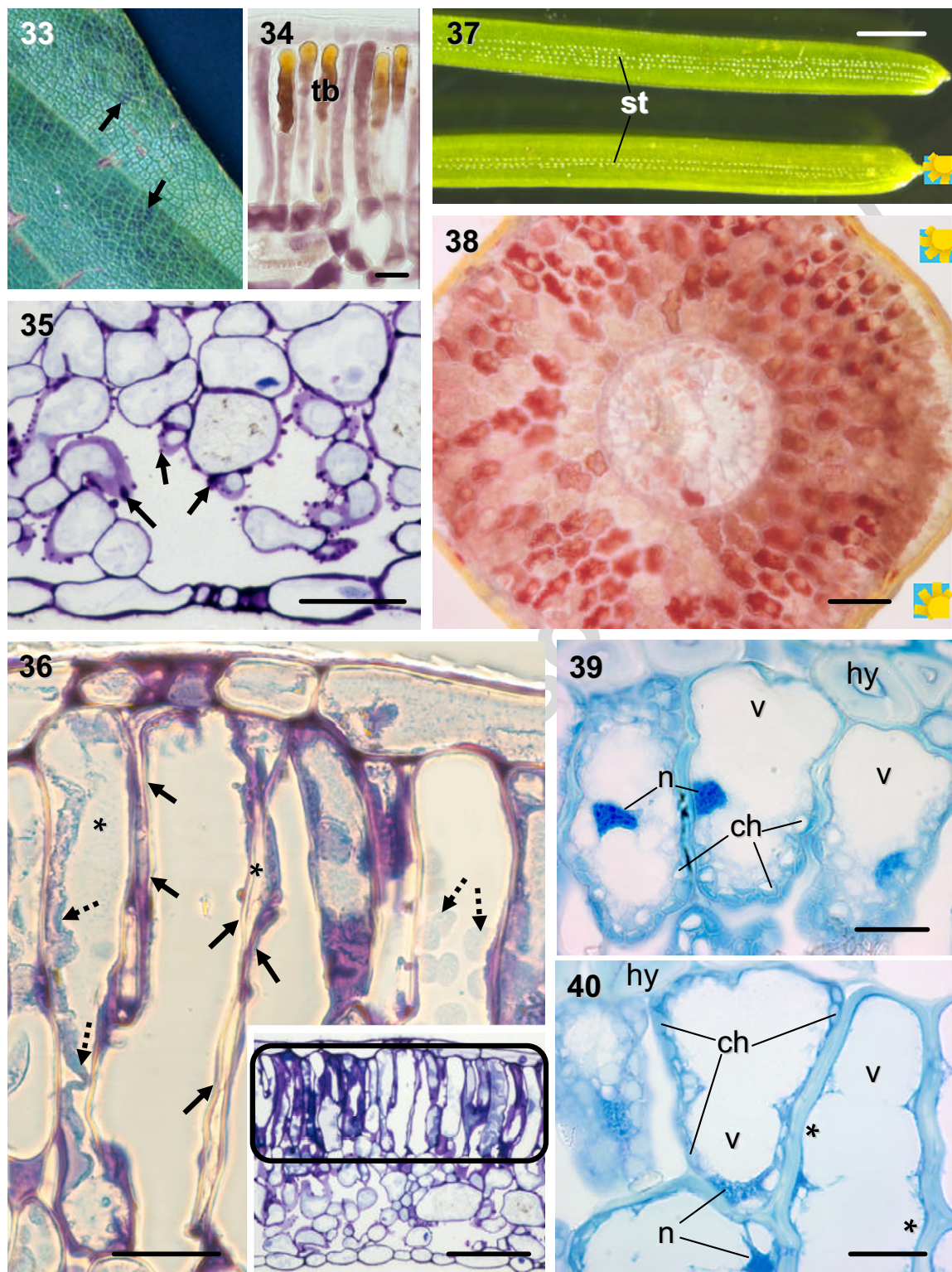


Fig. 5. Several visible and microscopical ozone symptoms in broadleaved and conifer species (field samples, ambient air). (33) Adaxial intercostal dark stippling (arrows) is a visible symptom characteristic for ozone. (34) The cause of which is the adaxial accumulation of oxidized condensed tannin bodies (tb) in the apical portion of the vacuole of underlying discrete groups of palisade parenchyma cells. (35) Pectic warts on cell walls (arrows) indicate oxidative stress in the apoplasm of the spongy parenchyma. (36) Cell structure inside a stipple. Discrete cell groups of variable size (framed in the inserted picture at lower magnification) show several HR-like features such as the partial or complete disruption (*) and condensation (dotted arrows show different stages of chloroplast condensation) of cell content, and collapse of cell walls (plain arrows). (37) Ozone stress in 3-year-old spruce needles as indicated by chlorotic bleaching of the light-exposed needle side (sun symbol). Notice the lower frequency of stomata (st) on the light-exposed vs. the shaded needle side. (38) Oxidation of the vacuolar condensed tannins increases progressively between the shaded (red tones, left) and the light exposed needle side (brownish tones, right) indicating growing oxidative stress in the symplasm. (39) and (40) Cells directly below hypodermis (hy) show ACS rather than senescence on the light-exposed (40) vs. the shaded (39) needle side. ACS is

cells adjacent to substomatal cavities are the target of SO₂ (early ultrastructural symptoms are swelling and separation of the thylacoid membranes) whereas the vascular bundles stay intact until a very late stage of injury (Fink, 1989). Specific responses to SO₂ and other gaseous pollutants are, however, difficult to individualize since much of the evidence published comes from field or controlled experiments where the effects of several stress factors were combined. SO₂ enhances microscopical symptoms with low stress specificity such as cytoplasm darkening, accumulation of cytoplasmic lipids or vacuolar tannin bodies (which are all markers for ACS) and the partial plasmolysis of cells (Zobel and Nighswander, 1991; Sutinen et al., 1998): the latter symptoms, for example, can also be found in conifers when trees are grown on acidic vs. calcareous soil conditions (Fig. 1(10 vs. 9)). The interaction between SO₂ and the needle and cell physiology therefore remains rather limited, as suggested by the localization of injury mostly next to the needle apertures and the rather high dose of pollutants needed to observe symptoms.

3.6. Response to ozone stress

The ozone concentrations originally used in the 1960s during short time fumigation treatments were too strong and induced spectacular and quickly developing necroses. These experiments have clearly established, however, that ozone injury mainly occurs in the mesophyll tissue whereas epidermal and stomatal cells and vascular tissues resist until the last stage before total leaf necrosis (Hill et al., 1961; 34 plant species, 0.13–0.72 ppm ozone for 2 h). Recent papers with illustration of macroscopic and microscopic ozone symptoms are presented in Tables 1 and 2. As ozone uptake and oxidative effects are similar whatever the species, the symptom display even in completely unrelated species presents striking similarities regarding the physiological changes and their localization (Tables 1 and 2). Whether in conifers or in broadleaved trees, visible ozone injury in the field occurs after periods of sunny weather and elevated ozone concentrations particularly at the light exposed tree, branch and leaf or needle parts and irrespective of the nutritional status of trees (Maurer et al., 1997; Günthardt-Goerg and McQuattie, 1998).

When periods with elevated ozone concentration occur early, during leaf formation, leaves can acclimate and show a reduced sensitivity during later ozone episodes (Pääkkönen et al., 1995b; Günthardt-Goerg et al., 1993). Broadleaved trees typically show interveinal bronzing (Fig. 5(33)), reddening, bleaching and interveinal stippling (groups of collapsed palisade cells forming tiny necrotic points) (see Innes et al., 2001; <http://www.ozone.wsl.ch>; <http://www.gva.es/ceam/ICP-forests/>). Although light-induced OS contributes to ozone symptom expression, ozone leaf injury can nevertheless occur on shaded foliage too (Vollenweider et al., 2003b) showing

similar macroscopic symptoms. It suggests that the stress signal perception, rather than the stress response, differs between both crown locations. Microscopical analyses (Table 1) have confirmed that the better light exposed assimilating cells, namely the palisade parenchyma in broadleaved trees (Vollenweider et al., 2003a) show the most severe injuries, whereas the conducting tissues are injured only in a late stage and/or at continuously applied elevated ozone concentrations (Günthardt-Goerg and McQuattie, 1998; Matyssek et al., 2002). Visible stippling in broadleaved species results from palisade cells undergoing HR-like reactions (Fig. 5(36)). The surrounding tissues show structural symptoms of ACS (reviewed by Pell et al., 1997) and OS. Protrusions on cell walls including pectin, polyphenolic or protein material (Günthardt-Goerg, 1996; Günthardt-Goerg et al., 1996, 1997, 2000; Fig. 5(35)) probably constitute reactions to apoplastic OS which occurs first following ozone uptake (Pellinen et al., 1999). Such symptoms can also appear in the shaded and asymptomatic foliage exposed to elevated ozone concentrations (Vollenweider et al., 2003a). Inner cell wall thickening with cellulosic or callose material frequently occurs, especially in palisade cells (Günthardt-Goerg et al., 1996; Vollenweider et al., 2003a; Bussotti et al., 2005). The inner and outer cell wall layer is generally thickened locally in response to ozone stress, and, as a consequence, the overall cell wall thickness becomes characteristically irregular, especially in the more light exposed cell portion of the outermost palisade cells (Vollenweider et al., 2003a). The condensed tannins constitute particularly remarkable markers of ozone stress for validation analyses: their amounts are increased in the more symptomatic mesophyll and they show gradients of oxidation at the tissue and cell level (Fig. 5(34)) according to the intensity of OS (Vollenweider et al., 2003a). Daily starch formation and translocation decline in symptomatic tissues, which lead to irregular starch accumulation in bundle sheath, epidermal and guard cells (Günthardt-Goerg et al., 1993, 1998). None of the above structural criteria is specific for ozone stress. However, an ascertained symptom validation is provided by the combination of several markers and by the close attention to their microlocalization in tissues and cells.

In conifers, visible symptoms in response to ozone stress include mottling (mostly in pine species) usually in older needle generations (Arbaugh et al., 1998; Dalstein et al., 2002; Sanz et al., 2000) and chlorophyll bleaching (Günthardt-Goerg and Vollenweider, 2001; Fig. 5(37), Table 2). Although ozone effects in conifers are the subject of many scientific papers, most often visible symptoms are not mentioned and photographs (without validation) are restricted to textbooks (Skelly et al., 1990; Hartmann et al., 1995). Mottles in pines show striking structural similarities with injuries in stipples of broadleaved trees (Dalstein et al., 2002; Vollenweider et al., 2003a; Table 2 vs. Table 1): they are also underlined by

shown by a larger vacuole (v) more nucleus (n) condensation and less and smaller chloroplasts (ch; * cell portions with missing chloroplasts). Broadleaved species: *Fagus sylvatica* (33) and (34); *Sambucus racemosa* (35) and (36). Conifer species: *Picea abies* (37)–(40). Revelation methods: (34) DMACA (modified from Gutmann and Feucht, 1991); (35),(36) and (39),(40) Toluidine blue (Feder and O'Brien, 1968) and *p*-phenylenediamine (Kivimäenpää et al., 2004); (38) Acid vanillin (Sarkar and Howarth, 1976). Bars: 15 µm ((34), (39), and (40)), 25 µm (36); 50 µm (35); 100 µm (detail in (36) and (38)); 1000 µm (37). (For interpretation of the references to colours in figure legends, the reader is referred to the web version of this article).

discrete groups of dead cells with a disrupted cell content whose remnants are compacted in apoptotic-like bodies. These cells are located just below the epidermal layers on the sun exposed needle side. In contrast to palisade in broadleaved trees, mesophyll cells in most conifer needles do not collapse from a realistic ambient ozone dose, probably because they are thicker and sturdier than in leaves (authors' observation). The term 'collapsed cells' in elevated ozone treatment (71 ppb, Evans and Fitzgerald, 1993) or multisymptomatic needles ("bone cells", Hasemann and Wild, 1990; Wenner and Merrill, 1998) appear to relate to dead cell content (no collapsed cell walls), or to fully necrotic needle tips. In one particular case (potted *Pinus halepensis*, exposed to ambient ozone, Soda et al., 2000) mesophyll cells, whose cell walls are ontologically thin in this species, collapsed but without showing other HR-like markers. Mottles as well as stipples are surrounded by senescing cells whose structure show ACS features (Table 2). Photobleaching, e.g. in *Picea abies* (Fig. 5(36)), is observed on the light-exposed needle side in trees experimentally exposed to ozone (Sutinen et al., 1990; Günthardt-Goerg and Vollenweider, 2001) or ambient ozone (Fig. 5(37); Kivimäenpää et al., 2004). Visible symptoms result from gradients of ACS between the better light-exposed outer cell layers and those remaining shaded, which progressively develop in aging needles of the light crown (Fig. 5(37–40), details see legends). The degeneration of chloroplasts (with gradients observable already at the cell level, Fig. 5(39)) plays a prominent role regarding the changes in needle color. Tannins also provide useful bioindication markers especially regarding their localization and level of oxidation (Dalstein et al., 2002; Fig. 5(38)). However, they reach lower concentrations than measured in some sensitive broadleaved species (e.g. 9.8 mg/g dw in asymptomatic field material from 1-year-old needles of *Pinus cembra*, vs. 37.0 mg/g dw in leaves of *Fagus sylvatica* sampled at the end of the vegetation season; unpublished data). Therefore, visible bronzing, which is structurally related to the accumulation of condensed tannins in symptomatic tissues (Vollenweider et al., 2003a), is not observed in conifers.

4. Conclusions

Compartmentation within organelles (Fig. 1(6–8)), cells, tissues, organs and entire trees plays a fundamental, organizational and functional role in plants. This role is not only static but also dynamic: golgi and endoplasmatic reticulum vesicles store and transport solute to different cell parts (Gunning and Steer, 1996). However, importation and exportation from the vacuole to cyto- and apoplasm and vice versa can also be mediated by vesicles as evidenced by different structural and ultrastructural studies (Echeverria, 2000) and not only by chaperons and other transporters, which became known through current approaches in molecular biology (Clemens, 2001). Proliferation of vesicles is not limited to ACS associated with different stress factors, as shown in this review, but also constitutes a fundamental part of daily cell physiology. Where defense reactions are concerned, segregation by

membranes allows the cell to store constitutive phenolic defense substances as non-toxic glycosides away from releasing aglucosidases (themselves confined to the relative site of action: apoplasm, plastids). Stress-induced decompartmentation thus generates toxic defense compounds contributing to the restriction of infections (reviewed with focus on periderm of agricultural plants by Beckman, 2000). During quantitative analysis, differences in compartmentation, noteworthy at the tissue level, are generally lost (Godbold et al., 1993) whereas precious bioindications about the microlocalization of each marker are obtained with microscopical studies. However, the microlocalization of the marker is generally more specific than its histochemical reactions. Therefore, structural and ultrastructural analyses applying the reviewed knowledge on macroscopic and microscopic symptoms provide choice methods for the causal diagnosis of stress in plants.

This review shows how, by combining insights on visible, microscopical and physiological reactions, the diagnosis of biotic or abiotic stress factors can be ascertained. This integrated diagnostic approach provides opportunities for cheap applications all over the world including the emerging countries. The diagnostic potential of visible symptom observation is far from being exhausted and still remains insufficiently used. Close attention to visible symptom morphology and distribution in the attached foliage provides numerous clues about the structural and physiological changes triggered by the responsible stress factor(s) in a very simple way. The message includes: (1) any change in the green hues of foliage signals stress affecting the chlorophyll and chloroplast machinery inside mesophyll tissues; (2) any increase in the red or bronze tones reflects an accumulation of vacuolar flavonoids in the form of anthocyanins and condensed tannins; (3) a strictly interveinal localization of the macroscopic symptoms indicates that the stress factor affects the leaf mesophyll in priority; (4) shading effects show that light stresses the mesophyll in synergy with another stress factor (most often ozone); (5) visual symptoms related to the vein system mean a disturbance in the water and/or mineral element supply; (6) injury symptoms sharply confined within adjacent green tissue shows efficient defense reactions as occurring with benign biotic injury. Microscopic symptoms can be detected before they become macroscopically visible, reveal unchecked visible symptoms and sharpen the observer's eye for less visible injuries. Using techniques from histochemistry, they allow the observer a physiologically based understanding of the structural changes. Synthesizing the observed changes in terms of plant responses help to cross barriers such as species specificities, lab specialties or the infinity of potential stress markers.

Ozone is probably the stress factor for which microscopical validation has been most successfully applied. A similar bioindication approach about HM stress in trees growing on contaminated sites would provide interesting monitoring and controlling opportunities but is still restricted by the lack of evidence concerning several toxic elements. Additional diagnostic criteria are needed to differentiate drought and elevated air temperature effects from other stress factors. Research in the last decade (as reflected in Tables 1 and 2) mainly focused

on the diagnosis of visible ozone symptoms in broadleaved trees and ultrastructural changes caused by different stress factors in conifer needles. To complete our understanding in stress physiology about trees, valuable insights would be obtained by applying similar research approaches to salt stress, nutrient deficiencies of several micro-elements and autumnal senescence. The efficiency of integrated diagnostic approaches would be improved specifying the diagnostic potential of the structural markers discussed here, the distinction between unspecific and bioindicative symptoms, and the differentiation between direct and indirect effects of stress, which have often been neglected. In an era during which the ongoing climate change may modify and increase the constraints affecting trees in the natural environment, improving and further developing new diagnostic tools to identify the origin of stress in trees represents an urgent challenge to analyze how ecosystems will cope with the forthcoming environmental conditions.

Acknowledgements

We gratefully acknowledge the collaboration of Claudia Cosio for Fig. 1(26–29), Michael Lautenschläger (microtechnical work) and Terry Menard (microtechnical work and language editing), and Angela Nunn (samples provided for Fig. 1(10) and Fig. 2(11,12)).

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